

Non-anonymous review offered by: Marc Redmile-Gordon (Rothamsted Research, UK)

### **General comments**

This paper presents an interesting and novel approach to cause biofilm detachment from soil minerals using enzyme digestion, followed by ultrasonic disaggregation and measurement of the particulate organic matter (POM) subsequently released. It is multidisciplinary and of broad international interest within soil and biofilm disciplines. The work represents a substantial contribution to scientific progress and fits comfortably within the scope of SOIL.

However, the manuscript is currently poorly presented and needs care. Over-emphasis and repetition of non-significant results and poor discussion detract from the valued approach and findings. Before I am able to recommend publication, there are numerous statements, assumptions and conclusions that I think need reconsideration. The discussion in particular needs substantial improvement on the version currently available online (1<sup>st</sup> April 2016). I have made several suggestions and would encourage the authors to attend to this at their earliest convenience for publication of this important manuscript.

I would be willing to conduct a repeat review of the revised version, and hope my comments are found useful.

### **Specific comments**

While most scientific questions/issues are given in line order under the 'technical corrections' section, I would initially draw attention to the following:

#### **1 - Title: *Enzymatic biofilm detachment causes a loss of aggregate stability in a sandy soil***

While this statement is probably true, the work done is not actually sufficient premise for the title. The release of POM is likely to be associated with greater disruption of aggregates, but, as I can see, aggregate stability *per se* was not measured directly, and the release of POM may have been caused by another factor(s). Since aggregate stability may or may not have been affected I suggest a more conservative replacement title: 'Enzymatic biofilm digestion in soil aggregates facilitates the release of particulate organic matter (POM) by sonication'. I think this title would also be more informative, useful, and accurate.

Accordingly, at line 264 I suggest heading renamed to 'Intra-aggregate POM' because 'Aggregate stability' suggests physical stabilities of aggregate size classes (not measured). There are many methods to quantify 'aggregate stability', none of which are described or referenced in the methods

of the manuscript. This is one example of rather too many leaps or assumptions at present that should be tamed.

There is circular overdescription of results. For example, at lines 284-288, contrary to the images of 'shift' and 'transfer' there has been no physical transfer of organic matter from the oLF fraction to the sediment. There were instantaneous observations of POM in different fractions that do not exist until one creates them in the laboratory. The different quantities of POM released simply indicate the effects of the enzyme treatment (and elegantly so). This *may* be due to a reduced aggregate stability for example, and/or additionally increased fragmentation of POM, possibly including biofilm fragments (including cells and EPS) from the soil that are large enough (or sticky enough) to be retained on the 1.5  $\mu$ m glass filter. This is a matter perhaps for the discussion. Nonetheless, the model proposed to explain the observed phenomenon (of increased POM release with enzyme addition and sonication) is one of reduced aggregate stability by EPS removal – I think this is reasonable and should be retained (but not stated as fact without harder evidence).

### **Further comments and general technical corrections**

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

13 please choose between either: 'shear and compression' or 'shearing and compressive'.

14 replace 'high stability results in less soil compatibility and erodability' with 'high stability results in less compaction and erosion, and has been linked to '.

15 delete 'a' in 'a dynamic'; replace 'regime' with 'regimes'.

24 delete 'which preserves aggregate structure': I do not think is correct to say this, physical aggregate structure was not measured directly; it was inferred indirectly via release of POM-C. Furthermore, some structure must have been changed enzymatically to result in the increased release of C. To avoid philosophical digression into definitions of 'biological', 'physical', and 'chemical' structure in the abstract, the manuscript is better without it: just delete.

26 replace 'effectivity' with 'efficacy'

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'.

### **Introduction**

37 replace compressive with compression

- 38 replace 'water caused lability' with 'disaggregation caused by water'
- 38 insert 'and' before 'is an integral'
- 39 replace 'Because' with 'Since'
- 46 delete 'the' from 'the water retention'
- 46 insert a comma after 'furthermore'
- 49 replace 'SOM promotes' with 'SOM is thought to promote'
- 56 delete '.'
- 61 – 63 awkward sentence, please rephrase
- 78 check spelling polysaccharide
- 79 replace 'exudated' with 'exuded'
- 80 insert 'being' before engineered
- 82 replace 'biofilm forming species and habitats:' with 'community composition and environmental cues:'
- 83 replace 'determined' with 'measured'
- 89 reverse word order, should be 'other diverse soils ranges between'
- 92 replace 'can amount to' with 'was measured at'
- 93 replace 'and often hold 60% of EPS dry mass' with 'but can contribute the largest fraction of EPS, e.g. 60%'
- 96 scientific notation check: I suspect this should be  $0.5 \times 10^6$  (and likewise throughout the rest of the manuscript).
- 101 'ranges'
- 105 should eDNA be eDNAase?
- 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."
- 110 replace 'hardly effecting other aggregate binding mechanisms' with 'with minimum impact on other types of aggregate bond'

- 112 replace 'coherence' with 'cohesion'
- 112 remove 'and aggregate stability' (I do not think you measured this) consider adding a statement like 'which is expected to contribute to aggregate stability' (then reference needed).
- 123 replace 'after' with 'occurs during'
- 123 replace 'that' with 'this'

## **Materials and methods**

- 138 replace 'receive' with 'obtain'
- 141-142 This is not a method to estimate soil microbial biomass, this is respiration, correct accordingly.
- 143 delete 'then'
- 144 replace 'have been' with 'were'
- 146 careful with pluralities, use 's' and 'were'
- 147  $\mu\text{g g}^{-1}$  is more appropriate
- 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)"
- 173 use large 'C' for carbon
- 181 Renamed to 'Intra-aggregate POM' (or similar) no method or results for a measurement of aggregate stability are presented or referenced: e.g. Cerli et al 2012 do not claim this method quantifies aggregate stability.
- 182 Accordingly, delete 'soil aggregate stability was measured at the macro scale'.
- 190 Why for 30 min? To allow NaPT diffusion? State your reason.
- 192 Unlike Cerli et al (2012), it is currently a little ambiguous if this fLF passed the filter or was captured on the filter. I expect the latter. Suggest you replace 'separate' with 'capture'.
- 195  $50 \text{ J ml}^{-1}$  given over what time period?
- 200 Total = sum of C in the sediment and filters? Be explicit (you did not quantify DOC, correct?)
- 210 'washed three times'
- 217 what volume of wash was used as an equivalent for the mass of soil stipulated in the FastDNA<sup>TM</sup> spin kit soil manual? (Can it really be used to extract DNA from a dilute wash and compare with soil?).

## RESULTS

263 rename section 3.1 as method above

264 delete 'the relative net SOC release shows' (you did not quantify DOC which is implied in 'SOC').

265 insert 'are shown' before '(Fig. 1)'

265 replace SOC with POM

266 replace 'organic carbon' with 'POM'

268 move 'data are shown as mean values and standard deviations of five parallels' to figure caption.

270 replace SOC with POM

272 replace SOC with POM

273 Replace 'SOC of the FLF' with 'quantity of POM released in the absence of sonication'

274-276 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)"

276-277 Unnecessary and confusing statement, we can see the standard deviation and Tukey test results on the figure, better to remove the statement.

277-279 Potentially misleading statement, yes, E2 and E3 have no difference compared to the control, but neither do E1 or E4. See comment above re 274-276.

280 Replace SOC with POM. Delete 'stock'. 'varying between variants'? Do you mean 'different among treatments'?

281 replace 'an in' with 'any'

283 replace 'is therefore differing between variants with' with 'therefore shows'

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  $\mu$ M 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 284-288.

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. Delete line 289.

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.

291        Clumsy sentence and repetition: delete first sentence.

291-293        Released POM data may be evidence of this, and may not be - this is a matter for the discussion. Delete these 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<sup>-1</sup>) than occurred with the addition of the lowest concentration (E1). This was statistically significant at ( $p < 0.05$ )."

end of section.

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.'

## DISCUSSION

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..."

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

339        Replace SOC with POM

340        Replace SOC with POM

342        Remove paragraph break

343        Replace 'SOC' with 'POM'; replace 'and' with 'with'; Replace 'enforced' with 'supported'

344        Full stop after (Fig. 3). replace 'which' with 'This'.

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'.

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  $\text{NH}_4\text{NO}_3$ , and added further  $\text{NH}_4\text{NO}_3$  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  $\text{NH}_4\text{NO}_3$ . In the present study,  $\text{NH}_4\text{NO}_3$  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<sup>-1</sup>, and the break in the trend for increasing POM release with increasing enzyme addition.”

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...”

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).

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<sup>-1</sup>; 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.

371 & 379 - Replace SOC with POM

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

380 – There is no ‘transfer’ other than in abstract operationally defined concepts - delete statement.

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’.

385 – results repetition.

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<sup>-1</sup> – and after more effectively separated from soil minerals by 50J sonication*).

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).

406 – Replace ‘most of this restrictions’ with ‘many of these uncertainties’

407 – better if you delete ‘a 9000 fold of the E1 enzyme activity calculated from actual soil biomass to remove approximately’

408 – 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’

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.’ (Add Kaiser and Berhe reference 2014)

410 - delete ‘nonetheless’

411 - replace ‘Loss of aggregate stability’ with ‘Release of entrapped POM’

413 - replace ‘stabilisation’ with ‘stabilising’

413 – Citation needed: suggest after ‘stabilising agent of soil aggregates’ to insert ‘as discussed in a comprehensive review by Or et al. (2007)’.

413 – 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.

414 – 417 Move this final part to the start of conclusions: “Our results suggest a change of biofilm composition due to a shift...”

## Conclusions

Much here currently appears needless repetition of the results already discussed.

419 – 420        Already discussed, is weak, better to delete.

422 – 423        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).



425 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."

425-427 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.

431 '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'.

## Figures and Tables

Renumber figures after deleting Figure 2

Figure 1: Replace SOC with POM

Figure 3: Check scientific notation (Y axis) and replace '.' With 'x' ?

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

Table 1: Check scientific notation under column 'Molar mass' ( e.g. should be  $2 \times 10^6$  ?)

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

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

## References

- Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: Current knowledge and future directions. *Soil Biology & Biochemistry* 58, 216-234.
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- Redmile-Gordon, M.A., Evershed, R.P., Hirsch, P.R., White, R.P., Goulding, K.W.T., 2015. Soil organic matter and the extracellular microbial matrix show contrasting responses to c and n availability. *Soil Biology and Biochemistry* 88, 257-267.
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