

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

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Final response to Marc Redmile-Gordon

Dear Mr Redmile-Gordon,
first I would like to express my sincere thanks to you for reviewing, especially for your detailed and very helpful suggestions and your forbearance concerning grammatical errors.

Title

Line 1: Title should change, I suggest: 'Enzymatic biofilm digestion in soil aggregates facilitates the release of particulate organic matter (POM) by sonication'.

We changed the title as suggested. Thank you very much.

General corrections

Lines 181, 200, 263, 264, 265, 266, 270, 272, 273, 280, 339, 340, 343, 371, 379, 426 and elsewhere: Renaming of SOC.

As (1) C is the actual measure and (2) SOC involves DOC, which is rejected during POM extraction, POM and SOC are not suitable to term the C release from aggregates. Instead, "particulate organic carbon" (POC) will be used. This also includes organic molecules, already adsorbed on the HF after ultrasonic treatment. When describing the extracted material as a whole, POM will be used.

Lines 13, 14, 15, 26, 37, 39, 46, 49, 78, 79, 80, 83, 89, 92, 93, 96, 101, 105, 110, 112, 123, 138, 143, 144, 146, 147, 192, 210, 265, 280, 281, 283, 342, 343, 344, 406, 410, 413: Diverse suggestions to improve orthography, grammar, lucidity and scientific notification.

All proposals are included. Thanks a lot.

Abstract

Line 24: delete 'which preserves aggregate structure'.

"... which preserves aggregate structure, ..." was removed, as additional influence on binding mechanisms such as surface charge of POM cannot be ruled out.

Line 30: This is overly confident and not quite accurate. Is it not true that enzymatic digestion of EPS polymers may have increased the abundance of EPS fragments released upon sonication? Therefore,

remove 'our results confirm, that EPS stabilises 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' and replace with 'our results suggest that EPS stabilises intra-aggregate particulate organic matter (POM) within soil aggregates'.

The samples have a C_{mic} of 0.352 mg g^{-1} dry soil aggregates and a C_{org} of 8.7 mg g^{-1} dry soil. total POC release amounts to 1.8 mg g^{-1} dry soil for E0 and 1.98 mg g^{-1} dry soil for E4 – the difference (0.18 mg g^{-1} dry soil) is half the C_{mic} . Therefore it is (mathematically) possible, that the whole difference in POC release is caused by release of biofilm fragments. The real share is unknown, but the small share of released bacterial DNA as well as visibly increased dark POM release in E4 after sonication reinforce additional non-biofilm POM release. However, we choose the more careful statement as you suggested "our results suggest that EPS stabilises intra-aggregate particulate organic matter (POM) within soil aggregates" and will revisit this in the discussion part.

Introduction

Lines 61-63: awkward sentence, please rephrase.

Done: "In addition, carbonates and phosphates as well as microbial precipitates force up aggregation."

Line 82: replace 'biofilm forming species and habitats:' with 'community composition and environmental cues:'

Sounds much better. Thank you and done.

Line 108: Unsubstantiated statement which leaves the reader wondering 'why'. I suspect the authors are drawing on the rationale presented Redmile-Gordon et al. (2014) and suggest this is expanded upon for clarity and to help build justification. Suggest the authors replace 'That is mainly due to methodological reasons' with 'This is mainly due to methodological reasons. For example, Tang et al. (2011) found no link between bacterial EPS extracted using sulphuric acid and aggregate stability. Redmile Gordon et al (2014) subsequently found in a comparison study that the techniques previously used to measure extracellular polysaccharide in soil co-extracted large quantities of 'random' soil organic matter which confounded estimates of EPS production."

I will add "Though [Tang et al. \(2011\)](#) showed a significant contribution of bacterial growth on aggregate stability, the observations could not definitely be attributed to soil microbial exopolysaccharide production. [Redmile-Gordon et al. \(2014\)](#) subsequently found that the techniques previously used to measure extracellular polysaccharide in soil co-extracted large quantities of 'random' soil organic matter which confounded estimates of EPS production."

Material and Methods

Lines 141-142: This is not a method to estimate soil microbial biomass, this is respiration, correct accordingly.

"To estimate the soil microbial biomass" refers to the whole paragraph. For clarification, the paragraph will be reshaped to "To estimate the soil microbial biomass, first $8 \times 10 \text{ g}$ 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. Then, based on DIN EN ISO 14240-2 ..."

Lines 163-172: This section takes some time to understand. Insert "sufficient enzymes were provided to digest the EPS content expected in five scenarios (E0 to E4)"

Line 165: "each" added before "with highest need of enzymatic units for the total biofilm detachment".

Line: 172: “five scenarios were design” is replaced with “sufficient enzymes were provided to digest the EPS content expected in five scenarios:”

Lines 181/193: ... e.g. Cerli et al 2012 do not claim this method quantifies aggregate stability
Cerli et al. (2012) was replaced by [Golchin et al. \(1994\)](#) as prime reference. Cerli et al. (2012) will appear in the discussion about light fraction release as indicator of aggregate stability.

Line 190: *Why for 30 min? To allow NaPT diffusion?*
“... to allow SPT diffusion into the aggregates” will be added.

Line 195: *50 J ml⁻¹ given over what time period?*
Time periods depend on the weight of sample+SPT solution and fluctuate around 1 min 15 sec.

Line 217: *What volume of wash was used as an equivalent for the mass of soil stipulated in the FastDNATM spin kit soil manual? (Can it really be used to extract DNA from a dilute wash and compare with soil?)*
[FastDNA™ SPIN KIT](#) (used for for liquid samples of 200 µl and pure cultures) and [FastDNA™ SPIN Kit for Soil](#) (normally used with “Up to 500 mg of soil sample” and for complicated samples) only differ

(1) in the first buffer,
(2) the point of time for the application of protein precipitation solution (PPS) and
(3) in the last incubation procedure (incubation in DES solution for 5 minutes in a heat block at 55°C after addition of SEWS-M instead of incubation at room temperature before addition of DES). Both methods are very similar. As we did a qualitative comparison of DNA release, variance of DNA release between methods is of minor importance.

Results

Line 268: *Move ‘data are shown as mean values and standard deviations of five parallels’ to figure caption*
Done.

Lines 274-279: *Incorrect (and potentially misleading) presentation of results. Suggest as replacement: “there was no increase or decrease relative to the control, however, there was a trend for increased POM release with increasing enzyme addition, and the difference between the lowest enzyme addition and the highest was statistically significant as indicated by the Tukey test. This trend was only broken by the control treatment (given no enzymes)” // Unnecessary and confusing statement, we can see the standard deviation and Tukey test results on the figure, better to remove the statement. // Potentially misleading statement, yes, E2 and E3 have no difference compared to the control, but neither do E1 or E4.*

Thank you very much for the proposal. We decided to desist from a specific significance level in the revision of this paper. A p-value of 0.05 is a convention underpinned only by practical but not scientific reason. E.g. visible differences are leveled by using it: E0, E2 and E3 appear to have similar mean values and variance, whereas E1 (p=0.6) and E4 (p=0.15) show visible differences to the control at 50 J/ml. Whereas E1 is not explained by the model and have to be discussed, E4 matches the forecast and is underpinned by the increase in bacterial cell release. That has to be carefully discussed. “There was a trend for increased POC release with increasing enzyme addition, and this trend was only broken by the control treatment (E0, given no enzymes).”

Lines 284-288: *There has been no physical transfer of organic matter between these analytical pools. A reduced aggregate stability may have for example, or increased release of biofilm fragments retained on the 1.5 µ glass filter, but this is a matter for discussion. It might be more useful to say here that it is reassuring that the SOC remaining in the sediment reflects what would be expected given the quantities extracted at 50*

J... but of course it would (because you present relative fractions in preference to absolute concentrations). I am struggling to find a reason to retain this section. I think it better to delete lines.

Our intention was to express that nearly the whole net POC differences E1-E0 and E4-E0 are related to variations in the HF, but not in fLF, oLF(100) and oLF(150). That will be included in lines 274-297.

Line 289: and Figure 2 These results have already been presented, it is not clear exactly what compounded estimate of error is being given, and besides, data were already presented in figure 1. Remove Figure 2.

Line 290: This has already been presented, that one can add the non-significant results to the significant, and finds the same thing is nothing surprising or worthy of comment. Delete. **Line 291:** Clumsy sentence and repetition: delete first sentence. **And lines 291-293:** Released POM data may be evidence of this, and may not be - this is a matter for the discussion. Delete these lines.

Lines 289-293 will be deleted. Fig. 2 will be changed to mg POC /g dry soil and shortly described.

Lines 293-296: Delete section starting "The lower aggregate stability is indicated by a steeper gradient and on average in an...". Replace with "The addition of the highest enzyme concentration (E4) caused the release of about 40% more POM by mild sonication (50J ml⁻¹) than occurred with the addition of the lowest concentration (E1). This was statistically significant at (p <0.05)." end of section.

Thank you very much. "At 50 J ml⁻¹ ultrasonic treatment results in an additional POC release of about 10% more POC compared to the control, whereas POC release is reduced by -18% in E1. The addition of the highest enzyme concentration (E4) caused the release of about 1/3 more POM by mild sonication (50 J ml⁻¹) than occurred with the addition of the lowest concentration (E1) (p=0.003).

Line 302: *In contrast here I think the relative increase in DNA release is a little understated. Yes it is useful to also give it as a percentage of total DNA extracted from the soil as you have done (Figure 3 - now rename to Figure 2), but perhaps in line 302 replace text "it is increased by about 3.5% to a value of 5.5% in the E4 scenario in comparison to the control" with "While there was no difference in DNA concentrations suspended in the wash of control and low enzyme additions, treatment E4 caused an increase to more than double the DNA content of either E0 or E1."*

Thank you. Replaced by: "While there was no difference in relative DNA release in the wash of control and low enzyme additions, treatment E4 caused an increase to more than double the DNA content of either E0 or E1, which amounts to 5.6% of total DNA".

Discussion

Lines 324-335: First paragraph disorganised: it is an unpleasant jump to the model in the first sentence. Build up to it. It would be smoother if begin with the main result result, followed by your description of enzyme transport into the unsaturated pore space and discussion of others work E.g. "We found that increasing the quantity of enzymes applied to aggregates led to increased release of POM when aggregates were sonicated. Then describe the pore system (currently lines 325 326), then give your model of explanation "we present a model to explain the observed findings ..."

Thank you. First paragraph was replaced by:

"We found that increasing the quantity of enzymes applied to aggregates led to increased release of POC when aggregates were sonicated. This detachment is explained by the transport of α -glucosidase, β -galactosidase, DNase and lipase into the unsaturated pore space. Consequently 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). We utilize a simple spacial model to explain the observed findings: The biofilm bridges gaps between primary particles, connects them and builds a restructured pore system inside the aggregate (Fig. 4). 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.”

Lines 336-337: *Delete the discussion of what is not being discussed.*

Done.

Line 345: ‘de facto’ is way too strong and encourages the reader think of examples to disprove this over-confident statement. E.g. it could have been caused by cell lysis. Delete ‘de facto’.

Done.

Line 352: This is not the only possible explanation and further discussion with relevant literature is required. Might some of the C released from occluded POM and/or biofilm not have been detected in the filtered light fraction? – e.g. may have been present as smaller particulates or DOC? Also, DNA/cells/POM may not have been released without sonication. Include this. Current literature has more to offer. Add “Furthermore, we pre-incubated soils given 0.2 mM NH₄NO₃, and added further NH₄NO₃ with the enzyme application. Redmile-Gordon et al (2015) proposed that low C/N ratios of substrates available to soil microorganisms reduces cell specific EPS production rates, and may trigger microbial consumption of EPS to acquire C for cell-growth. The observations leading to this proposed dynamic were also found by addition of NH₄NO₃. In the present study, NH₄NO₃ was applied with all treatments including the control (which also received no C from enzyme provision). The resulting lowest C/N ratio in the control soils may itself have decreased 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.

We now write: “Decreased POC release in E1 could be explained by pre-incubation of soil aggregates given 0.2 mM NH₄NO₃ and further addition of NH₄NO₃ with enzyme application. [Redmile-Gordon et al. \(2015\)](#) proposed that low C/N ratios of substrates available to soil microorganisms reduces cell specific EPS production rates, and may trigger microbial consumption of EPS to acquire C for cell-growth. The observations leading to this proposed dynamic were also found by addition of NH₄NO₃. In the present study, NH₄NO₃ was applied with all treatments including the control (which also received no C from enzyme provision). The resulting lowest C/N ratio in the control soils may itself have decreased 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.”

Further “Probably high enzyme concentrations dissolve biofilm structures that remain part of the coarse POM at low enzyme treatment, which results in underestimation of E4 POC release.” was added in this paragraph.

Lines 350-352, 390: *Discussion about link between biofilm digestion and aggregate stability.*

Sentence in lines 350-352 “The incomplete biofilm digestion suggests, that the influence of biofilms on aggregate stability is larger than demonstrated in scenario E4.” shifted to a later part of discussion. Previous reference to aggregate stability is replaced by biofilm digestion/POC release context, except in the spacial model.

Line 353: *Replace “The incomplete ... ambiguously” sentence with “Nonetheless, biofilm detachment caused by E4 is still likely to be incomplete.” And continue with “Slow enzyme diffusion...”*

Line 348-350 is replaced by “Hence, biofilm detachment caused by E4 is still likely to be incomplete.” Continued with “Slow enzyme diffusion...”.

Lines 352, 356-367: *This paragraph contains some useful information that should be retained for comparison of enzyme quantities added. However, the explanation drawing on enzyme activities in natural soils is not clear and needs re-thinking and re-writing. Actually, it seems the argument is flawed. You only observed effects when you increased enzyme activities well above ‘natural’ levels so on the contrary seems to support the hypothesis that diffusion factors ARE limiting (e.g. sorption to active surfaces). Suggest you cite the excellent review by (Burns et al., 2013) (see section 3.3; page 220).*

“Based on our calculations enzyme concentrations of mix E1 should be 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; Acosta-Martinez and

Tabatabai, 2000], such factors might be reasonably assumed. This is underpinned by our results, that show the only increase in POC release in scenario E4 attended by only an incomplete cell release. After addition to the soil sample, enzymes must enter the EPS matrix by diffusion. Therefore it is assumed that 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. Due to this boundary conditions, quantification of the relation of enzyme concentration and POC release was not possible in this work, although there is a tendency for enhanced POC release.” This information will be included in lines 356-367.

Lines 368-370: *It does not reinforce this, and if it does it conflicts with your model. If your model is correct it would only be found after disruption of aggregates to release the oLF (as you observed at 50 J ml⁻¹; congruent with your model). It could also have been lost as soluble C, as mentioned above in reference to line 352 above. Delete 368 – 370.*

Correct. I referred to a POM occlusion only mediated by EPS, but in the model it seems very implausible to assume occluded POM, that is not bound by physico-chemical interactions. Deleted.

Lines 378-383: *Not statistically significant therefore remove this speculation. Statistically it is built on observations that can be reasonably expected by chance.*

Done.

Line 384 *replace ‘cumulation of LF carbon release overall energy level clarifies the alteration of soil aggregate stability’ with ‘The trend for increased of LF carbon release over increasing enzyme additions demonstrates an alteration of soil aggregate stability’.*

Thank you. Done.

Line 385 – *results repetition. And lines 386-389 Careful, you are discussing SOC (POM) release and aggregate stability as if you measured both independently, and focus drifts. I recommend you instead discuss the connection you propose (POM release being due to digestion of EPS which seems to prevent POM release by sonication alone up to 150 J ml⁻¹ – and after more effectively separated from soil minerals by 50J sonication).*

See “New line of argument” at the end of this document.

Line 395: *Good point re enzyme metabolism, although 1 hour is not a lot of time for it, it would be useful to include a reference for rapid metabolism of enzymes/proteins. Add that the large additions of enzyme-C could be used as a C-source for microbial growth which is known to stabilise soil aggregates, e.g. (Watts et al., 2005). This is why total enzyme-C added should be included in your manuscript (suggest this is added to Table 3).*

“The applied enzymes have no relevant mass input to extractable POM. Even in case of complete adsorption to POM in only one fraction, highest enzyme concentration (E4) would result in additional 13.5 µg enzyme /g dry soil being <0.4% of the smallest extracted POM fraction.

Although enzyme concentration has no influence on extracted POC, addition of enzyme-C could be used as microbial metabolic C-source which is known to lead to soil aggregate stabilization ([Watts et al., 2005](#); [Tang et al., 2011](#)). Soil turn-over rates of enzymes are not assessed ([Burns et al., 2013](#)). Fast metabolism of enzymes within 1 hour would hinder quantification of the relation of biofilm digestion and POC release by influencing aggregate stability during the experiment.” This content will be connected to point (**Line 352**).

Lines 407, 408: *better if you delete ‘a 9000 fold of the E1 enzyme activity calculated from actual soil biomass to remove approximately // suggest replace ‘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’ with*

'5.5% biofilm removal indicated by DNA measurements coupled with no increase in fLF release, may suggest that the pooled influence of the disregarded boundary conditions on enzymatic detachment efficiency is large'.

As the role of fLF C is discussed regarding lines 368-370, the paragraph is replaced with: "Most of these restrictions are owed to the high complexity of the soil ecosystem. 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. Considering maximum 5.5% biofilm removal indicated by DNA measurements may suggest that the pooled influence of the disregarded boundary conditions on enzymatic detachment efficiency is large." Orders of magnitude are still noted to illustrate the probable range of influence of the disregarded boundary conditions.

Lines 410-413: delete 'nonetheless' // replace 'Loss of aggregate stability' with 'Release of entrapped POM' // replace 'stabilisation' with 'stabilising' // Citation needed: suggest after 'stabilising agent of soil aggregates' to insert 'as discussed in a comprehensive review by Or et al. (2007)'. // Subsequent sentence, why limit to just natural ones? I suggest you replace 'Aggregate stability is influenced by the digestion of EPS components. Adapting this relation to natural soil ecosystems,'" with 'The apparent loss of aggregate stability caused by the digestion of EPS components in the present study suggests biofilm relevance in soil ecosystems.' And finish the discussion there.

Paragraph replaced with "These results give insight in fundamental processes underlying aggregate stability. Release of occluded POM coupled with increased bacterial DNA release after treatment with high enzyme concentrations underpin the assumption that biofilm is a stabilising agent of soil aggregates as discussed in a review by [Or et al. \(2007\)](#). The apparent loss of aggregate stability caused by the digestion of EPS components in the present study suggests biofilm relevance in soil ecosystems e.g. in terms of soil-aggregate related functions like soil water dynamics, mechanical stability as well as rootability."

Conclusion

Lines 414-417, 419-420, 422-423, 425, 425-427, 427, 431: Move this final part to the start of conclusions: "Our results suggest a change of biofilm composition due to a shift ..." // Already discussed, is weak, better to delete. // delete "and thereby enhances aggregate stability". Already discussed and now superseded by your two important sentences above this (first one suggested to be taken from discussion, lines 414 – 417). // Delete 'fLF' (these abstract technical distinctions are not appropriate for this statement). Continue with the condition i.e. "not to an increase in fLF release without physical disruption of aggregates by sonication." // replace SOC with POM (should already be defined) 427 delete the sentence starting "The bacterial DNA..." as discussed already; this does not withstand logical critique. // 'microbial communities' already are for various reasons, I think you mean the biofilm or EPS, EPS being relevant even when no biofilm can be observed ... suggest you replace 'communities' with 'EPS dynamics'.

New conclusion: "It was shown that EPS is a factor of aggregate stability. Our experimental results suggest that extracellular polymeric substance (EPS) contributes to occlusion and attachment of particulate organic matter (POM) in soil aggregates. The application of a highly concentrated mix of α -glucosidase, β -galactosidase, DNase and lipase is related to a detachment of POM from a stable to a more fragile binding structure, but not to an increase in POM release without physical disruption of aggregates by sonication. The pattern of measured POC release and additional bacterial DNA release points to an intra-aggregate fixation of POM by enzyme targets. A loss of EPS integrity could therefore cause a detachment of soil organic matter, not only in the laboratory but also in natural soil ecosystems. Our results further suggest that a change of biofilm composition probably due to a shift in microbial population structure may alter soil aggregate stability. On macro-scale this could affect soil compactibility, erodibility, water transport, retention and aeration regime, rooting depth and the occlusion of soil organic carbon. This, in conclusion, invites to behold soil EPS dynamics as a factor of sustainable land use."

Figures and Tables

Figure 4: edit caption – you are not showing ‘biofilm structure’ – this is ‘aggregate structure’ replace accordingly.

Caption changed to “Proposed model of aggregate structure: ...”

Table 3: Add quantity of enzyme-C added to enable judgement of substrate utilisation by soil microbial biomass.

Quantities added.

Table 3: column E0: should the q value not be zero? Otherwise why are the enzyme activities different from column E1?

Yes. Thanks.

Furthermore ...

... there are also some points I have to answer back.

Line 38: *insert ‘and’ before ‘is an integral’*

That doesn't fit in this place.

Line 56: *delete ‘.’*

There is an end of sentence and the references are related to the whole paragraph.

Line 173: *use large ‘C’ for carbon*

c in C_{cell} means “concentration”

Line 409: *Insert sentence: ‘Conversely, or in addition to the above, complete biofilm removal may have been achieved, however as the model (figure 4 – now figure 3) proposes, POM would not be released until the retaining aggregates were disrupted by disruptive physical forces such as those caused by sonication.’ (Kaiser and Berhe, 2014)*

As only 5.5% of the bacterial DNA are removed after enzymatic treatment, it seems implausible to expect complete biofilm detachment. Further, point (Lines 368-370).

New line of argument

Line of argument will be restructured in the following way (e.g. to avoid repetitions): Discussion of POM release (increase in E4, decrease in E1, tendency, p-values but no significance level) – discussion of bacterial DNA release – discussion of of the relation of both (EPS as enzyme target) – discussion of the explanatory power of (small) POM release and of its usability for aggregate stability measurement in similar soil samples – “A more quantitative analysis of the relation of enzymatic EPS detachment and POM release would require more replicate samples and probably inclusion of soils from different land use. However, this was beyond the scope of the present study.”

*Best regards,
Frederick Büks*