Soil aggregates by design: Manufactured aggregates with defined microbial composition for interrogating microbial activities in soil microhabitats

Harry J. Harvey, Ricky D. Wildman, Sacha J. Mooney, Simon V. Avery

School of Life Sciences, University of Nottingham, Nottingham, UK
Faculty of Engineering, University of Nottingham, Nottingham, UK
School of Biosciences, University of Nottingham, Nottingham, UK

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ABSTRACT

Differences in the structure of microbial communities are reported to exist between the inside and outside of soil aggregates, but the impacts of soil aggregation on microbial activity in soils, essential for soil health, have proven difficult to study in a controlled manner. We have developed a new method to manufacture soil macroaggregates in the laboratory with the ability to introduce microorganisms of choice to the endo- or exo-aggregate environments, offering new avenues for experimental research. X-ray Computed Tomography imaging confirmed the manufactured aggregates had similar overall porosity, pore size and pore connectivity to naturally-formed aggregates. We exploited this new approach to test the hypothesis that microorganisms within aggregates are protected from environmental stresses, in comparison to organisms located near aggregate surfaces. Soil isolates of yeasts introduced to the interior or exterior of manufactured soil aggregates showed no significant difference in the survival of metal- or anoxic-stresses, but organisms within aggregates were protected from heat stress in a time-dependent manner. The results indicate that microbial communities may be protected from particular environmental perturbations by the complex porous architecture that arises from the aggregated soil structure, and underscore the value of this new approach for improving our understanding of the interactions between the soil physical environment and the associated soil biology.

1. Introduction

Soil aggregation refers to the formation of a larger collection of particles from the smaller constituents of soil including clay, silt, and sand, that adhere to each other more strongly than to surrounding particles (Martin et al., 1955; Nimmo, 2005). Soil aggregates are in a constant state of deformation, reposition, and reformation due to several soil based processes including surface weathering, wetting and drying cycles, freeze-thawing cycles, soil-root interactions, as well as microbial interactions (Hillel, 2003), with aggregate turnover rates varying from weeks to months (De Gryze et al., 2006). These processes impact both the overall soil structure, which varies seasonally based on the aforementioned environmental cycles, and the extent to which aggregates can act as a habitat for microorganisms. The use of soils for agriculture also impacts on aggregate stability. For example, under zero-tillage, where soils are not cultivated prior to planting, aggregates tend to be more stable and resilient, often due to an increase in soil organic matter content (Sasal et al., 2006).

Soil aggregation can impact the soil microbiota as aggregates may contain chemical (e.g. oxygen) gradients (Sexstone et al., 1985) as well as physical barriers to surrounding pore space or other soil particles (Rabbi et al., 2016). These differences can contribute to the creation of contrasting soil microenvironments, leading to heterogeneity in the microbial community composition and structure over small spatial scales (Carson et al., 2010; Blaud et al., 2014; Ebrahimi and Or, 2016; Upton et al., 2019). In addition, there can be local adaptation to specific microenvironments in circumstances where aggregates are more stable and aggregate breakdown and formation is relatively slow (Almás et al., 2005; Upton et al., 2019).

Previous work describing the microbiota of the interior versus exterior soil-aggregate environment has relied on isolating organisms from either location retrospectively, using soil samples taken from natural environments (Mummey and Stahl, 2004; Blaud et al., 2014; Garbuz et al., 2016). While this approach offers a snapshot of the microorganisms present from the particular soils examined, it does not allow researchers to ask other questions concerning aggregate...
located outside aggregates, resulting in locally-adapted individuals over
small distances, potentially of the same species (Rillig et al., 2017). This may
result in different selection pressures to those on microorganisms
variation or predation by other organisms (Rillig et al., 2017). This may
considered as

H.J. Harvey et al.
aggregate size, as microaggregates (<250 μm) are reported to harbour
relatively dynamic and diverse microbial communities, whereas mi-
crobial communities of macroaggregates (>250 μm) are more stable
(Upton et al., 2019).

Here, we aimed to develop a new method for producing field-
representative soil aggregates in a laboratory environment, while
enabling the incorporation of known organisms and quantities of or-
ganism selectively to the inside or outside of the aggregates. This was to
enable a more controlled approach for investigating the influence of
diverse parameters on soil aggregates as microhabitats, with the ability
to fine tune the microbial composition and location as required. As an
exemplar of the potential of this new approach, we compared the environ-
mental stress response of a common soil yeast localised to the
surface or within the aggregates, to test the hypothesis that the aggre-
gate interior confers a protective effect.

2. Materials and methods

2.1. Yeasts and culture conditions

The haploid Saccharomyces cerevisiae strains W303 (MATa ura3-1
ade2-1 trp1-1 his3-11,15 leu2-3,112) and BY4741 (MATa his3-1 leu2-0
met15-0 ura3-0) were maintained and grown in YPD medium [2% pep-
tone (Oxoid), 1% yeast extract (Oxoid), 2% D-glucose] or YNB me-
dium [0.69% yeast nitrogen base without amino acids, (Formedium)]
supplemented with 2% (w/v) D-glucose and amino acids or nucleobases
as appropriate for strain auxotrophies (Moreno-Martinez et al., 2015).
Saitomyza podzolica, identified by ITS sequencing and RAPD PCR as
described in Holland et al. (2014), was a wild isolate recovered from soil
near a disused metal smelting works in the north-east of the UK (http://
twww.twsitelines.info/SMR/4192). S. podzolica was maintained and
grown in MYP medium (7% malt extract (Sigma), 0.5% yeast extract
(Oxoid), 2.5% soytone (BD Bacto)). Where required, media were solid-
ified with 2% (w/v) agar. For experiments, single colonies were used to
incubate 10 ml of medium in 50 ml Erlenmeyer flasks and incubated
with orbital shaking (New Brunswick Scientific) at 120 rev min
−1, either at 30 °C for S. cerevisiae or 24 °C for S. podzolica.

2.2. Soil preparation

A sandy silt loam soil (1% clay, 39% sand, 60% silt) classified as a
luvisol, was collected from agricultural land in Rutland, U.K.
(52.6448051, −0.00671415) and used for manufacturing soil ag-
gregates. The soil had an organic matter content of 4.2% (determined by
Hounsfield Facility, University of Nottingham. Each projection image
was an integration of 3 images with a skip setting of 2 discarded images.

2.3. Production of manufactured soil aggregates and incorporation and
recovery of microorganisms

For demonstrating step wise-aggregate production, first 7 μl of sterile
water was mixed with ~20 mg of sterile ground soil. The moist soil was
vortexed (Vortex Genie 2, Scientific Industries Inc) at speed setting 3
(unless otherwise stated) for 15 s in a sterile 15 ml centrifuge tube
(Scientific Laboratory Supplies) causing the loose soil to bind into a
single cohesive aggregate. A large tube of this shape was used because it
allowed the aggregate to “roll” around the inner circumference of the
tube when vortexed, collecting soil particles and collating them into a
single aggregate. The aggregate was then transferred to a clean tube,
vortexed with a further ~10 μl of sterile soil, to create a barrier be-
tween interior and exterior layers, and transferred to a clean tube again.
For the outer layer of the aggregate, ~10 mg of sterile soil mixed with 7
μl of sterile water was added to the tube and vortexed for 15 s (unless
otherwise specified) together with the existing aggregate to produce a
single aggregate. To incorporate different organisms into the interior
and exterior of single aggregates, the procedure was performed exactly
as described above except sterile water was replaced with the same
volume of water containing cell suspensions (OD600 ~1) of either
S. cerevisiae W303 (first water addition) or S. cerevisiae BY4741 (final
water addition). These organisms were used as they can be selected
using different media supplements (see below), enabling the interior and
exterior organisms to be distinguished after isolation.

For determining independent recovery of organisms from the exter-
ior or interior of aggregates, aggregates were transferred to a Fish-
erBrand X50 cell strainer (mesh size 40 μm) and submerged in 1 ml
sterile water with manual agitation for 1 min to recover surface or-
ganisms in the water fraction. Aggregates were then bathed for 1 min in 1 ml
of 20% (v/v, diluted in sterile water) electrolysed water (Ozo In-
novations) to disinfect the aggregate surfaces. Aggregates were then
disrupted by vigorous vortexing for 1 min in sterile water to release
organisms from the aggregate interior. The two isolated fractions were
subsequently streak-plated to selective YNB agar, supplemented as
appropriate to select for the different auxotrophies of the introduced
S. cerevisiae BY4741 (Met auxotrophy) and W303 (Trp auxotrophy)
strains.

To assess the relative stress resistance of organisms located to the
surface or inside macroaggregates, aggregates were manufactured with
defined quantities of yeast cells provided at the relevant process steps
(Fig. 1). In this case, each aggregate was manufactured to contain or-
ganisms (S. podzolica) either on the outside or inside, not both, to enable
quantitative recovery of all cells of each aggregate. To achieve this,
the same procedure as above was used except that a ~7000 cells μl
−1 suspension (OD600 ~0.03) was used as inoculum for the interior-
aggregation step (~4900 cells). These aggregates were then processed
exactly as above except that the second yeast inoculum was omitted at
the exterior-aggregation stage. To generate aggregates with sterile in-
teriors and organisms in the exterior layer, the same process was used
but the inoculum was omitted for the interior-aggregation stage but
included (7 μl of a 7000 cells μl
−1 suspension) for the exterior-aggregation
stage. Aggregates were used for experiments immediately after
manufacturing.

2.4. X-ray Computed Tomography (CT) and image analysis

The pore structures of natural and manufactured soil aggregates were
imaged using a Phoenix Nanotom S X-ray CT scanner at the
Hounsfield Facility, University of Nottingham. Each projection image
was an integration of 3 images with a skip setting of 2 discarded images.

disrupt any minor aggregation that occurred during the autoclave pro-
cess. Sterility of autoclaved soil was supported by the absence of
contaminant colonies after plating soil suspensions to MYP or YNB agar
and incubation at 24 °C for 21 days.
Voxel resolution was set at 2 μm, potential energy at 90 kV and current at 65 μA. The total scan time was 63 min per aggregate and a total of 1440 projections were captured for each aggregate. VGSStudio MAX was used to generate and calculate the 3D pore volumes of CT imaged aggregates as well as pore connectivity. The images were then exported as LGF files and subsequently analyzed using the BoneJ plugin (Doube et al., 2010).

2.5. Stress survival of cells located within and outside of aggregates

Approximately 400 mg sterilised, ground soil was transferred to each well of a 24-well microtiter plate (Greiner Bio-One) and a single aggregate, prepared as described above, was then placed on the centre of each soil sample, before covering with a further 400 mg sterilised, ground soil. For lead (Pb) stress treatments, each well was then treated with 500 μl of Pb(NO3)2 solution and then aggregate disruption by vortexing in sterile water was sufficient to produce aggregation of soil particles. A single cohesive aggregate per assay was achieved reproducibly at an intermediate vortexing vigour, whereas low or high vortex speeds tended to produce smaller aggregates of varying number and size (Fig. 2A). The volume of water added to the sample was a strong determining factor in how well the soil aggregated. At 5 μl water, not all soil particles aggregated (producing a smaller aggregate), whereas at 9 or 11 μl water, the soil tended to smear on the tube wall and single, cohesive aggregates did not form reproducibly (Fig. 2B). Single aggregates were readily formed at 7 μl water. The aggregates were approximately 1.5 mm in diameter. It is likely that soils of a different particle size composition would react differently than described above, as particle size is known to affect granulation processes (Iveson et al., 2001).

3.2. Manufactured aggregates are characteristically similar to natural soil aggregates

To compare the properties of the aggregates manufactured in the laboratory with natural aggregates, examples of both, from the same batch of soil, were X-ray CT scanned (Fig. 2). The manufactured aggregates exhibited very similar % pore volume and pore connectivity compared to natural aggregates (Fig. 3). The pores of manufactured aggregates had a slightly smaller (c. 10 μm difference) average equivalent spherical diameter than the natural aggregates but this was not significant (p = 0.084). The mean pore volume and equivalent spherical diameter were also very similar between the inner and outer volumes of the manufactured aggregate (p = 0.840, p = 0.371 respectively) (data not shown), suggesting that the two-step manufacturing process did not produce different physical micro-environments in these fractions.

3.3. Organisms can be independently recovered from the aggregate interior and exterior

In order to study aggregate-associated microorganisms, aggregates were produced containing S. cerevisiae W303 cells within the aggregate interior, and S. cerevisiae BY4741 on the aggregate exterior. These strains were chosen as they could be distinguished by cultivation (after recovery from aggregates) on media selective for respective auxotrophies. Bathing the aggregate with gentle agitation in water was sufficient to recover BY4741 cells from the aggregate surface but not the W303 cells from the aggregate interior (Fig. 4). After subsequent disinfection of the aggregate surface with diluted electrolysed water solution and then aggregate disruption by vortexing in sterile water, colony forming units were counted after incubation at 24 °C either under anaerobicity [Whitley DG250 anaerobic workstation; Don Whitley Scientific (10% CO2, 80% N2)] or in ambient air. For heat stress, 24-well plates were incubated for 30 min at 50 °C or 3 h at 40 °C. In either case, incubation for the corresponding duration at 24 °C served as the control. After treatment, each aggregate was recovered using a sterile metal spatula, deposited into 3 ml sterile water and vortexed vigorously for 1 min to break up the aggregate and release cells. After allowing soil to settle for ~20 s, 1 ml of supernatant was taken and centrifuged at 4500 g, 7 min. The supernatant was removed and the cell pellet re-suspended in 500 μl sterile water before spread plating 100 μl aliquots to Petri plates containing MYP agar. Colony forming units were counted after incubation at 24 °C for 7 d.

2.6. Statistical analysis

Comparisons between treatments were analysed by unpaired t-test. The normality of datasets was tested using the Shapiro-Wilk test. All statistical analyses were performed with GraphPad Prism version 8.2.1.

3. Results

3.1. Manufacture of discrete soil aggregates mimicking natural aggregates and introduction of organisms to aggregate-interior or -exterior

In order to enable the study of soil microorganisms both within and on the surface of soil aggregates under controlled conditions, we sought to develop a method to manufacture artificial soil aggregates in the laboratory. Gentle vortexing of ground samples of the soil collected in this study with sterile water was sufficient to produce aggregation of soil particles. A single cohesive aggregate per assay was achieved reproducibly at an intermediate vortexing vigour, whereas low or high vortex speeds tended to produce smaller aggregates of varying number and size (Fig. 2A). The volume of water added to the sample was a strong determining factor in how well the soil aggregated. At 5 μl water, not all soil particles aggregated (producing a smaller aggregate), whereas at 9 or 11 μl water, the soil tended to smear on the tube wall and single, cohesive aggregates did not form reproducibly (Fig. 2B). Single aggregates were readily formed at 7 μl water. The aggregates were approximately 1.5 mm in diameter. It is likely that soils of a different particle size composition would react differently than described above, as particle size is known to affect granulation processes (Iveson et al., 2001).
followed by plating, it was apparent that this second fraction comprised W303 cells that had been introduced to the interior. There appeared to be negligible mixing of cells from the aggregate exterior and interior when isolated with this method: only one yeast strain was recovered from each of the two fractions, as anticipated from the manufacturing process. This showed that organisms could be selectively introduced to and/or recovered from the exterior or interior of aggregates, and that no movement of organisms was detectable between the fractions (*S. cerevisiae* and *S. podzolica* are non-motile).

We next adapted the procedure to study a single soil organism, *S. podzolica*, in the aggregate; that is, the same organism at both exterior and interior locations. As the use of the single organism did not enable routine verification of purity of the interior or exterior fractions, as done above with the *S. cerevisiae* strains, the manufacture protocol was amended to incorporate *S. podzolica* into either the interior or exterior of different aggregate preparations. This was done by introducing cell-free sterile water instead of inoculum at the appropriate stage of manufacture (see Materials and Methods). Similar numbers of cells could be reproducibly recovered from aggregates where *S. podzolica*, at equivalent inoculum size, had been introduced either to the interior (4164 ± 767 colony forming units (CFU) recovered) or exterior (4116 ± 605) of the aggregate (Supplementary Fig. S1).

### 3.4. Cells within aggregates show stressor-dependent, differential survival compared with cells at aggregate surfaces

We applied this new procedure to test the hypothesis that soil aggregation may buffer organisms from environmental stress. Aggregates were manufactured as above with the soil yeast *S. podzolica* localised either to the aggregate-interior or -exterior. These aggregates were then mixed in with a larger amount of the soil (twice-autoclaved) from which the aggregates had been derived before testing a small panel of soil-relevant environmental stresses. Treatment with lead (a common soil pollutant near mining sites and major roads, for example) caused between 30 and 40% loss of viability of aggregate-associated yeasts within 1 h, according to percentage CFUs recovered from aggregates. However, there was no significant difference in survival of Pb by cells at the interior or exterior of aggregates (Fig. 5A).

Soil samples were also incubated under anoxia as anoxia is a common issue in soils prone to seasonal or permanent waterlogging.
This caused some loss of S. podzolica viability over time as this yeast is an obligate aerobe (Fig. 5B). Moreover, similar to the acute Pb-stress, there was no evidence that the aggregate interior buffered cells from anoxic stress compared to the exterior aggregate measurements.

High temperature, as an alternative physical stress (e.g. associated with forest fires, soil solarisation, etc.), caused some loss of yeast viability within 30 min at 50 °C and within 3 h at 40 °C. In the former treatment, and in contrast to the other stresses, relative survival of heat stress at the aggregate exterior was <50% of that by cells in the aggregate interior (Fig. 5C). This difference was reproducible over three independent replicate experiments. However, the difference in survival between cells in the aggregate interior or exterior was lost after incubation for a longer period of 3 h at high temperature (40 °C). Collectively the data indicated that soil aggregates may buffer microbial communities from environmental stress only in particular circumstances, and this is discussed further below.

4. Discussion

4.1. Manufacturing aggregates

In this study, we developed a simple and reproducible method for manufacturing realistic soil aggregates that could be readily adopted by other laboratories. The aggregates can be manufactured with a microbial composition that is defined qualitatively (i.e. choice of organisms), quantitatively, and spatially (i.e. localisation to aggregate-interior or -exterior), with organisms also being selectively recoverable from aggregates. These features enable the study of the impact of soil aggregation on microbial activities in soils, for example, which we exploited here to demonstrate selective buffering by aggregates of key environmental stresses. The morphological similarity of the manufactured and naturally formed macroaggregates from the same parent soil material was reflected in similar measurements of pore volume and connectivity. These pore properties are important determinants of gas and liquid accessibility into aggregates and of the available space for microorganisms to occupy and modify (Sexstone et al., 1985; Carson et al., 2010). A key parameter in manufacturing these aggregates is using wet granulation and, as such, this method may be less suitable for very sandy soils that are not cohesive when wet, although for soils that contain a greater proportion of silt or clay then the role of micro-aggregates not destroyed by sieving may need to be considered. Regarding cell recovery from aggregates, the technical variation was low and similar for cells recovered from the inside or surface of aggregates. This suggests that similar numbers of cells are incorporated to each aggregate and that their recovery rates post-experiment are relatively constant. We believe that this robust, quantitative tool provides new capability for probing soil structure and the associated soil biology, in a defined and reproducible manner.

4.2. Recovery of organisms from manufactured aggregates

Selective recovery of yeast cells previously introduced to either the inside or outside of aggregates only required a simple wash to collect exterior organisms then aggregate-disruption to release yeast from the aggregate interior, with an intervening surface sterilisation step. This corroborates that the aggregate manufacturing process does generate spatially discrete environments for organisms, with only one of the two environments readily accessible (to a disinfecting solution) from the aggregate surface. Manufactured aggregates appeared stable, remaining intact following submersion in water. Whereas the procedure for recovering organisms from inside and outside of the same aggregate lacks the quantitative recovery from aggregates carrying cells only either internally or externally, it does allow assay of both microhabitats in the same aggregate. This could be valuable for experimental evolution applications, for example, where absolute recovery rates can be less important.

4.3. Stress response of organism with respect to aggregate location

We applied the system to tackle the important exemplar question...
Soil Biology and Biochemistry 148 (2020) 107870

6

concerning how soil structure may impact perturbation of microbial communities by environmental stress. Several studies have examined interactions between soil microbial communities and soil structure at different scales (Sessitsch et al., 2001; Frey et al., 2008; Rabbi et al., 2016), but few have addressed the interplay between soil aggregation, in particular, and phenotypes like environmental stress impacts. Specifically, we investigated whether environmental resilience of the common soil yeast S. podzolica is affected by localisation within or on an aggregate during stress challenge. Stressors with different actions were selected for this study (e.g. chemical stress requiring stressor (Pb) uptake from the environment, versus physical stress from a heated environment), in order to assess a range of potential environmental perturbations on interior versus exterior organisms.

Regarding metal stress, previous work has suggested that strongly attached organisms located within micropores or biofilms are less exposed to metals in the soil matrix (Almås et al., 2005), whereas other work suggests some metals tend to concentrate in the aggregate interior (Ilg et al., 2004), highlighting the difficulty of disentangling these relationships. We examined Pb stress, relevant to soils polluted with lead as a result of mining and smelting activity (Toth et al., 2016). As the results indicated no evidence for significant protection from Pb by cell localisation within the aggregate interior, they suggest that the dissolved metal may equilibrate at a similar available concentration within the aggregate pore space as near the aggregate surface.

Soils are also known to present marked, spatially heterogeneous oxygen gradients, even potentially within a single aggregate (Sexstone et al., 1985; Schlüter et al., 2018). S. podzolica is unable to grow in anaerobic conditions, but some (not all) cells may survive and resume growth, after a short delay, if restored to an oxygenated environment. Using this phenotype, we showed that the encapsulation of the yeast cells within aggregates did not reduce the impact of external anoxic stress on cell viability. This evidence suggests oxygen gradients which may arise over the spatial scales of soil-aggregate microenvironments are not sufficient to impact oxygen-sensitive viability.

We also examined heat stress, as soils are naturally exposed to a wide range of temperatures; across different environments, between varying

Fig. 5. The survival of S. podzolica within or on the surface of soil aggregates in response to different stresses. (A) Survival in response to acute (60 min) exposure to 12 mM lead nitrate (p = 0.2335, two-sample t-test). (B) Survival after incubation in anoxic conditions for 9 days (p = 0.4630). (C) Survival in response to acute heat stress, at 50 °C for 30 min (p = 0.0408) or 40 °C for 3 h (p = 0.4903). Percentage survival was calculated relative to stressor-free control assays, from counts of colony forming units (CFU). Error bars represent SEM. Individual points represent biological replicates.
Soil aggregates can provide other benefits to microorganisms beyond the scope of this study, such as protection from predation (e.g. by soil nematodes) (Vargas, 1986), isolation from environmental fluctuation (e.g. nutrient and toxic fluxes) of the bulk soil (Rillig et al., 2017) and compartmentalisation to support discrete micro-communities and associated species resilience (Mummey and Stahl, 2004). Although poorly explored, there are also likely advantages of localisation at the aggregate exterior, such as greater access to carbon and nutrient flow from the bulk soil. Furthermore, factors such as aggregate size may influence microbial community composition and activity (Schlüter et al., 2018; Upton et al., 2019), while the time between aggregate formation and breakup can be variable in the natural environment (De Gryze et al., 2006). These factors could also be considered for study with adaptions to the present method, such as by varying aggregate diameter or the time elapsed between aggregate manufacture and aggregate disruption/assay. These parameters warrant further investigation and the new method presented here provides a tool to support such efforts.

5. Conclusions

Soil aggregation is an important process in governing distribution and activity of soil microbiota. The method for manufacturing soil aggregates developed in this study now enables key questions to be tackled in a controlled manner, not available previously. By focusing on one such question here, we showed that aggregate-associated microbial communities can be differentially affected by key environmental perturbations according to their relative localisation in or on the aggregates, in a time dependent manner. On the other hand, we demonstrate certain environmental stress scenarios which produce no such effect of localisation. This new insight into interactions at the soil structure/biology interface opens the door to addressing further related questions such as how soil aggregation may influence microbial community composition and longer-term adaptations in response to environmental pressures.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.107870.

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