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# Enzymatic biofilm detachment causes a loss of aggregate stability in a sandy soil.

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**Abstract.** The stability of soil aggregates against shear and compressive forces as well as water caused dispersion is an integral marker of soil quality. High stability results in less soil compactibility and erodibility, enhanced water retention, a dynamic water transport and aeration regime, increased rooting depth and protection of soil organic matter (SOM) against microbial degradation. For decades the importance of biofilm extracellular polymeric substances (EPS) regarding aggregate stability has been canonical because of its distribution, geometric structure and ability to link primary particles. However, experimental proof is still missing. This lack is mainly due to methodological reasons. Thus, the objective of this work is to develop a method of enzymatic biofilm detachment for studying the effects of EPS on soil aggregate stability. The method combines an enzymatic pre-treatment with different activities of α-glucosidase, β-galactosidase, DNAse and lipase, which preserves aggregate structure, with a subsequent sequential ultrasonic treatment for disaggregation and density-fractioning. Soil organic carbon (SOC) releases of treated samples were compared to an enzyme-free control. To test the effectivity of biofilm detachment the ratio of bacterial DNA from sessile and suspended cells after enzymatic treatment was measured by quantitative real-time PCR. Although the enzyme treatment was not sufficient for total biofilm removal, our results confirm, that EPS stabilizes soil aggregates predominantly by a strong intra-aggregate fixation, and enzymatic biofilm digestion caused a shift of occluded particulate organic matter (POM) to more fragile binding patterns. This suggests that an effect of agricultural practices on soil microbial populations could influence aggregate stability and thereby soil quality.

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## 1 Introduction

The stability of soil aggregates against shear and compressive forces *(Skidmore and Powers, 1982)* as well as water caused lability (Tisdall and Oades, 1982) is an integral marker of soil quality (Bronick and Lal, 2005). Because aggregate stability implies pore stability, it results in less soil compactibility (Baumgartl and Horn, 1991; Alaoui et al., 2011) and a more dynamic water transport regime in the macropores that reduces erosion caused by surface runoff (Barthes and Roose, 2002). Other benefits in comparison to compacted soils are a higher aeration (Ball and Robertson, 1994) and lower penetration resistance (Bennie and Burger, 1988) causing increased rootability and rooting depth (Bengough and Mullins, 1990; Taylor and Brar, 1991). In addition, micropores within the aggregates enhance the water retention by increased soil-moisture tension. Furthermore physical isolation protects soil organic matter (SOM) including particulate organic matter (POM) against microbial degradation (Six et al., 2002; Lützow et al., 2006) while on the other hand this SOM promotes soil aggregation (Bronick and Lal, 2005).

The structural stability of soil aggregates depends on the properties of the attached components. The mineral part of the solid soil matrix is composed of siliceous sand, silt and clay particles, oxides and hydroxides of Fe, Al and Mn as well as diverse minor minerals. Sticking together, pervaded and coated with multivalent cations and organic constituents like soluble metabolic products, humic substances, black carbon and other POM, macro-aggregates (>250 µm) are formed by direct coagulation or built of micro-aggregates (<250 µm). (Bronick and Lal, 2005; Brodowski et al., 2006; Lützow et al., 2006) The structure-bearing primary particles, precipitates and adsorbed molecules cohere by physico-chemical interactions between (i) permanent charge of mainly the clay mineral fraction, (ii) multivalent cations with small hydrate shells such as $Ca^{2+}$, $Fe^{3+}$ and $Al^{3+}$, (iii) variable charges of various minerals and SOM and (IV) variable and permanent dipoles of different soil components. Also in part biologically precipitated oxides and hydroxides as well as carbonates and phosphates force up aggregation. (Jastrow and Miller, 1997; Bronick and Lal, 2005)

In addition since a few decades biological structures like bacterial colonies, bacterial pseudomycelia, algae, fungal hyphae and their exudates (e.g. glomalin), roots and soil fauna are accepted as a major factor of soil aggregation (Tisdall, 1991; Oades, 1993; Wright and Upadhyaya, 1998; Brown et al., 2000; Chenu and Stotzky, 2002; Rillig, 2004; Bronick and Lal,

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2005). The role of extracellular polymeric substance (EPS) of bacterial biofilms as an adhesive between soil particles is also seen to be of major importance (Baldock, 2002; Ashman et al., 2009).

The biofilm itself is a viscous microenvironment mainly built up of 90-97% water (Zhang et al., 1998; Schmitt and Flemming, 1999; Pal and Paul, 2008). The remaining dry mass contains differing ratios of polysaccharides, extracellular DNA (eDNA), proteins and lipids besides 10-50% cell biomass (More et al., 2014). In contrast to 'biofilm', EPS terms the extracellular polymeric matrix excluding cells. Extracellular polysaccharides cause the EPS structural stability by means of entanglement and $Ca^{2+}$ bridging between molecules. So does eDNA (Das et al., 2014). Proteins function as enzymes and structural links stabilizing the polysaccharid matrix, while lipids act as biosurfactants for bacterial attachment on surfaces. (Flemming and Wingender, 2010) The extracellular matrix is not only exudated by soil bacteria and archaea, but also by fungi and algae as well as engineered by protozoa and small metazoa (Battin et al., 2007; Flemming and Wingender, 2010).

The composition of EPS is highly variable depending on biofilm forming species and habitats: Redmile-Gordon et al. (2014) determined a natural habitat extracellular polysaccharide concentration of 401 µg $g^{-1}$ dry soil in grassland and 169 µg $g^{-1}$ in fallows. Diverse single- and multi-species biofilms show a proportion of polysaccharides on dry EPS of up to 95% (Pal and Paul, 2008; More et al., 2014). Different single- and multi-species biofilms in laboratory cultures and natural soils show a dry EPS eDNA content up to 10% (More et al., 2014). For forest soils values of 1.95 up to 41.1 µg $g^{-1}$ dry soil are known (Niemeyer and Gessler, 2002; Agnelli et al., 2004). Extracellular DNA content of diverse other soils is ranging between 0.03 and 200 µg $g^{-1}$ dry soil (Niemeyer and Gessler, 2002; Pietramellara et al., 2009), whereas concentrations in soils explicitly used for agriculture are unknown. Extracellular matrix protein concentration can amount to 163 µg $g^{-1}$ dry soil in grassland and 43 µg $g^{-1}$ dry soil in fallow (Redmile-Gordon et al., 2014) and often hold 60% of EPS dry mass (More et al., 2014) and even up to 75% in *P. putida* biofilms in laboratory cultures (Jahn et al., 1999). The typical proportion of lipids in the EPS dry-mass of different non-soil biofilms amounts up to 10% (More et al., 2014). Sparse molar mass data from different environments comprise $0.5 \cdot 10^6$ to $2 \cdot 10^6$ Da for polysaccharides (Flemming and Wingender, 2010), $7.75 \cdot 10^4$ to $2.32 \cdot 10^7$ Da for eDNA (DeFlaun et al., 1987) and 750 to 1,500 Da for lipids (Munk, 2008). Data from literature are compiled in Table 1.

The activity of EPS degrading enzymes in natural soils spans up to two orders of magnitude:

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The α-glucosidase and β-galactosidase acitivity of various soils range from 0.00011 U $g^{-1}$ to 0.0011 U $g^{-1}$ and from 0.00017 to 0.0094 U $g^{-1}$, respectively (Eivazi and Tabatabai, 1988; Acosta-Martinez and Tabatabai, 2000). The lipase activity in coarse mineral soils shows values from 0.3 U $g^{-1}$ in a sandy soil (Cooper and Morgan, 1981) to 2.09 U $g^{-1}$ in a Luvisol (Margesin et al., 2000) and up to 5 U $g^{-1}$ in a Leptosol (Margesin et al., 1999). Data for eDNA activity in soils are not available.

Not much is known about the contribution of EPS to aggregate stability in relation to other aggregate stabilizing factors. That is mainly due to methodological reasons. Owing to the widespread interest in the role of biofilms on soil fertility, the objectives of this work are (i) to design a selective method for enzymatic biofilm detachment hardly effecting other aggregate binding mechanisms in soils and (ii) to apply the method to an agricultural soil to proof and estimate the influence of biofilm coherence on POM fixation and aggregate stability.

The method combines a modified enzymatic pre-treatment (Böckelmann et al., 2003) with α-glucosidase, β-galactosidase, DNAse and lipase, a determination of the DNA ratio of sessile to suspended cells after enzymatic treatment and an ultrasonication of soil aggregates followed by density-fractioning and soil organic carbon (SOC) measurement (Kaiser and Berhe, 2014). The ultrasonication/density-fractionation separates SOM into three fractions: non-occluded free light fraction POM (fLF), aggregate-embedded occluded light fraction POM (oLF) and colloidal as well as (macro)molecular SOM, which is not detachable from mineral surfaces by the chosen fractioning method and subsumed under heavy fraction (HF) (Kaiser and Berhe, 2014).

We hypothesize a destabilization of EPS matrix after enzymatic treatment. That should result in an increased cell detachment from aggregates. We also expect an increased free light fraction (fLF) release from destabilized aggregates compared to the control and a shift of occluded light fraction (oLF) ratio from higher to lower binding strength represented by ultrasonic energy levels.

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## 2 Materials and methods

### 2.1 Soil properties and microbial biomass

Well aggregated silty sand (Su3) of a plowed topsoil from a cropland near Berge (Brandenburg/Germany) was air-dried and sieved to receive a particle size of 0.63 to 2.0 mm containing mainly macro-aggregates. The aggregates have a $pH_{CaCl2}$ of 6.9, $C_{org}$ of 8.7 mg $g^{-1}$ and a carbonate content of 0.2 mg $g^{-1}$.

To estimate the soil microbial biomass, 8 x 10 g of soil aggregates have been adjusted to 70 vol% soil water content and incubated for 70 hours at 20°C in the dark to attain basal respiration. Based on DIN EN ISO 14240-2 then half of the samples were fumigated with ethanol-free chloroform in an evacuated desiccator for 24 h. Afterwards all samples have been extracted with 40 ml of 0.5 M $K_2SO_4$ solution by 30 min of horizontal shaking and filtered through 0.7 µm glass fiber filters. The DOC concentration of all filtrates was measured by use of a TOC Analyzer (TOC-5050A, Shimadzu). 176 mg microbial carbon $kg^{-1}$ dry soil ($C_{mic}$) were derived from the difference between DOC contents of fumigated and non-fumigated samples multiplied by a conversion factor of 2.22 (Joergensen, 1996). Soil bacterial biomass was derived from $C_{mic}$ as 352 mg $kg^{-1}$ assuming 0.5 as a ratio of $C_{mic}$ to total cell dry mass (Bratbak and Dundas, 1984).

### 2.2 Detachment scenarios

Four enzymes were selected on the basis of soil pH and temperature used for catalytic unit definition ($T_{def}$): α-glucosidase from *S. cerevisiae* (Sigma-Aldrich, $pH_{opt}$ 6 to 6.5, $T_{def}$=37°C) hydrolyzes terminal α-1,4-glycosidic linkages in polysaccharides as β-galactosidase from *E. coli* (Sigma-Aldrich, $pH_{opt}$ 6 to 8, $T_{def}$=37°C) does with β-glycosidic bonds. Lipase from porcine pancreas (Sigma-Aldrich, $pH_{def}$ 7.7, $T_{def}$=37°C) splits fatty acids from lipids via hydrolysis and DNAse I from bovine pancreas ($pH_{def}$ 5, $T_{def}$=25°C) breaks the phosphodiester linkages between nucleotides of DNA as an endonuclease. Proteases were not used because of their promiscuity and therefore incalculable influence on the other applied enzymes.

According to the data in Table 1 maximum EPS component concentrations can be estimated for soil samples with known or assumed microbial biomass. To construct scenarios with highest need of enzymatic units for the total biofilm detachment, maximum

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percentage of EPS $(\xi_{EPS}^{max})$ and enzyme target dry masses $(\xi_{target}^{max})$ as well as minimum percentage of bacterial dry mass $(\xi_{cell}^{min})$ and minimum enzyme target molar masses $(M_{target}^{min})$ were adopt from literature (Table 2). Any other boundary conditions such as ion activity, diffusion rates or metabolization of enzymes by soil organisms were disregarded. Calculated by Eq. (1)

$$Unit_{target} = \frac{c_{cell} \cdot q \cdot \xi_{EPS}^{max} \cdot \xi_{target}^{max} \cdot m_{sample}}{\xi_{cell}^{min} \cdot M_{target}^{min} \cdot t} \quad (1)$$

with variables listed in Table 2 and Table 3, five scenarios were designed: In the E1 scenario $c_{cell}$ was given by the results of fumigation-extraction. In the E2 scenario a bacterial dry mass of 500 g $m^{-2}$ in the upper 30 cm is considered, which is assumed to be the maximum for middle and northern European soils (Brauns, 1968). Supposing a soil bulk density of 1.4 g $cm^{-3}$, a $c_{cell}$ of 1190.5 µg $g^{-1}$ dry soil is given. The E3 scenario uses a 100-fold excess (q=100, Table 3) of the enzyme activities applied in the E2 scenario, whereas the E4 scenario contained the 2,820-fold, which is slightly higher than activities used in Böckelmann et al. (2003). Enzyme-free samples (E0) were used as a control.

### 2.3 Aggregate stability

Soil aggregate stability was measured on the macro-scale. Fifteen g of air-dried soil aggregates were incubated in 5 replicates per scenario with 3.4 ml of highly concentrated artificial rainwater (ARW: 0.2 mM $NH_4NO_3$, 0.3 mM $MgSO_4$ x $7H_2O$, 0.5 mM $CaCl_2$ x $2H_2O$, 0.5 mM $Na_2SO_4$, 15 mM KCl, pH 5.7) for 3 days at 20°C in the dark to establish basal respiration and avoid slaking in the following preparation steps. After incubation 2.5 ml of ARW containing enzymatic units according to Table 3 were added to the samples. By means of a following incubation at 37°C, enzymes were let to work near their catalytic optimum for 1h. After this enzymatic pretreatment, 67.2 ml of 1.67 g $cm^{-3}$ dense sodium polytungstate (SPT) solution were added, and samples were stored for 30 minutes. Then samples were centrifuged for 26 min with 3,569 G. Sodium polytungstate solution with floating fLF was filtered through an 1.5 µm pore size glass fibre filter to separate LF particles. Afterwards following Cerli et al. (2012) aggregate samples were consecutively disaggregated in four steps by application of each 50 J $ml^{-1}$ of ultrasonic energy (Branson© Sonifier 250). The energy output was determined by measuring the heating rate of water

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inside a dewar vessel (Schmidt et al., 1999). Every treatment cycle consisted of ultrasonication, centrifugation for 26 min with 3,569 G and filtering of SPT solution through an 1.5 µm pore size glass fibre filter to separate the LF. Afterwards the LFs and the remaining soil matrix ('sediment', consisting of oLF bonded >150 J $ml^{-1}$ and the HF) were freezed, lyophilized, ground and dried at 105°C. Total amount of C was determined using an Elementar Vario EL III CNS Analyzer.

**2.4 Effect of enzyme addition on the release of bacterial cells**

The release of bacterial cells into the solution was quantified using a FastDNA™ SPIN Kit for Soil and quantitative real-time PCR.

Therefor 45 µl of ARW were added directly to 0.1 g of air-dried aggregates. The samples were sterilely incubated in duplicate at 20°C for 3 days in the dark in a closed FastPrep Lysing Matrix E tube during run to basal respiration. Then 30 µl of ARW containing enzymatic units according to Table 1 were distributed equally to the aggregates' surfaces. The samples were incubated for 1 h at 37°C in a heating block and afterwards 3x washed in 1 ml of ARW not by shaking but gently rotating along the tube's longitudinal axis to separate detached and planktonic cells from the soil matrix. Supernatants of all three washing steps were removed carefully with a pipette, pooled, centrifuged at 13.000 G for 15 minutes, supernatant was discarded, the pallet resuspended in 200 µl ARW and transfered to another FastPrep Lysing Matrix E tube. Both soil and washing ARW samples were extracted and purified following the FastDNA™ SPIN Kit for Soil manual. All DNA samples were stored at -20°C for further use.

Amplification of 10-fold diluted DNA samples was performed using a C1000 Touch Thermal Cycler (BioRad). According to the reference for SG qPCR Master Mix (Roboklon) thermocycling comprised an initial denaturation at 95°C for 10 min as well as 55 cycles of 15 sec of denaturation at 95°C, 20 sec of annealing at 49°C and 30 sec of elongation at 72°C. The reaction mix contained 1 µl PCR-$H_2O$, 12.5 µl SG qPCR MasterMix, each 0.75 µl of a 20 µmol $l^{-1}$ solution of the universal bacterial primers 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 341r (5'-CTGCTGCCTCCCGTAGG-3') (Muyzer et al., 1993; Marchesi et al., 1998) and 10 µl template DNA. *Escherichia coli* 16s DNA solution containing 10,000 copies $µl^{-1}$ was used as qPCR standard in steps of tenfold diluted concentration from $10^6$ to $10^2$ copies $µl^{-1}$.

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### 2.5 Statistics

For evaluation of light fraction carbon release, mean values as well as standard deviations were calculated. Parallels of each variant were positively tested to provide normal distribution and evidence of variance homogeneity (Shapiro Wilk test, Levene test, both $p>0.05$, data not shown). One way analysis of variance (ANOVA) was applied followed by Tukey test to clarify significant differences in C release between variants of each energy level. Results of bacterial DNA release were presented as duplicates.

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## 3 Results

### 3.1 Aggregate stability

The relative net SOC release shows the different effects of the enzymatic treatments on POM detachment from soil aggregates (Fig. 1). The relative net SOC release is defined as $C_{frac}\ C_{\Sigma}^{-1}$, in which $C_{frac}$ is the release of organic carbon per energy level or – in case of the sediment – the organic carbon remaining in the soil matrix. $C_{\Sigma}$ is the total SOC of all seprated LFs and the sediment. Data are shown as mean values and standard deviations of 5 parallels.

Averaging all treatments, around 79% of SOC remain in the sediment, whereas the bulk of light fraction SOC is released as weakly bound oLF (50 J $ml^{-1}$) and fLF. Only around 4.5% of SOC is detached at 100 J $ml^{-1}$ and 150 J $ml^{-1}$.

None of the enzymatic treatments altered the SOC of the fLF (0 J $ml^{-1}$).

In contrast, compared with the control an increased net detachment of oLF carbon is measured at 50 J $ml^{-1}$ for the highest enzyme activity (E4), whereas it is decreased at very low enzyme activity (E1). However, due to high variance these differences in oLF carbon release reside sharply above the significance level ($p<0.05$). On the other hand, E1 and E4 scenario show a significant difference from each other in net SOC release. E2 and E3 have no difference compared to the control.

Released SOC stock at 100 and 150 J $ml^{-1}$ is not varying between variants. Only the E2 scenario shows an in tendency increased release of oLF carbon at 100 J $ml^{-1}$.

The sediment represents the SOC remaining unextractable at ≤150 J $ml^{-1}$ and is therefore differing between variants with a trend to decrease with increasing enzyme activity.

Comparison between a treatment and the control allows to identify net shifts of organic matter between sediment, oLF and fLF: SOC stock remaining in the sediment after ultrasonication mainly corresponds to a net transfer *from* the oLF at 50 J $ml^{-1}$ in the E1 scenario, a net transfer *to* the oLF at 100 J $ml^{-1}$ in the E2 scenario and a net transfer *to* the oLF at 50 J $ml^{-1}$ of the E4 scenario. For E3 no explicit shift is noticeable.

Cumulative data of relative net SOC release in E0, E1 and E4 are pictured in Fig. 2. Compared to the control, E1 data show a reduction in SOC release over all energy levels. The E4 treatment exhibit an increasing net SOC release. That data illustrate a reduced aggregate stability of the E4 samples compared with the control, whereas the aggregate

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stability is increased in the E1 scenario. The lower aggregate stability is indicated by a steeper gradient and results on average in an additional final C release of about 2.2% in the E4 scenario compared with the control. In the E1 scenario, the increased aggregate stability is related to an additional C bonding of about 3.3%.

### 3.2 DNA release

The relative DNA release after enzymatic treatment, as pictured with the variants E0, E1 and E4 in Fig. 3, is defined as the ratio of extracted DNA from suspended bacterial cells ($DNA_{susp}$) to the sum of DNA extracted from suspended and sessile bacterial cells ($DNA_{\Sigma}$) multiplied by 100. It is increased by about 3.5% to a value of 5.5% in the E4 scenario in comparison to the control. The E1 variant has no increase compared to the control. Inside each variant, duplicates of E0, E1 and E4 are homogeneous.

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## 4 Discussion

In our spatial model, the biofilm bridges gaps between primary particles, connects them and builds a restructured pore system inside the aggregate (Fig. 4). The pore system has influence on physical properties like aggregate stability and permeability (Taylor and Jaffé, 1990). The applied enzyme treatment leads to a transport of α-glucosidase, β-galactosidase, DNAse and lipase into the unsaturated pore space. Subsequently enzymes diffuse into the biofilm matrix, where structural components like polysaccharides, eDNA and lipids are digested as approved for diverse enzymes and enzyme targets in ecological and medical studies (Böckelmann et al., 2003; Walker et al., 2007). As macromolecular biofilm components yield EPS as a viscoelastic structure (Sutherland, 2001), their digestion causes a loss in EPS viscosity and thereby should reduce aggregate stability. The effect is expected to grow with increasing enzyme activity until the whole EPS matrix is dispersed.

The scenarios E2 and E3 show no difference to the control at any energy level and are not further discussed.

In accordance with the model, the relative SOC releases of scenario E4 and control (Fig. 1) indicate a shift of SOC from stable (sediment) to more fragile binding patterns (oLF). The E4 scenario shows an increased relative SOC release of +2.2 % at 50 J $ml^{-1}$ compared to the control, but at 0 J $ml^{-1}$, 100 J $ml^{-1}$ and 150 J $ml^{-1}$ its release is similar to the mean of the other treatments.

The relation of SOC release and enzymatic biofilm digestion is enforced by the comparison of bacterial DNA releases between treatments (Fig. 3), which indicates that applied enzymes de facto are targeting biofilm components: The E4 scenario shows an additional DNA release of 3.5% compared with the control. However, considering that most of the soil bacteria are expected to live in biofilms (Davey and O'toole, 2000), the total DNA release of only 5.5% in the E4 scenario is too low for total biofilm digestion. Hence, the loss in aggregate stability and the related SOC release of E4 compared to the control only results from a partial soil biofilm detachment. The incomplete biofilm digestion suggests, that the influence of biofilms on aggregate stability is larger than demonstrated in scenario E4.

The incomplete detachment under application of the 9,538-fold of the E1 enzyme activity is still caused ambiguously. Slow enzyme diffusion may be one reason: (Macro)molecules

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penetrate biofilms or interfere with EPS components depending on molecular size, charge and biofilm structure (Stewart, 1998; Lieleg and Ribbeck, 2011). In contrast, enzyme activities of natural soils suggests that the low efficiency is not exclusively caused by a diffusion rate insufficient for total biofilm penetration within 1 hour of incubation: The enzyme activities in coarse mineral soils span concentrations in the magnitude of the scenarios E1 to E2 for α-glucosidase, E1 to E3 for β-galactosidase and of the scenario E3 for lipase (Cooper and Morgan, 1981; Eivazi and Tabatabai, 1988; Margesin et al., 1999; Acosta-Martinez and Tabatabai, 2000; Margesin et al., 2000). That is 2 to 3 orders of magnitude over the activity calculated for EPS digestion deduced from $C_{mik}$ of the used soil sample. A natural soil EPS being continuously exposed to that enzyme activity, would be digested after a certain time, if only protected by low diffusion rates. Therefore, additional mechanisms, e.g. an equilibrium between biofilm production and decomposition, which is unbalanced by the E4 enzyme activity, should be point of future research.

No difference in fLF carbon release between all treatments is observed, which also reinforces the assumption of incomplete biofilm digestion. A total biofilm detachment should have lead to an additional fLF release due to detachment of parts of the oLF.

The increase of the relative SOC release in the E4 scenario is predominantly related to an equally lower C content of the sediment but no decrease in the 100 J $ml^{-1}$ and 150 J $ml^{-1}$ fractions. That points to a strong (>150 J $ml^{-1}$) intra-aggregate fixation of POM due to enzyme targets, which is weakened by enzymatic treatment. However, opposite reallocation of POM between fractions due to converse physico-chemical effects can only be observed in sum. Therefore shifts are depicted as net C transfer between stability fractions.

In contrast to scenario E4, scenario E1 shows a stabilization indicated by -2.8 % of relative SOC release at 50 J $ml^{-1}$, whereas the DNA release remains unchanged compared to the control. The stabilization, shown by the transfer of oLF from the 50 J $ml^{-1}$ level to the sediment, cannot be explained by the model (Fig. 4). That phenomenon suggests a stabilizing effect of low enzyme activities on soil aggregates in the lower end of the scale of natural soil enzyme activities.

Cumulation of LF carbon release over all energy level clarifies the alteration of soil aggregate stability after treatment: The mean aggregate stability of E4 decreases in comparison to the control and the soil aggregates are destabilized even at low mechanical

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stress (50 J $ml^{-1}$). The E4 scenario only caused an additional LF carbon release of +2.2% compared to the control, wich is entirely related to the SOC release at 50 J $ml^{-1}$. In the E1 scenario the stabilization is indicated by an additional cumulative SOC release of -3.3% compared to the control.

Although our results give a qualitative evidence for the influence of biofilms on aggregate stability, in face of an incomplete biofilm detachment, results have to be recognized with restrictions to full quantifiability: The enzyme concentration hypothetically needed to disperse the whole soil sample EPS matrix depends on diverse boundary conditions like the concentration of enzyme targets, environmental conditions such as pH, temperature as well as ion activity and delay factors such as low diffusion, kinetic influence or metabolization of enzymes by soil organisms. Released organic cytoplasm molecules of lysed cells can be excluded to be an additional enzyme target due to their low concentration. On the other hand, enzyme specificity to EPS targets in face of the organic soil matrix is unbeknown. Underlying enzyme kinetics were measured by the producer using pure targets for unit definition, while biofilm targets are much more diverse and soil matrix could interfere. Regarding DNA release measurement as well, data are semi-quantitative, since quantification of the detachment effect is limited by a potential adherence of detached cells to soil particles after washing (Absolom et al., 1983; Li and Logan, 2004). Thus, cell release could be underestimated as biofilm detachment increases.

Most of this restrictions are owed to the high complexity of the soil ecosystem. Considering a 9,538-fold of the E1 enzyme activity calculated from actual soil biomass to remove approximately 5.5% of the biofilm and no increase in fLF release, the pooled influence of the disregarded boundary conditions on enzymatic detachment efficiency is large.

Nonetheless, these results give insight in fundamental processes underlying aggregate stability. Loss of aggregate stability coupled with increased bacterial DNA release after treatment with high enzyme concentrations strongly underpin the assumption of biofilm as a stabilization agent of soil aggregates. Aggregate stability is influenced by the digestion of EPS components. Adapting this relation to natural soil ecosystems, a change of biofilm composition due to a shift in microbial population structure could alter soil aggregate stability. On macro-scale this could affect soil compactibility, erodibility, water transport, retention and aeration regime, rooting depth and the recalcitrance of soil organic carbon.

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## 5 Conclusions

Although the chosen enzyme activities weren't sufficient for total biofilm detachment and effectivity of enzymatic biofilm detachment is limited by soil system properties, experimental results show that extracellular polymeric substance (EPS) contributes to occlusion and attachment of particulate organic matter (POM) in soil aggregates and thereby enhances aggregate stability: Application of a highly concentrated mix of α-glucosidase, β-galactosidase, DNAse and lipase is related to a shift of POM from a stable to a more fragile binding structure, but not to an increase in fLF release. The pattern of measured soil organic carbon (SOC) release points to a strong (>150 J $ml^{-1}$) intra-aggregate fixation of POM by enzyme targets. The bacterial DNA release after enzyme treatment was used to identify EPS components as an actual enzyme target. It was shown that biofilms are a factor of aggregate stability. A loss of biofilm integrity could therefore cause a detachment of soil organic matter, not only in the laboratory but also in natural soil ecosystems and agricultural soils and invites to behold soil microbial communities as a factor of sustainable land use.

### *Author contribution*

The experiments were designed, carried out and data were evaluated by F. Büks. The manuscript was prepared by F. Büks with contributions from M. Kaupenjohann.

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***Data availability***

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## *Tables*

**Table 1:** Concentrations and molar masses of biofilm stabilizing macromolecules (polysaccharides=PS, eDNA, lipids and proteins) in different environments. Values generated from different sources are labeled with (*).

| Conc. | Proportion | Molar mass | Comment | Reference |
|---|---|---|---|---|
| µg (g soil)$^{-1}$ | µg (100 µg EPS)$^{-1}$ | Da | | |
| *PS* | | | | |
| 169 | | | µg g$^{-1}$ bare fallow | [Redmile-Gordon et al., 2014] |
| 401 | | | µg g$^{-1}$ grassland | [Redmile-Gordon et al., 2014] |
| | 95 % | | % of EPS dry-mass* | [More et al., 2014] |
| | 40-95 % | | % of EPS dry-mass* | [Pal and Paul, 2008] |
| | | $2 \cdot 10^6$ | | [Chenu and Roberson, 1996] |
| | | $0.5\text{-}2 \cdot 10^6$ | | [Flemming and Wingender, 2010] |
| *eDNA* | | | | |
| 2.2-41.1 | | | µg g$^{-1}$ forest soil | [Agnelli et al., 2004] |
| 0.08 | | | µg g$^{-1}$ Luvisol | [Niemeyer and Gessler, 2002] |
| 1.95 | | | µg g$^{-1}$ forest podzol | [Niemeyer and Gessler, 2002] |
| 0.03-200 | | | µg g$^{-1}$ unnamed soil | [Pietramellara et al., 2009] |
| | 10 % | | % EPS dry-mass* | [More et al., 2014] |
| | | $7.75 \cdot 10^4\text{-}2.32 \cdot 10^7$ | estuarine and oceanic environments | [DeFlaun et al., 1987] |
| *Lipids* | | | | |
| | 10 % | | % of EPS dry-mass* | [More et al., 2014] |
| | | 750-1500 | | [Abröll and Munk, 2008] |
| *Proteins* | | | | |
| 43 | | | µg g$^{-1}$ bare fallow | [Redmile-Gordon et al., 2014] |
| 163 | | | µg g$^{-1}$ grassland | [Redmile-Gordon et al., 2014] |
| | < 75 % | | % of *Ps. Putida* biofilm | [Griebe and Nielson, 2000] |
| | 60 % | | % EPS dry-mass* | [More et al., 2014] |

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**Table 2:** Variables used for the calculation of enzyme Units needed for biofilm target decomposition and scenario parameters shared by all variants, [a] More et al., 2014; [b] Pal and Paul, 2008; [c] Flemming and Wingender, 2010; [d] Abröll and Munk, 2008; [e] DeFlaun et al., 1987.

| | | |
|---|---|---|
| $c_{cell}$ | [µg $g^{-1}$] | bacterial dry mass per g dry soil |
| $q$ | [-] | enzyme concentration multiplier |
| $\xi_{EPS}^{max}$ | [-] | maximum ratio of EPS dry mass per total biofilm dry mass ( $\xi_{EPS}^{max}=0.9^{[a]}$ ) |
| $\xi_{target}^{max}$ | [-] | maximum ratio of enzyme target per EPS dry mass ( $\xi_{polysaccharides}^{max}=0.95^{[b]}$ , $\xi_{lipids}^{max}=0.1^{[a]}$ and $\xi_{eDNA}^{max}=0.1^{[a]}$ ) |
| $m_{sample}$ | [g] | sample mass |
| $\xi_{cell}^{min}$ | [-] | minimum ratio of bacterial dry mass per total biofilm dry mass ( $\xi_{cell}^{min}=0.1^{[a]}$ ) |
| $M_{target}^{min}$ | [µg µmol$^{-1}$] | minimum molar mass of enzyme target ( $M_{polysaccharides}^{min}=0.5\,x\,10^{6[c]}$ , $M_{polysaccharides}^{min}=700^{[d]}$ , $M_{eDNA}^{min}=7.75\,x\,10^{4[e]}$ ) |
| $t$ | [min] | incubation time |

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**Table 3:** Specific scenario parameters of the variants E0, E1, E2, E3 and E4.

| | | E0 | E1 | E2 | E3 | E4 |
|---|---|---|---|---|---|---|
| $c_{cell}$ | [µg g$^{-1}$ dry soil] | 352 | 352 | 1191 | 1191 | 1191 |
| $q$ | [-] | 1 | 1 | 1 | 100 | 2,820 |
| $U^{max}_{a-glucosidase}$ | [U g$^{-1}$ dry soil] | 0.00000 | 0.00010 | 0.00034 | 0.03393 | 0.95683 |
| $U^{max}_{b-galactosidases}$ | [U g$^{-1}$ dry soil] | 0.00000 | 0.00010 | 0.00034 | 0.03393 | 0.95683 |
| $U^{max}_{lipids}$ | [U g$^{-1}$ dry soil] | 0.00000 | 0.00754 | 0.02551 | 2.55102 | 71.93876 |
| $U^{max}_{eDNA}$ | [U g$^{-1}$ dry soil] | 0.00000 | 0.00007 | 0.00023 | 0.02304 | 0.64973 |

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***Figures***

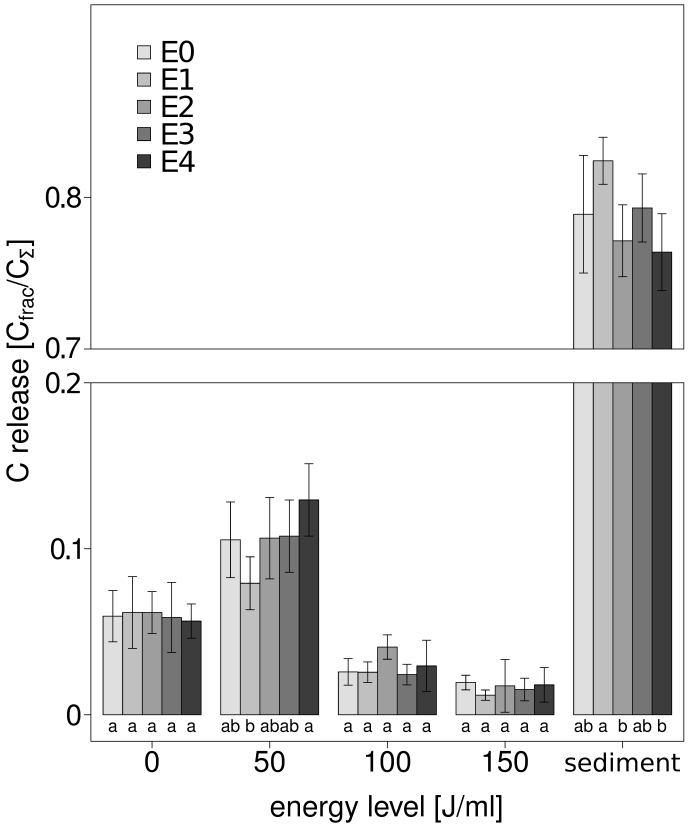


**Fig. 1:** Relative SOC release of treatments (E0, E1, E2, E3, E4) at different energy levels (0, 50, 100, 150 J $ml^{-1}$, sediment), illustrated by Tukey test characters (a, ab, b).

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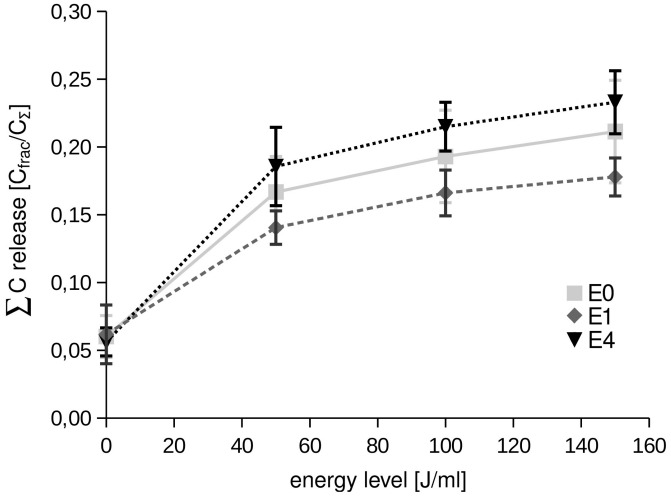


**Fig. 2:** Cumulative data of relative net SOC release mean values for the treatments E0, E1 and E4 as a function of applied energy (standard deviations not shown).

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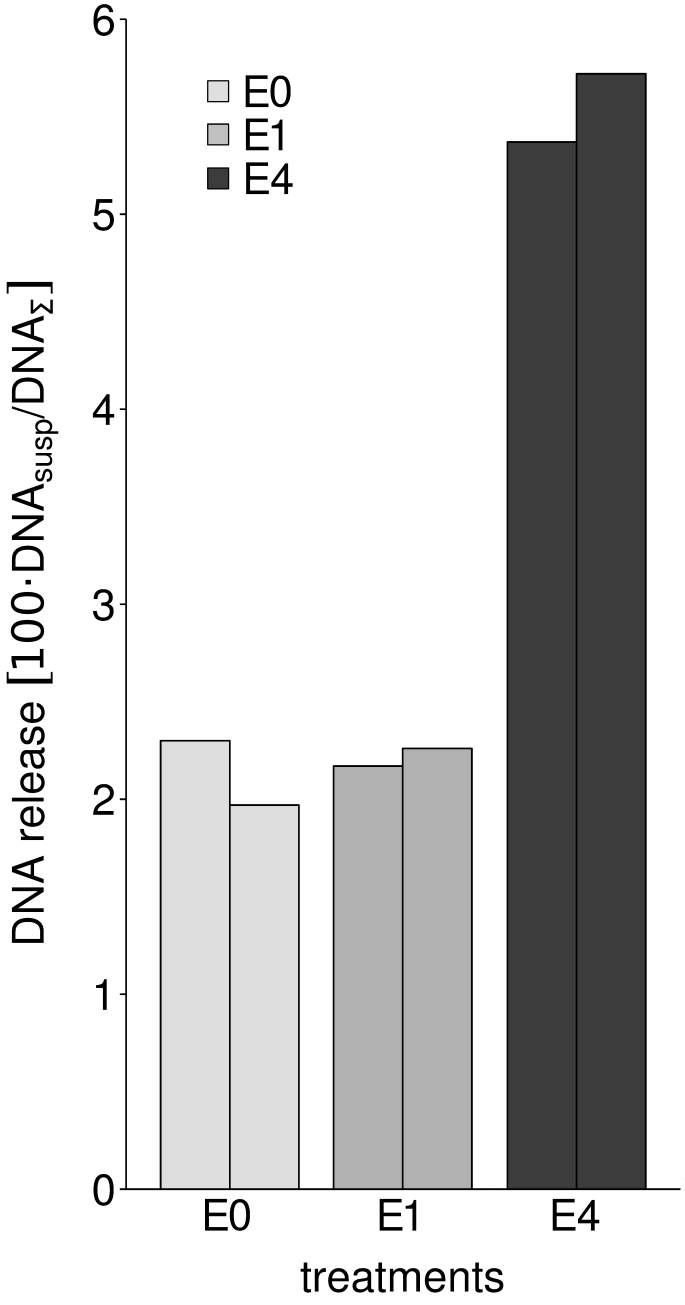


**Fig. 3:** Relative bacterial DNA release from soil aggregates after treatments E0, E1, and E4 defined as 100x ratio of bacterial DNA from suspended cells ($DNA_{susp}$) to total bacterial DNA from suspended and sessile cells ($DNA_{\Sigma}$).

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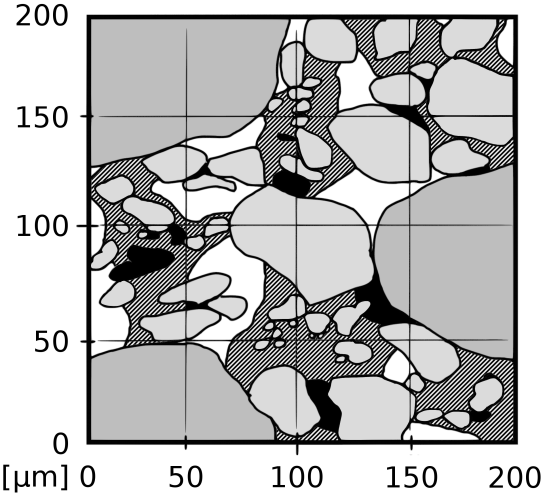


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**Fig. 4:** Proposed model of biofilm structure in a soil aggregate: Sand and silt (both grey) and organic particles (black) stick together by physico-chemical interactions and are bridged by EPS (striped), which additionally stabilizes the soil aggregate structure and the pore space (white).