The role of macro-aggregation in regulating enzymatic depolymerization of soil organic nitrogen

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The role of macro-aggregation in regulating enzymatic depolymerization of soil organic nitrogen.

Author names and affiliations

Jumpei Fukumasu\textsuperscript{a,b}, Liz J. Shaw\textsuperscript{h,*}

\textsuperscript{a} Graduate School of Environmental and Life Science, Okayama University, 3-1-1 Tsushimanaka, Okayama 700-8530, Japan

\textsuperscript{b} Soil Research Centre, Department of Geography and Environmental Science, School of Archaeology, Geography and Environmental Science, University of Reading, Whiteknights, P. O. Box 233, Reading RG6 6DW, UK

* Corresponding author. Tel.: +44 (0) 118 3786971; fax: +44 (0) 118 3786666. E-mail address: e.j.shaw@reading.ac.uk (L.J. Shaw).

Highlights

- Aggregation protection of polymeric organic N (PON) from enzyme attack explored
- Dis-macroaggregation significantly increased net anaerobic N mineralization rate (Nmin)
- PON depolymerase-Nmin relationships distinguish mechanisms responsible
- Role of disaggregation-increased accessibility of substrate to enzymes revealed
- Factors promoting net Nmin on disaggregation may differ with land use
Abstract

Extracellular enzymatic depolymerization of polymeric organic nitrogen (PON) is a rate-limiting step in N mineralization. However, enzymatic accessibility to PON might be regulated by physical occlusion of the PON resulting from the architectural packing of soil minerals during aggregate formation. To examine the extent to which enzymatic accessibility to PON is regulated by soil aggregation, we put forward a new approach involving the comparison of relationships between potential N depolymerase activity (protease and β-glucosaminidase; as an estimate of the potential to produce depolymerized products) and net N mineralization (as a bioassay for actual low molecular weight dissolved ON production) in aggregated and corresponding disaggregated soil. Soils were sampled from grassland (GL) and arable land (AL), separated by dry sieving into fractions (4.75-2, 2-0.25 and 0.25-0.063 mm) and fractions mixed (4:4:1 by mass, respectively) to obtain constructed aggregated soils. Corresponding disaggregated soils were prepared using a mortar and pestle. This procedure mainly disrupted the 4.75-2 mm (large macro-aggregate) fraction. Disaggregation significantly promoted (p<0.05) net N mineralization rates by 1.3 times and 1.5 times in GL and AL soil, respectively. When net N mineralization - potential N depolymerase relationships for GL were examined, a greater slope parameter for disaggregated compared to aggregated soil (p=0.001; ANCOVA) quantified the extent to which this promoted N mineralization could be attributed to disruption of macroaggregate-increased enzymatic accessibility to PON. For
AL, which had low protease and β-glucosaminidase activity, promoted N mineralization rate could not be attributed to increased protease + β-glucosaminidase accessibility to PON reflecting a possible role for other N depolymerases and/or osmolyte/lysate effects. By proposing how differences between mineralization-depolymerase relationships for soils differing in aggregation status might, with assumptions, be interpreted to identify the role of physical occlusion in protection of PON, we give new insight on the regulation of enzymatic depolymerization by physical protection through macro-aggregation for soils from contrasting land use.

Keywords: Nitrogen mineralization, extracellular enzymes, soil macro-aggregation, bioaccessibility, enzymatic depolymerization

1 Introduction

Nitrogen (N) availability is the most important factor for ecosystem productivity, and soil organic matter (OM) is a sink and source of nitrogen for plants (Schulten and Schnitzer, 1998). In surface soil, up to 90% of nitrogen is stored as organic N in soil OM (Olk, 2008). The transformation of polymeric organic N (PON) to plant available forms depends initially on depolymerization mediated by extracellular enzymes (Geisseler et al., 2010) to yield monomeric/lower molecular weight dissolved organic N (LMW DON) which already may be plant-available (Schimel and Bennett, 2004; Jones et al., 2005) and also readily mineralizable to inorganic N (Schimel and Bennett, 2004). These extracellular
enzymes may be of microbial, plant and animal origin (Vranova et al., 2013) and the
depolymerization process appears to be the rate-limiting step in N mineralization
(Schimel and Bennett, 2004; Jan et al., 2009).

However, depolymerization of PON could be regulated not only by the biochemical
reactions described above but also by physical and chemical factors that alter the
accessibility of PON substrates to the extracellular enzymes that act on them. While
representing a chemical continuum of structures derived from the progressive
decomposition of organic macromolecules, soil OM (with constituent N) has been
conceptualised as belonging to discrete pools differing in their susceptibility to
decomposition and the mechanisms by which the OM is stabilized, namely: (i) physical
inaccessibility through occlusion within soil mineral or aggregate architecture; (ii)
chemical interaction between OM and inorganic constituents (e.g., sorption, organo-metal
cheletion) (Sollins et al. 1996). Polymeric OM could also be biochemically inaccessible
to enzymatic attack through inherent or acquired recalcitrance of chemical structure (Six
et al. 2002) but the importance of biochemical stabilization through molecular
recalcitrance of soil OM has been questioned quite recently and greater importance given
to the influences of physical occlusion and chemical interaction (Six et al., 2004; Schmidt
et al., 2011; Dungait et al., 2012; Lehmann and Kleber, 2015). Much of the discussion of
the mechanisms of persistence of soil OM have been focused on organic carbon, however,
the accessibility of soil PON to enzymatic depolymerization might also be viewed within
the same framework (Olk, 2006; Brzostek and Finzi, 2011). It is well established that
soils contain significant potential activity of depolymerases that are involved in the breakdown of the proteinaceous and chitinaceous OM (Allison and Jastrow, 2006; Geisseler et al., 2010; Vranova et al., 2013) that represents a significant proportion of soil PON (Geisseler et al., 2010). However, the extent to which physical occlusion and mineral associations prevents this activity from being realized with respect to N mineralization has not been explicitly examined (Benbi and Richter, 2002).

A significant mechanism for the physical occlusion of OM results from the architectural packing of soil minerals during aggregate formation (Golchin et al., 1994), which traps OM within pores created. Previous studies have reported that disaggregating soil structure, either through soil tillage or by soil physical treatments imposed in the laboratory, promotes N mineralization (Cabrera and Kissel, 1988; Balesdent et al., 2000). This disaggregation-promoted N mineralization might be consistent with the suggested role that physical occlusion within aggregates plays in limiting the accessibility of PON for decomposition. However, this promotion might also occur due to the physiological release of mineralizable osmolytes by microbial cells in response to disaggregation, for example, on exposure of cells that were previously inside aggregates to dehydration and rewetting (Navarro-García et al., 2012; Borken and Matzner, 2009; Halverson et al., 2000; Fierer and Schimel et al., 2002) or as a result of the rupture of macroaggregate-binding fungal hyphae (Jastrow et al., 2007; Hobbie and Hobbie 2012). Quantifying the contribution of the release of PON from physical constraints to depolymerisation to the promotion of N mineralization on disaggregation, to our knowledge, has not previously
been attempted, potentially due to lack of approaches to untangle this contribution from that of the mineralization of osmolytes/lysates produced as a result of disaggregation.

Accordingly, our overall aim is to better understand the extent to which the promotion of N mineralisation following the disruption of soil aggregates can be explained by release of PON from physical constraints to depolymerisation rather than by osmolyte/lysate release. To do this, we put forward an approach involving the comparison of relationships between potential N depolymerase activity (as an estimate of the potential to produce depolymerized products) and net N mineralization (as a bioassay for actual LMW DON production) in aggregated and corresponding disaggregated soil. We apply this analysis to grassland and arable soil with the additional aim of understanding how the contribution of PON release to the flush in N mineralization on disaggregation varies with land use.

2 Materials and Methods

2.1 Soil sampling and construction of “aggregated” and “disaggregated” soils

Soil samples (0 to ~20 cm depth) were taken from random locations within grassland (GL; N=6) and arable (AL; N=5) fields from the University of Reading farm (Sonning, Berkshire, U.K.; NGR: SU765765) on 15/05/2015. Following air-drying, “constructed aggregated” soils were prepared by sieving to obtain 4.75-2 mm, 2-0.25 mm and 0.25-
0.063 mm size fractions and then by mixing these fractions, on a mass basis, in the following respective proportions: 4:4:1 (to approximately represent the proportions initially present in GL soil, Supplementary Fig. 1). The size classes were chosen to represent macro-aggregate (2-0.25 mm) and micro-aggregate (0.25-0.063 mm) fractions (Six et al., 2000) and large macro-aggregates (4.75 to 2 mm) and the same proportions of these size classes were used for both soils so that we could examine land use effects on the nature of the protection provided by aggregates with the same initial size distribution. Corresponding “constructed disaggregated” soils were prepared by disruption of a subsample of the constructed aggregated soil by grinding using a pestle and mortar until no further disaggregation could be achieved, as judged by eye. Selected properties of the constructed soils are shown in Table 1. Fig. 1 shows the percentage, on a mass basis, of the four different fractions (4.75-2mm, 2-0.25mm, 0.25-0.063mm and <0.063mm) in the constructed soils prior to and after disaggregation. The constructed soils were kept in the air-dried state at room temperature until sub-sampled for use in N mineralization and enzyme assays. Sub-samples for enzyme assays were processed within 14 days of the commencement of the net N mineralization assay.

2.2 Net anaerobic N mineralization

 Constructed aggregated and disaggregated soils (54g) were put into 100mL flasks and the water content adjusted to 100% of water filled pore space (WFPS) as calculated using the bulk density and a soil particle density of 2.6 g cm$^{-3}$. After the flasks were flushed with
N$_2$ gas for 2 minutes, the flasks were sealed with rubber stoppers and incubated at 26°C for 10 days. At the end of the incubation, inorganic N was extracted with 1M KCl (200 ml, 30 min). The net N mineralization rate was determined by subtracting NH$_4^+$ measured at the beginning of the incubation (Day 0; Table 1) from NH$_4^+$ concentration measured on Day 10 (Hart et al., 1994) and expressed as mmol N kg$^{-1}$ OD-soil$^{-1}$ 240 h$^{-1}$.

2.3 Potential N-acquiring enzyme activity assays

Protease activity was determined by measuring the concentration of tyrosine produced through depolymerization of Na-caseinate as described by Ladd and Butler (1972) and Geisseler and Horwath (2008). Briefly, aggregated or disaggregated soils (1 g air-dried basis) in autoclaved glass vials were amended with Tris buffer (2.5mL, pH 8.0 modified with 1M HCl) and Na-caseinate (2.5mL, 2%) and incubated at 50°C for 2 hours. Trichloroacetic acid (TCA, 5mL, 10%) was then added to stop the reaction and a 1.5mL aliquot centrifuged (16000 x g, 2 min.). Na$_2$CO$_3$ (0.9mL, 1.4 M) and diluted Folin-Ciocalteu reagent (0.3mL, water: Folin-Ciocalteu = 3:1; Sigma-Aldrich) were added to an aliquot (0.6mL) of the resulting supernatant and the absorbance at 680 nm determined after 5 min. using a spectrophotometer. Blank incubations followed the above procedure except Na-caseinate was added to the samples after the incubation and addition of TCA. Blank readings provided an estimate of concentrations of tyrosine and other Folin-Ciocalteu-reactive compounds native to soil (e.g. cysteine, tryptophan; Everette et al., 2010) and were subtracted from the readings from the caseinate-incubated samples to
express protease activity as $\mu$ mol tyrosine equivalents g$^{-1}$ OD-soil hour$^{-1}$ after comparison of absorbance 680 nm readings to a tyrosine calibration curve (0 to 2.76 $\mu$mol tyrosine). A preliminary experiment showed that protease activity was linear with incubation time (0-4 h).

$\beta$-glucosaminidase activity was determined by measuring the amount of $\rho$-nitrophenol produced from the cleavage of $\rho$-Nitrophenyl-N-acetyl-$\beta$-D-glucosaminide ($\rho$NNAG) as described by Parham and Deng (2000). Briefly, constructed aggregated air-dried soils (1g) were amended with acetate buffer (4mL, 100 mM, pH 5.5) and $\rho$NNAG (1mL, 10mM) substrate solution and incubated at 37°C for 1 hour. After the incubation, 1mL of CaCl$_2$ (0.5 M) and 4mL NaOH (0.5 M) were added, the samples centrifuged (1000 x g, 10 min.) and the supernatant taken for determination of absorbance at 405nm using a spectrophotometer. Blank incubations followed the above procedure except that $\rho$NNAG was added after the incubation. Incubations including substrate but no soil were also included. For constructed disaggregated soils the same procedure was followed except that the assay was based on 0.5 g soil, with the volumes of buffer, substrate and extractant solutions also reduced by a half. $\beta$-glucosaminidase activity was expressed as $\mu$ mol $\rho$ -nitrophenol g$^{-1}$ OD-soil hour$^{-1}$ through comparison of spectrophotometer readings to a $\rho$-nitrophenol calibration curve (0 - 1.08 $\mu$ mol $\rho$ -nitrophenol).

2.4 Sample size and statistical analysis
Soils from 6 and 5 locations for GL and AL, respectively, were sampled and the analysis of soil properties, mineralization rates and enzymatic activities for constructed soils within each location were conducted in triplicate from which a mean value for each location was derived and used as the basis for statistical analysis.

Statistical analysis was conducted using IBM SPSS 22.0 STATISTICS and Statsmodels package within Python™ 3.5. To compare the difference in soil properties between GL and AL, Welch’s t-test or t-test was used. To test for effect of physical treatment (aggregated versus disaggregated) on (i) the production of native Folin-Ciocalteu - reactive compounds, (ii) individual and total enzymatic potential and (iii) net N mineralization rate, paired t-tests, or, where data did or did not satisfy the assumption of normality (Shapiro Wilk test), One sample Sign test of median was used. $P = 0.05$ was adopted as the significance level. Ordinary least squares regression models were established for total enzyme activity (protease + $\beta$-glucosaminidase) versus net N mineralization rate for GL, AL and GL + AL datasets, respectively. For datasets showing a significant relationship (GL and GL+AL), ANCOVA was used to examine if slope parameters for aggregated and disaggregated soils differed statistically under a model assuming common intercepts and different slopes, which was the preferred specification using both Akaike and Bayesian information criteria along with adjusted $R^2$. F and Breush-Pagan tests were used to verify assumptions of equality of error variances and homoscedasticity, respectively. The normality of residuals was confirmed for regression analysis.
Results and Discussion

We sought to better understand the role that physical occlusion of PON plays in regulating N mineralization. To do this, we quantified net N mineralization activity and PON depolymerase potential in soils from two different land uses differing in aggregation status (Table 2). Initial examination of the net N mineralization data for soil from both land uses verified the expectation that disaggregation would significantly increase net N mineralization (Table 2) as has been reported in many other studies (Cabrera and Kissel, 1988; Balesdent et al., 2000). The magnitude of the disaggregation-promoted increase (1.3 times and 1.5 times in GL and AL soil, respectively) we recorded is within the range (0.74 to 3.49 times) reported in a review of previous related studies (Balesdent et al., 2000).

3.1 The efficacy of the disaggregation treatment

The disaggregation treatment was imposed by grinding with a pestle and mortar which resulted in the complete destruction of large macro-aggregates (4.75-2 mm) (Fig. 1) in soil from both land uses with concomitant redistribution of soil mass to the 0.25-0.063 mm and <0.063 mm size fractions. We did not distinguish primary particles from aggregates in the resulting size fractions, but, the <0.063 mm fraction, by definition, would consist of silt- and clay-sized primary particles and micron-sized aggregates (Six et al., 2000). From comparison of the size fraction distribution data (Fig. 1) with initial soil textural information (Table 1), we deduce that the 2–0.25 mm and 0.25–0.063 mm
fractions together could not have been comprised solely of primary particles (medium to very coarse sand, very fine to fine sand, respectively) and therefore that some macro- and/or micro-aggregates (produced following macro-aggregate disruption) remained after the disaggregation treatment. In recognition of the predominant role that micro-aggregates are suggested to play in physical protection of OM (Six et al., 2002), we initially considered the use of a ball mill rather than a pestle and mortar to achieve greater levels of dis-(micro)-aggregation (Pulleman and Marinissen, 2004). However, ball-milling might alter soil particle properties such as specific surface area and reactivity (Vdović et al., 2010) and therefore chemical and physicochemical binding between PON/enzymes and soil mineral surfaces (Zimmerman and Ahn, 2011). Ball-milling might also significantly reduce the particle size of PON. Such alterations would confound isolation of the role of aggregation in PON protection through occlusion within aggregate architecture, and therefore crushing with a pestle was chosen as a gentler method that might also result in a level of dis-(macro)-aggregation that more closely resembles that brought about on soil disturbance by tillage (Six et al., 2004).

3.2 Understanding the role of physical occlusion of PON in regulating N mineralization.

As previously discussed (Section 1), the disaggregation-promoted mineralization we recorded (Table 2) might be due not only to increased accessibility of PON (i.e. release from occlusion) to depolymerizing enzymes but also due to mineralization of microbial compounds that were released on disaggregation as a result of physiological adaptations.
to dehydration (osmolyte production) by microbes previously protected within aggregates
or rupture of fungal hyphae (lysate production) on disaggregation.

In order to distinguish between osmolyte/lysate- and accessibility-related mechanisms,
we examined the relationships between potential N (combined protease and β-
glucosaminidase) depolymerase activity (as an estimate of the potential to produce
depolymerized LMW DON) and net N mineralization (as a proxy or bioassay for actual
LMW DON production) in aggregated and corresponding disaggregated soils (Fig. 2).
We suggest that intercept and slope parameters derived from linear regressions between
these variables for aggregated and disaggregated states (Table 3) can be interpreted and
compared to help distinguish between the mechanisms responsible for disaggregation-
promoted N mineralization. Our assumptions (section 3.2.1) and interpretations of the
regression parameters (section 3.2.2; Fig. 2a) are discussed below.

3.2.1 Assumptions

The use here of net N mineralization as a bioassay for the production of LMW DON
(whether by depolymerization of PON or as osmolytes/lysates) assumes that, firstly,
LMW DON production (and not microbial uptake of, and release of inorganic N from,
DON) is the rate-limiting step to net N mineralization (Schimel and Bennet, 2004;
Kuzyakov et al., 2009), i.e. as soon as LMW DON is produced, it is rapidly mineralized
and detected as ammonium N. The validity of this assumption is supported by studies
showing that free amino acids do not accumulate in soil, implying rapid microbial
turnover (Jones et al., 2004), and also that the mineralization rate of protein added to soil is significantly slower than that of amino acid (Jan et al., 2009). Both these studies suggest that the bottleneck of the soil N cycle is the production of LMW DON, not its uptake and mineralization.

Secondly, by using net N mineralization as a bioassay for the production of LMW DON in the context of examining the effect of aggregation on enzymatic accessibility to PON, we also make an assumption about the ability of the bioassay to bioreport on DON production with an efficiency that is not affected by the aggregation status of the soil. This efficiency of bioreporting is related to the relative contributions of the processes of gross N mineralization and gross N immobilization in defining the concentration of ammonium quantified as net N mineralization in our bioassay. Out of the various mineralization-immobilization pathway schemes previously conceptualized (Manzoni and Porporato, 2009), we adopt the model that gross N mineralization occurs following the cellular assimilation of LMW DON and is a result of the subsequent release of N to the mineral pool that is surplus to requirements. The ammonium production that is measured in our net N mineralization assay reflects the balance between the production of this surplus N and gross immobilization and it is this balance we assume that is not affected by soil aggregation status. In addition to the decomposition flux of LMW DON substrate (most simply considered as a function of substrate concentration and rate of decomposition), this balance is a function of the substrate C:N ratio and the critical substrate C:N ratio (which depends on characteristics of the microbial biomass: biomass
C:N and the efficiency with which substrate C is respired) (Manzoni and Porporato, 2009).

Thus, underlying the assumption that the efficiency of the bioreporting of LMW DON production by the net N mineralization assay is not affected by soil aggregation status, are the assumptions that the following properties are not affected: (i) the C:N quality of the available substrate and (ii) biomass characteristics (C:N and C use efficiency).

Studies that have employed fractionation to isolate OM associated with different soil physical locations have shown that the C:N of particulate OM to be fairly constant, regardless of its physical location (i.e. whether it was free or within macroaggregates (Leifeld and Kögel-Knabner, 2005; Liao et al., 2006; Marriott and Wander, 2006)). Such findings are potentially supportive of the assumption (i) of unaltered substrate quality on disaggregation. With regards to assumption (ii), as previous research has shown effects of soil physical disruption, in this case sieving, on microbial community structure (Thompson et al. 2010), we cannot rule out that changes in microbial community composition on disaggregation occurred in our experiment and that this changed community had altered characteristics with respect to biomass C:N and C use efficiency.

In addition, the above discussion has assumed that changes in biomass size (growth or decay) are negligible. These last uncertainties should be kept in mind when judging our later interpretations (section 3.2.2). Further development of the methodological concept introduced here should involve quantification of the gross process of mineralization and dynamics of the microbial biomass throughout the mineralization incubation.

A final assumption underpinning our interpretation is that the potential N depolymerase
assays employed determine the same pool of potentially active enzymes regardless of aggregation status, i.e. that the active enzyme pool had access to saturating substrate concentrations during the assay incubation. This, as is the basis for all soil depolymerase assay methods, was facilitated here through addition of excess and freely dissolved substrate and incubation under slurry conditions to limit diffusional constraints (Wallenstein and Weintraub, 2008). To support this assumption, comparison of depolymerase activities between aggregated and disaggregated soil (Table 2) reveals no effect of aggregation on individual (protease and β-glucosaminidase) and total (protease plus β-glucosaminidase) activities, with just one exception (protease in GL soil). Potential explanations for why protease activity in disaggregated GL soil was decreased are discussed in the supplementary material.

3.2.2 Interpretation of regression parameters to distinguish accessibility-related (slope) from other (intercept) contributions to disaggregation-promoted net N mineralization.

As depicted in Fig. 2a, the intercept term extrapolates the relationship between PON depolymerase potential and net N mineralization to the case where PON depolymerase (protease + β-glucosaminidase) potential is zero. The magnitude of the intercept can thus be interpreted to represent the production of LMW DON (and its subsequent net mineralization) that is independent of protease + β-glucosaminidase potential. An intercept that is significantly different from zero might reflect the role of ‘other’ depolymerases whose activity was not quantified. Whilst chitin and protein are
considered major PON sources for soil N supply (Geisseler et al., 2010) and therefore, together, protease and β-glucosaminidase reflect important activity degrading polymeric N, there are other enzyme classes that might be involved in PON depolymerisation in soil, such as nucleases. In addition, a non-zero intercept might reflect a contribution from the mineralization of non-polymeric N (e.g. amino acids, N-acetylglucosamine), but, this contribution in at least the aggregated soils would not be significant under the assumption of depolymerisation-limited N mineralization, as just discussed (section 3.2.1). The difference between intercept terms for the aggregated versus disaggregated states quantifies the impact of the physical disruption of aggregates on protease + β-glucosaminidase-independent N mineralization (Fig. 2a). For illustration, applying this interpretation to the regression analysis of data for AL and GL soils combined (Fig. 2b, Table 3) reveals that, for aggregated soil, the intercept term was insignificant, supporting the importance of protease and β-glucosaminidase potential for net N mineralization. However, the intercept term for disaggregated soil indicates that a significant amount of N mineralization occurred independently of the potential activity of proteases and β-glucosaminidases. An increase in the intercept on disaggregation might reflect an increased role for ‘other’ depolymerases in N mineralization (i.e. non-protease/β-glucosaminidase enzymes or new proteases and β-glucosaminidases produced during the incubation) in the disaggregated soil, or, the mineralization of LMW DON compounds that were released (independently of depolymerase activity) in response to disaggregation. This latter might have occurred as a result of osmolyte/lysate production discussed above, or, as a result of the release of physically sequestered labile N that was previously not
accessible (Darrouzet-Nardi and Weintraub, 2014). Enhancement of the F-C reactive compound pool (which represents concentrations of N-containing monomers such as cysteine, tryptophan, tyrosine, guanine alongside a variety of other antioxidant compounds (Everette et al. 2010; Table 2) by such a release of non-polymeric N on disaggregation would not necessarily be expected due to rapid monomer turnover (Jones et al., 2004) and therefore we do not have evidence to support one explanation over another for the protease+β-glucosaminidase – independent N mineralization suggested by the regression analysis.

As also depicted in Fig. 2a, the slope parameter quantifies the extent to which net N mineralization increases for a given increase in PON depolymerase potential (protease+β-glucosaminidase). It is suggested that the magnitude of this parameter represents the extent to which PON depolymerase potential (protease+β-glucosaminidase) is realized for the production of LMW DON, as bioreported by the net N mineralization assay. Critical to our original aim, it follows that the difference between slope parameters for soils differing in aggregation status can be used to quantify the role of aggregate occlusion, and, in our case mostly macroaggregate (section 3.1) occlusion, of PON in constraining PON depolymerization and subsequent net mineralization. Applying this interpretation to the combined GL+AL data (Fig. 2b, Table 3), it can be seen that the slope for the disaggregated soils is statistically greater (according to ANCOVA, p<0.001) than that for the aggregated soil. Thus, more depolymerase potential is realized for mineralization in disaggregated soil and we interpret that this greater realization of potential is due to
greater accessibility of PON following its release from physical protection. We believe that the disaggregation treatment disrupted and homogenized the within- (mainly macro-) aggregate pore network, particularly through opening pore ‘throat’ restrictions to accessibility (Mayer et al., 2004; Ewing et al., 2006). There is a possibility, however, that our (manual pestle and mortar) method of disaggregation also resulted in some reduction in particle size of PON. This possibility and the subsequent consequences for net N mineralization and the slope parameter for disaggregated soil remain to be tested for our samples. However, previous work has inferred that breakdown of soil structure and not fragmentation of plant residues explains the mineralization flush in crushed soils (Chevallier et al., 2011). Additional studies on the effect of plant residue particle size on decomposition and mineralization produce variable conclusions (Ambus and Jensen, 1997; Bending and Turner, 1999; Vestergaard et al., 2001; Bhupinderpal et al., 2006; Toenshoff et al., 2014) with some studies suggesting no effect of residue particle size on decomposition and N dynamics depending on interactions with other factors such as residue quality and incubation time (Ambus and Jensen, 1997; Bending and Turner, 1999; Vestergaard et al., 2001; Toenshoff et al., 2014). Consequently, in our system, we favour the breakdown of soil structure as a significant contributor to the increased slope for the disaggregated soils.

It is relevant to note here that since our net N mineralization assay was conducted at a moisture content of 100% WFPS, the access of N depolymerases to their substrates would not be constrained by lack of hydrological connectivity within the soil and therefore that the (release from) physical protection that was assayed for here was a
function solely of the structure (connectivity) of the pore network. This situation of constant moisture content is distinct from dynamic wetting and drying cycles likely encountered under field conditions where variable hydrological disconnectivity in addition to pore network disconnectivity would play a role in protecting PON from enzymatic attack.

3.3 The impact of land use.

Initial comparison of net N mineralization and potential N depolymerase activities between GL and AL (Table 2, comparisons done for aggregated soils) revealed that net N mineralization activity and potential β-glucosaminidase activity were significantly higher in GL than in AL soil and this presumably reflects the higher total C and N contents in GL soil (Table 1). In particular, β-glucosaminidase activity was approximately ten-fold higher in GL than in AL, suggesting that chitin concentrations, as a major substrate for β-glucosaminidase, are low in AL soil, possibly because of tillage effects on soil fungal populations (Jastrow et al., 2007; Gupta and Germida, 2015). The magnitude of the land use effect on β-glucosaminidase contrasts to that of protease ($P=0.059$, only ~1.6 fold increase in GL) and, given that enzyme production is regulated in response to the availability of substrates (Geisseler et al., 2010), this contrast suggests that PON quality differs between AL and GL.

To understand the impact of land use on the N depolymerase-accessibility of PON, the
relationships between net N mineralization and N depolymerase potential for GL and AL soils were examined individually (Fig 3a and b; Table 3). For disaggregated GL soil (Fig 3a), there was a strong significant relationship between net N mineralization and depolymerase potential (P=0.005, R²= 0.86) while for aggregated GL soil the evidence for a positive relationship was weaker (P=0.081, R²= 0.47) with the slope coefficient significantly (p=0.001, ANCOVA) lower than that for disaggregated soil. The intercepts for both aggregated and disaggregated GL soil are not significant. Applying the interpretation already discussed (section 2.3.2; Fig. 2) suggests that in the GL soil, the disaggregation-promoted net N mineralization might be explained as a function of increased accessibility of PON to proteases and β-glucosaminidase rather than other mechanisms such as osmolyte/lysate production or an increased role of ‘other’ enzymes in depolymerization. As also already discussed (section 3.2.1), this interpretation assumes that there is no difference in biomass turnover contributions to the measured net N mineralization between physical treatments. It is possible, for example, through disaggregation-enhanced trophic interactions (i.e. increased access to prey for bacterial predators in disaggregated soil; Young and Ritz, 2000) that this assumption was not met. As cell debris provides a source of PON (Miltner et al., 2012) which would comprise substrates (N-acetylglucosamine/proteins) and non-substrates for the enzymatic potential we determined, any differences in cell turnover between physical treatments might be reflected in differences between both gradient and intercept terms, respectively.

In contrast to the GL soils, the relationship between net mineralization and depolymerase
potential was not significant for either aggregated (P=0.435) or disaggregated (P=0.241) AL soils (Fig 3b, Table 3). A larger sample size might have increased statistical power to detect relationships, but, the data obtained suggests that depolymerization through protease and β-glucosaminidase is not important for N mineralization in AL soil, irrespective of aggregation status. As discussed above, the quality of PON in AL soil may differ to that in GL. Different PON quality may be partly attributed to different aggregate cycles between land use soil types (Six et al., 2000; Balesdent et al., 2000). Because of likely shorter longevity of macro-aggregates in AL as a result of tillage, PON in AL might have been exposed to a greater degree of microbial processing to forms that are not accessible or not substrates for β-glucosaminidase and protease. For example, such microbial processing may have led to: (i) a more intimate association of proteinaceous and chitinaceous microbial residues with mineral phases and thereby their protection through chemical interaction (Miltner et al; 2012; Bingham and Cotrufo, 2016); or, (ii) creation of organic N structures (e.g. heterocyclic N, Leinweber et al., 2013) that are not recognized as substrates by β-glucosaminidase and protease. That potential β-glucosaminidase and protease activity could be detected in AL, even though it was apparently uncoupled from current availability of suitable substrates, might be explained by the relative longevity of extracellular enzymes in the soil environment, their potential activity thus integrating historical substrate conditions (Burns et al., 2013). Due to the lack of significance for AL, we are not able to interpret the mechanisms responsible for the disaggregation-promoted N mineralization flush seen for this soil (Table 2) in the context of increased access of β-glucosaminidase / protease to substrates (Fig. 3b).
speculate in this case that the flush is a function of either osmolyte/lysate production or release of non-proteinaceous/chitinaceous PON for ‘other’ depolymerase attack or a combination of both.

4 Conclusions

In the present study, net N mineralization rates for GL and AL soils were promoted significantly by disruption of mainly large macro-aggregates (4.75-2mm). We hypothesized that these increased net N mineralization rates would be attributable to increased accessibility of PON to extracellular enzymes (protease and β-glucosaminidase) with the assumption that enzymatic depolymerization is a rate-limiting step in overall N mineralization. It has been pointed out that micro-aggregate structure is more important in protecting SOM (Six et al., 2002). However, we present evidence to suggest that in the short term (e.g. 10 days), macro-aggregates in a grassland soil contribute to the regulation of enzymatic accessibility to their substrates. For an arable soil, the situation was less clear; with low concentrations of protease and β-glucosaminidase, other depolymerase enzymes or increased availability of LMW DON could be important in the promotion of N mineralization upon disruption of macro-aggregates. More research on regulation of enzymatic depolymerization by soil structure is useful for improved understanding of N dynamics through empirical studies and for models incorporating enzymatic depolymerization as a key process in the N cycle (e.g. Schimel and Weintraub, 2003). Here we suggest how differences between mineralization-depolymerase relationships for soils differing in aggregation status might,
with assumptions, be interpreted to identify the role of physical occlusion in protection
of PON from mineralization (Section 3.2; Fig. 2). The same approach might also be
useful for understanding physical constraints to organic carbon mineralization in soil.
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**Figure Legends**

**Figure 1.** The percentage mass of each fraction in constructed aggregated soils and corresponding disaggregated soils used for the net N mineralization and potential enzyme activity assays. Aggregated GL and AL soils were constructed by mixing 24g of 4.75-2mm, 24g of 2-0.25mm and 6g of 0.25-0.063mm fractions. Corresponding disaggregated soils were prepared by disrupting the aggregates using a pestle and mortar. Data for disaggregated soils are mean ± standard errors (n=6 for GL and n=5 for AL). There were no significant differences between GL and AL for 2-0.25mm ($P=0.115$; Welch’s t-test), 0.25-0.063mm ($P=0.066$; Welch’s t-test) and <0.063mm ($P=0.925$; t-test).

**Figure 2. a:** Interpretation of intercept and slope parameters derived from linear relationships between N depolymerase activity (combined protease and β-glucosaminidase) (as an estimate of the potential to produce depolymerized LMW DON products) and net N mineralization (as a bioassay for actual production of LMW DON) and their comparison between aggregated and disaggregated states to distinguish between the mechanisms responsible for disaggregation-promoted N mineralization.

**Figure 2. b:** Linear regression models between N mineralization rate and total enzyme activity for the GL+AL dataset (n=11). Circles are GL soils and diamonds are AL soils. Regression parameters are given in Table 3.

**Figure 3.** Relationship between N mineralization rate and total enzyme activity for aggregated and corresponding disaggregated GL (a, n=6) and AL (b, n=5) soil.
Table 1 Selected initial mean properties of the constructed grassland (GL) and arable (AL) soils used for N mineralization incubations and enzyme assays.

Concentrations of NH$_4^+$-N and NO$_3^-$-N were determined for soils both prior to (A, aggregated) and after disaggregation (D). Soil properties were determined for aggregated soil with the exception of Total C and N for AL (determined for disaggregated soil) and soil pH (determined for soils passing through a 2 mm sieve). It was assumed that properties for the disaggregated soil, given its derivation, were the same as for the aggregated soil. Figures in parentheses are standard errors.

<table>
<thead>
<tr>
<th>Land Use details</th>
<th>GL soil (n=6)</th>
<th>AL soil (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Land Use details</td>
<td>&gt; 20 years under permanent pasture; in Entry Level Stewardship Scheme.</td>
<td>&gt;10 years under arable (maize/ winter wheat) rotation.</td>
</tr>
<tr>
<td>N fertilizer and tillage</td>
<td>Limited inorganic N fertilizer (&lt; 50 Kg ha$^{-1}$) and no organic N inputs other than addition by grazing heifers.</td>
<td>Regular tillage (ploughing/power harrow) and N fertilizer additions as farm yard manure (~40 t ha$^{-1}$) and foliar feeds.</td>
</tr>
<tr>
<td>Gravimetric Water Content (air-dried soil; %)</td>
<td>6.7 (1.2)</td>
<td>0.8 (0.006)</td>
</tr>
<tr>
<td>Soil pH (1$_{soil}$: 2.5 H$_2$O)</td>
<td>5.95 (0.0946)</td>
<td>6.15 (0.0107)</td>
</tr>
<tr>
<td>NH$_4^+$ (mg-N / kg OD-soil)$^a$</td>
<td>A: 4.03 (0.532)</td>
<td>A: 1.52 (0.104)</td>
</tr>
<tr>
<td></td>
<td>D: 4.48 (0.800)</td>
<td>D: 1.68 (0.109)</td>
</tr>
<tr>
<td>NO$_3^-$ (mg-N / kg OD-soil)$^a$</td>
<td>A: 17.8 (1.76)</td>
<td>A: 27.2 (3.10)</td>
</tr>
<tr>
<td></td>
<td>D: 18.0 (1.70)</td>
<td>D: 26.1 (2.64)</td>
</tr>
<tr>
<td>Total C (g / kg OD-soil)$^b$</td>
<td>58.2 (8.18)</td>
<td>20.8 (0.231)</td>
</tr>
<tr>
<td></td>
<td>$P=0.006^c$</td>
<td></td>
</tr>
<tr>
<td>Total N (g / kg OD-soil)$^b$</td>
<td>6.24 (0.895)</td>
<td>2.00 (0.0311)</td>
</tr>
<tr>
<td></td>
<td>$P=0.005^c$</td>
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</tr>
<tr>
<td>C to N ratio</td>
<td>9.33 (0.142)</td>
<td>10.4 (0.0570)</td>
</tr>
<tr>
<td>Soil texture$^d$</td>
<td>Silt Loam</td>
<td>Sandy Loam</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
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</tr>
<tr>
<td>Clay (%)</td>
<td>3.75 (0.297)</td>
<td>3.48 (0.104)</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>31.72 (3.03)</td>
<td>51.37 (0.670)</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>64.53 (2.74)</td>
<td>45.15 (0.592)</td>
</tr>
</tbody>
</table>

^a^ determined by 1 M KCl extraction and colorimetric continuous flow analysis (Scalar SAN++).

^b^ determined by elemental analysis (Thermo Flash 2000)

^c^ Welch’s t-test

^d^ determined by Laser Granulometry (Mastersizer 3000)
Table 2. The effect of aggregation status on net N mineralization activity, individual (protease and β-glucosaminidase) and total (protease plus β-glucosaminidase) potential N-acquiring enzyme activity and native Folin Ciocalteau (FC)–reactive compounds (i.e. phenolic and other antioxidant chemicals, Everette et al., 2010) in constructed soils. Data are mean ± standard error. Aggregated soils with a capital letter in common do not differ significantly when mean values for GL and AL are compared. For AL protease activity, one replicate out of the five was below detection limits and not significantly different from 0, thus that value was treated as 0.

<table>
<thead>
<tr>
<th>Land use</th>
<th>GL soil (n=6)</th>
<th>AL soil (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aggregate</td>
<td>Disaggregate</td>
</tr>
<tr>
<td>Net N mineralization</td>
<td>7.99 (0.782) A^a</td>
<td>10.6 (1.33)</td>
</tr>
<tr>
<td>(μmol N g⁻¹ soil 240 h⁻¹)</td>
<td>P = 0.016^b</td>
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<tr>
<td>Protease activity</td>
<td>0.298 (0.0324) A^c</td>
<td>0.164 (0.0316)</td>
</tr>
<tr>
<td>(μmol tyrosine equivalents g⁻¹ soil h⁻¹)</td>
<td>P = 0.002^d</td>
<td></td>
</tr>
<tr>
<td>β-glucosaminidase activity</td>
<td>1.09 (0.154) A^a</td>
<td>1.10 (0.183)</td>
</tr>
<tr>
<td>(μmol p-nitrophenol g⁻¹ soil h⁻¹)</td>
<td>P = 0.924^d</td>
<td></td>
</tr>
<tr>
<td>Protease+β-glucosaminidase activity</td>
<td>1.39 (0.180) A^a</td>
<td>1.27 (0.214)</td>
</tr>
<tr>
<td>(μmol (tyrosine equiv. + p-nitrophenol) g⁻¹ soil h⁻¹)</td>
<td>P = 0.232^d</td>
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<tr>
<td>FC-reactive compounds</td>
<td>0.590 (0.0376) A^a</td>
<td>0.642 (0.0469)</td>
</tr>
<tr>
<td>(μmol tyrosine equivalents g⁻¹ soil h⁻¹)</td>
<td>P = 0.191^d</td>
<td></td>
</tr>
</tbody>
</table>

^a Welch’s t test
^b One sample Sign test of median = 0.00 versus < 0.00
^c t-test
^d Paired t-test
Table 3. Coefficients and their P values for regression models shown in Figs. 2b and 3.

<table>
<thead>
<tr>
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<td>Gradient</td>
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<tr>
<td></td>
<td>Intercept</td>
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<td>GL</td>
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<td></td>
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<td>AL</td>
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<td>Intercept</td>
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