Minor revision

Enzymatic biofilm digestion in soil aggregates facilitates the release of particulate organic matter by sonication

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Dear Mr Redmile-Gordon.

Thank you again for your helpful suggestions. Please pardon the puzzling corrections of my last revision and let me know if I missed a point.
Best regards,

Frederick Büks

Contents

1) “Whereas E4 matches the forecast of releasing more POM than the control, scenario E1 shows a reduced release by -2.8% and the DNA release remains unchanged compared to the control. This decrease in the 50 J ml^{-1} fraction is related to an increase in the sediment fraction and cannot be explained by the model (Fig. 1).” deleted. Next sentence: “ Probably this could be explained ...”

2 and 3) Lines 429-31 were changed to “Enzyme C in E1 to E4 could be used as microbial C source. The addition of C increases the C/N ratio and has been shown to lead to soil aggregate stabilization (Watts et al., 2005; Tang et al., 2011).” and moved behind “... increasing enzyme addition” (Line 435).

4) “in contrast, the retention of ...” and “However ...” were deleted.

Also) Written as a separate paragraph: “Enzyme C in E1 to E4 could be used as microbial C source. The addition of C increases the C/N ratio and has been shown to lead to soil aggregate stabilization (Watts et al., 2005; Tang et al., 2011). Decay rates of enzymes in soil are unknown but needed for a more accurate estimation of enzyme C as a fast energy and carbon source.”

Revised manuscript

4 Discussion

We found that increasing the quantity of enzymes applied to aggregates led to increased release of LF-SOC when aggregates were sonicated. This detachment is explained by the following mechanism: The enzyme mix flows into the unsaturated pore space. From there α-glucosidase, β-galactosidase, DNAse and lipase 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). We propose a simple spacial model to explain the observed findings: The biofilm bridges gaps between organic and mineral primary particles, connects them in addition to other physico-chemical bondings and builds a restructured pore system inside the aggregate (Fig. 1). As macromolecular biofilm components yield EPS as a viscoelastic structure (Sutherland, 2001), their digestion causes a loss in EPS viscosity and thereby should reduce forces involved in the occlusion of POM. The effect is expected to grow with increasing enzyme activity until the whole EPS matrix is dispersed.

In the following, LF-SOC is interpreted as SOC from released POM, since the share of both adsorbed DOM and colloids on captured dry mass is considered to be negligible after SPT treatment. Furthermore, LF-SOC transferred from the sediment fraction to light fractions due to enzymatic treatment is also interpreted as POM, as in contrast mineral associated organic matter of the HF is not assumed to be extractable at the applied energies (Cerli et al., 2012).

In accordance with the model, measured oLF-SOC releases indicate a trend for increased POM release with increasing enzyme addition (Fig. 2). The E4 scenario shows that relative oLF-SOC release increased by 63% (5% of C_{Σ}) compared to E1 at 50 J ml^{-1}, but its release is similar to the mean of the other treatments at 0 J ml^{-1}, 100 J ml^{-1} and 150 J ml^{-1}. Noticeable deviations of E1 and E4 from the control do not match the usual significance criteria ($p<0.05$). However, the increase of the relative oLF-SOC release in the E4 scenario compared to the control 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 (oLF >150 J ml^{-1}) intra-aggregate fixation of POM due to enzyme targets, which is weakened by enzymatic treatment.

The relation of LF-SOC release with enzymatic biofilm digestion is supported by the comparison of bacterial DNA releases between the treatments (Fig. 3). This indicates that applied enzymes are targeting biofilm components and release bacterial cells: The E4 scenario shows EPS digestion and additional cell release leading to a doubled relative

DNA release compared with the control and E1. 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 5.6% in the E4 scenario is too low for total biofilm digestion. Hence, biofilm detachment caused by E4 is still likely to be incomplete and the increased oLF-SOC release of E4 only results from a partial soil biofilm detachment. We conclude a slight influence of enzymatic treatment on the occlusion of POM at enzyme concentrations exceeding natural concentrations. This conforms to results of Böckelmann et al. (2003), which indicate that a treatment with enzyme concentrations of near that of E4 is sufficient to destabilize biofilms within 1 hour.

The incomplete biofilm detachment can be explained by the reduction of enzyme activity due to interaction with the soil matrix. Based on our calculations enzyme concentrations of mix E1 should have been sufficient for total biofilm digestion within time of application (1h) – as far as there are no other factors reducing enzyme efficiency. As surveys of natural soils show enzyme concentrations up to mix E3 (Cooper and Morgan, 1981; Eivazi and Tabatabai, 1988; Margesin et al., 1999; Acosta-Martinez and Tabatabai, 2000; Margesin et al., 2000), such factors might be reasonably assumed. After addition to the soil sample, enzymes must enter the EPS matrix by diffusion. Therefore parts of the enzymes probably do not reach the biofilm due to inhibited diffusion. Beside diffusion, sorption and decomposition could play a major role in reducing enzyme efficiency. Whereas turn-over rates of soil enzymes are not yet assessed, extended stabilization of active enzymes over time on soil mineral and organic surfaces is reported (Burns et al., 2013). This mechanism could explain immobilization of enzymes off the biofilm and high measured soil enzyme concentrations from literature in face of still existing biofilms. After penetration of biofilms (macro)molecules interfere with EPS components depending on molecular size, charge and biofilm structure (Stewart, 1998; Lieleg and Ribbeck, 2011) which is strongly influencing decay rates of enzymes. Due to these boundary conditions, quantification of the relation of enzyme concentration and POM carbon release was not possible in this work.

The trend for increased POM release with increasing enzyme addition was only broken by the control treatment. Probably this could be explained by pre-incubation of soil aggregates given 0.2 mM NH_4NO_3 and further addition of NH_4NO_3 with enzyme application: Redmile-Gordon et al. (2015) proposed that low C/N ratios of substrates available to soil microorganisms reduce cell specific EPS production rates, and may trigger microbial consumption of EPS to acquire C for cell-growth, which could weaken the biofilm. The observations leading to this proposed dynamic were also found by addition of

NH_4NO_3. In the present study, NH_4NO_3 was applied with all treatments including the control (which also received no C from enzyme provision). The lowest C/N ratio in the control soils may itself have sustained EPS consumption and repressed reconstruction of the EPS, contributing to the higher than expected release of POM from the control soil with sonication at 50 J mL^{-1} and the break in the trend for increasing POM release with increasing enzyme addition.

Enzyme C in E1 to E4 could be used as microbial C source. The addition of C increases the C/N ratio and has been shown to lead to soil aggregate stabilization (Watts et al., 2005; Tang et al., 2011). Decay rates of enzymes in soil are unknown but needed for a more accurate estimation of enzyme C as a fast energy and carbon source.

Under certain conditions POM carbon release is indicative for soil aggregate stability. Generally, aggregate stability is characterized by determining the reduction in aggregate size after application of mechanical force. The commonly used methods are dry and wet sieving. However, the destruction of soil aggregates by ultrasonication has an advantage over these methods, which is the quantification of the applied energy (North, 1976). It is used for studying reduction of aggregate size (Imeson and Vis, 1984) as well as detachment of occluded POM carbon (Golchin et al., 1994). Kaiser and Berhe (2014) reviewed 15 studies using ultrasonication of soil aggregates in consideration of its destructiveness to the soil mineral matrix and occluded POM. They found destruction of POM at applied energy levels >60 J/ml, destruction of sand-sized primary particles at >710 J/ml and of smaller mineral particles at even higher energy levels. We used this method of gentle POM detachment from soil aggregates to measure the oLF-SOC release as a result of mechanical force and linked it to aggregate stability. Since Cerli et al. (2012) have shown that the release of free and occluded light fractions strongly depends on soil properties like mineralogy, POM content, composition and distribution, this method is restricted to comparison of soils being similar in these properties. Having regard to this restriction, the trend for increase of oLF-SOC release over increasing enzyme additions demonstrates an alteration of soil aggregate stability.

Although our results give a slight evidence for the influence of biofilms on aggregate stability, they have to be recognized with restrictions to full quantifiability: (1) 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. (2) 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. (3) Alternative enzyme targets might be reasonably assumed within the complex chemism of the soil matrix. 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. (4) The decrease of extracted POM mass due to biofilm erasement from surfaces is suggested to be low, but could cause underestimation of POM release especially in scenario E4. In contrast, a direct contribution of enzyme C to the POM carbon release can be refused. Even in case of complete adsorption to the POM of only one fraction, the highest enzyme concentration (E4) would result in additional 13.5 µg enzyme /g dry soil being <0.4% of the smallest extracted POM fraction (Table 3). (5) 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.

Many of these uncertainties are owed to the high complexity of the soil system. Enzymes were applied in concentrations four orders of magnitude higher than calculated from actual C_{mic} and even 1-2 orders of magnitude higher than values from literature. Incomplete biofilm removal indicated by the release of maximum 5.5% DNA from the soil matrix may suggest that the pooled influence of the disregarded boundary conditions on enzymatic detachment efficiency is large.

However, these results give a first though still vague insight in fundamental processes underlying POM occlusion. A slight release of occluded POM coupled with increased bacterial DNA release after treatment with high enzyme concentrations underpin the assumption that biofilm is involved in POM occlusion being a stabilizing agent of soil aggregates as proposed in a review by Or et al. (2007). The apparent increase of POM carbon release caused by the digestion of EPS components suggests biofilm relevance in soil ecosystems e.g. in terms of soil-aggregate related functions like soil water and C dynamics, mechanical stability as well as rootability. However, the statistical power of this introductory work is low and a more quantitative analysis of the relation of enzymatic EPS detachment and POM release would require deeper knowledge of enzyme dynamics in soil, more replicate samples, additional enzyme concentrations and probably inclusion of soils from different land use. However, this was beyond the scope of the present study.

Former version

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