Physical influence of microbial communities on the structure and occlusion of particulate organic matter in a sandy agricultural soil

vorgelegt von
Dipl.-Ing.
Frederick Büks
geb. in Berlin

an der Fakultät VI – Planen Bauen Umwelt
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften
- Dr. rer. nat. -

genehmigte Dissertation

Promotionsausschuss:

Vorsitzende: Prof. Dr. Eva Paton
Gutachter: Prof. Dr. Martin Kaupenjohann
Gutachterin: Prof. Dr. Liliane Rueß
Gutachter: Prof. Dr. Ulrich Szewzyk

Tag der wissenschaftlichen Aussprache: 3. Mai 2018

Berlin 2018

“Selbst um auf einem Stein zu sitzen – drei Jahre.”

ZEN-WEISHEIT

Table of contents

List of figures 8

List of tables 9

Abbreviations 11

Acknowledgements 13

Abstract 14

Zusammenfassung 15

1 General introduction 1
1.1 A short scientific history of aggregate structure and genesis 1
1.2 Soil aggregation 5
1.2.1 Binding mechanisms within soil aggregates 5
Physico-chemical interactions 5
Biochemical interactions I: Bacterial extracellular polymeric substances (EPS) 6
EPS and bacterial habitats 9
Aggregates scaffolded by filamentous microorganisms 12
Biochemical interactions II: Fungal glomalin 13
1.2.2 Carbon occlusion within soil aggregates 17

2 Measurement of aggregate stability and C occlusion 21
2.1 The diversity of methods for aggregate stability measurement 21
2.2 Measuring aggregate stability by use of ultrasonication 23
2.3 Measurement of C occlusion 26

3 Aim of this work 30

4 Enzymatic biofilm digestion in soil aggregates facilitates the release of particulate organic matter by sonication 31
4.1 Abstract 31
4.2 Introduction 32
4.3 Materials and methods 36
4.3.1 Soil properties and microbial biomass 36
4.3.2 Detachment scenarios 37
4.3.3 Release of POM carbon 39
4.3.4 Release of bacterial DNA 40
4.3.5 Statistics 41
4.4 Results 41
4.4.1 Release of POM carbon 41
4.4.2 Release of bacterial DNA 43
4.5 Discussion 44
4.6 Conclusions 48
4.7 Acknowledgements 49

5 POM occlusion within sandy soil macroaggregates is not affected by feeding and motion of the nematode *Acrobeloides buetschlii* 51
5.1 Abstract 51
5.2 Introduction 52
5.3 Materials and methods 54
5.3.1 Soil sample 54
5.3.2 Basal respiration 54
5.3.3 Preparation of the inoculum 55
5.3.4 Nematode population development 55
5.3.5 Measurement of POC release 56
5.3.6 Phospholipid fatty acid (PLFA) extraction and analysis 56
5.3.7 Mercury intrusion 58
5.3.8 Statistics 59
5.4 Results 59
5.4.1 Soil respiration 59
5.4.2 Population development 60
5.4.3 POC release 61
5.4.4 PLFA analysis 62
5.4.5 Mercury intrusion 64
5.5 Discussion 64
5.6 Conclusions 68
5.7 Acknowledgements 68

6 Two different microbial communities did not cause differences in occlusion of particulate organic matter in a sandy agricultural soil 69
6.1 Abstract 69
6.2 Introduction 70
6.3 Materials and methods 73
6.3.1 Preparation of soil and soil extracts 73
6.3.2 Incubation and sampling 74
6.3.3 DNA extraction and qPCR 75
6.3.4 Disaggregation of soil aggregates and quantification of POC 77
6.3.5 Statistical analyses 77
6.4 Results 78
6.4.1 Microbial population analysis 78
6.4.2 POC release 81
6.5 Discussion 82
6.6 Conclusions 85
6.7 Acknowledgements 85

7 Synthesis and conclusions 87
7.1 Single experiments 87
7.2 Statistical restrictions 89
7.3 General conclusion 90
7.4 Transferability to other soils 92
7.5 Future research 92

References 95

List of figures

Fig. 1: Idealized binding pattern of hierarchical and non-hierarchical soil aggregates 4

Fig. 2: Extracellular polysaccharides, DNA, proteins and lipids within soils 8

Fig. 3: A model of soil aggregate structure on different scales 16

Fig. 4: Proposed model of inner soil aggregate structure including EPS 33

Fig. 5: Relative POC release after enzyme treatments 42

Fig. 6: Relative bacterial DNA release from soil aggregates after enzyme treatments …. 43

Fig. 7: Soil respiration of samples with 0, 50, 70 and 80% water holding capacity 60

Fig. 8: Population development of nematodes 61

Fig. 9: Relative POC release of samples with *A. buetschlii* and controls 62

Fig. 10: Amount of group specific PLFAs in soil aggregate samples 63

Fig. 11: Cumulative pore space diagram of macro-aggregate samples 64

Fig. 12: pF-bioreactor with its components 74

Fig. 13: DNA conc. of phylogenetic classes and domains in SP_{soil} and SP_{air} 80

Fig. 14: Relative POC release of the variants SP_{soil}, SP_{air}, $SP_{control}$ 82

List of tables

Table 1: Collection of frequently used and/or promising methods for aggregate stability measurement 23

Table 2: Concentrations and molar masses of biofilm stabilizing macromolecules in different environments 35

Table 3: Variables, that are used for the calculation of enzyme units needed for biofilm target decomposition 38

Table 4: Specific scenario parameters of the enzyme treatments 39

Table 5: List of group specific PLFAs used in the present study 58

Table 6: Target classes and domains, appropriate primer pairs, annealing temperatures (AT) and standard organisms for qPCR 76

Table 7: Applied primer sequences for class- and domain-specific qPCR 76

Table 8: Measured eubacterial class DNA of SP_{air} and SP_{soil} variant in relation (%) to total eubacterial DNA 81

Abbreviations

Abbreviation	Full term
Å	Ångström (0.1 nm)
AMF	Arbuscular mycorrhizal fungi
ANOVA	One way analysis of variance
ARW	Artificial rainwater
C	Carbon
COM	Colloidal organic matter
CPOM	Co-precipitated organic matter
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
eDNA	Extracellular DNA
EEG	Easily extractable glomalin
EPS	Extracellular polymeric substances
Eub	Eubacteria(l)
FID	Flame ionization detector
fwd	Forward (Primer)
GRSF	Glomalin related soil fraction
fLF	Free light fraction
GC/MS	Gas chromatography/mass spectroscopy
GRSP	Glomalin related soil protein
HF	Heavy fraction
IREEG	Immonoreactive easily extractable glomalin
LF	light fraction
MOM	Mineral-associated organic matter
MWD	Mean weight diameter
N	Nitrogen
oLF	Occluded light fraction
OC	Organic carbon
OM	Organic matter
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PLFA	Phospholipid-derived fatty acids
POC	Particulate organic carbon
POM	Particulate organic matter
qPCR	Quantitative real-time PCR
R2A	Reasoner's 2A growth medium
rev	Reverse (Primer)
rpm	Rounds per minute
SOC	Soil organic carbon
SOM	Soil organic matter
SPT	Sodium polytungstate
Su3	Silty sand (Bodenkundliche Kartieranleitung, 2005)
TEM	Transmission electron microscopy
U	Enzyme unit (conversion of 1 µmol substrate per minute)
UDF	Ultrasonication/density fractionation
UV	Ultraviolet
WSA	Water stable aggregates

Acknowledgements

I am deeply grateful to

- our laboratory assistants Claudia Kuntz, Kotan Yildiz, Maike Mai, Monika Rohrbeck, Ruth See, Sabine Dumke and Sabine Rautenberg
- Prof. Martin Kaupenjohann, who supervised this work
- Prof. Ulrich Szewcyk for the unbureaucratic possibility to use the laboratories of the chair of environmental microbiology
- the helpful staff of the chair of soil protection (TU Berlin)
- Prof. Liliane Rueß for her knowledge about nematodes
- Dr. Peter Lentzsch and M.Sc. Philip Rebensburg for their perseverance
- M.Sc. Patrick Reger and B.Sc. Lara Schneider for their theses and our former students Marcus Bork, Kathrein Fischer, Christine Hellerström, Katja Jung, Annabelle Kallähne, Paula Nitsch, Susann-Elisabeth Schütze, Anne Timm and Karolin Woitke for their help in soil sampling, preparation and pre-trials.
- Tom Grassmann for his enthusiasm to explain me how to handle R
- the Leibnitz-Gemeinschaft who financially supported this project (SAW Pact for Research, SAW-2012-ATB-3)
- and all the people, who have been patient with me talking again and again about soil aggregates and bacterial slime between primary particles.

They all were responsive and kind to help me each time I had problems or need for discussion.

Abstract

Soil aggregate stability is an integral marker of soil fertility. Well aggregated soil contributes to high rootability, a proper water and aeration regime, resistance against compaction and erosion as well as storage capability of organic carbon. The aggregation of soil primary particles and microaggregates is promoted not only by a large variety of physico-chemical but also biological interactions, that are based on e.g. the excretion of bacterial extracellular polymeric substance (EPS) or fungal glomalin, concretion of biomineralization products or adsorption of hydrophobic cell debris on soil particle surfaces. Hence, microbial growth, exudation and grazing of bacterial-feeding organisms on sessile prokaryotes – most living within a viscouse EPS matrix (biofilms) – are assumed to play a regulating role in the occlusion of POM within soil aggregates. In consequence, microbial communities would have physical influence on organic matter cycling e.g. in agricultural soils.

In the present doctoral thesis, a sandy agricultural soil was treated with EPS degrading enzymes, the bacterial feeding nematode *Acrobeloides buetschlii* or two different non-converging microbial communities. After these treatments, respective changes of POM occlusive strength were measured.

Results show no or little influence of microbial communities living in pores above the lower mesopore scale (~10 µm) on the POM occlusive strength, whereas the applied methods were not suitable to affect organisms within smaller pores. If there is a markable stabilizing effect of microbial processes, it is located within the finer mesopores as indicated by current literature. The present work highlighted this indication from a new point of view. Future research should focus on the role of EPS as an aggregation agent within small mesopores.

Zusammenfassung

Die Aggregatstabilität in Böden ist ein integrales Merkmal von Bodenfruchtbarkeit. Gut aggregierter Boden unterstützt Durchwurzelbarkeit, Wasser- und Luftversorgung, die Widerstandsfähigkeit gegen Verdichtung und Erosion sowie seine Fähigkeit organischen Kohlenstoff zu speichern. Zur Aggregierung von Primärpartikeln und Mikroaggregaten trägt eine große Vielfalt nicht nur physico-chemischer sondern auch biologischer Wechselwirkungen bei, die z.B. auf die Ausscheidung bakterieller extrazellulärer polymerer Substanzen (EPS) oder pilzlichen Glomalins, auf Biomineralisationsprodukte oder die Adsorption hydrophober Zellfragmente an Bodenpartikel zurückgehen. Daher kann angenommen werden, dass mikrobielles Wachstum, Exsudation und die Beweidung sessiler Prokaryoten, von denen die meisten in einer viskosen EPS-Matrix leben (Biofilm), einen regulierenden Einfluss auf die Stärke der POM-Okklusion in Bodenaggregaten haben. Folgerichtig hätten mikrobielle Gemeinschaften einen physikalischen Einfluss auf den Kohlenstoffkreislauf z.B. in Ackerböden.

In dieser Arbeit wurde sandiger Ackerboden mit EPS-verdauenden Enzymen, der auf bakteriellen Biofilmen weidenden Nematodenart *Acrobeoloides buetschlii* sowie verschiedenen nicht konvergierenden mikrobiellen Gemeinschaften behandelt. Nach der Inkubation wurde die jeweilige Veränderung der POM-Okklusionsstärke gemessen.

Die Ergebnisse zeigen wenig bis keinen Einfluss der im oberen Mesoporenbereich (< 10 µm) lebenden microbiellen Gemeinschaften auf die POM-Okklusionsstärke, während die verwendeten Methoden nicht geeignet waren, Organismen in feineren Poren zu erreichen. Sofern ein bedeutender stabilisierender Effekt durch mikrobielle Prozesse existiert, ist dieser im unteren Mesoporenbereich verortet, wie in der aktuellen Literatur angedeutet wird. Diese Arbeit hebt jenen Effekt aus einem neuen Blickwinkel hervor. Zukünftige Arbeiten sollten ihren Fokus auf die Rolle von EPS als Aggregierungsmittel im unteren Mesoporenbereich legen.

1 General introduction

1.1 A short scientific history of aggregate structure and genesis

For nearly 90 years soil structural stability gained attention in research on soil properties. Early works concerning “soil friability”, “soil consistency” and “soil aggregate stability” were aimed at a more precise assessment of soil plowability for the enhancement of plant production (Russell, 1928; Christensen, 1930). In the following decades, soil aggregate stability attained increasing importance as positive condition for plant growth and resistance against soil compaction e.g. by large agricultural machinery (Rosenberg, 1964). In some of the first trials focusing on aggregates, the crushing strength of dry soil aggregates was determined by Martinson et al. (1950), and Greacen (1960) provided evidence for increasing plastic deformation in soil aggregates with increasing water content leading to reduced aggregate stability.

In a cutting-edge work Edwards and Bremner (1967a) postulated, that large soil aggregates (>250 µm) are composed of smaller aggregates, which again are formed by mineral and organic primary particles and molecules. With the underlying experiment the authors could show, that small mechanical stress leads to disaggregation of large soil aggregates, whereas aggregates <250 µm have a higher structural resistance, which is, however, reduced by the removal of polyvalent cations and oxidation of water-insoluble organic substance. Edwards and Bremner (1967a) postulated, that microaggregates are stabilized by clay-humus interaction and are bricks of less stable macroaggregates. This hypothesis became the foundation of later works about aggregate stability and structure.

From another point of view, based on a “theory of statistical brittle fracture”, Braunack et al. (1979) hypothesized a decreasing stability of soil aggregates with increasing volume. If mechanical stress reaches a critical level, the structure of a soil volume element is no longer able to deflect forces (e.g. by plastic deformation) and thereupon cracks in its weakest flaws. As large aggregated soil volumes statistically contain more weak points, their stability would be reduced compared to smaller volumes. The resulting proportional

correlation between aggregate volume and susceptibility to mechanical stress was found for soils observed in the named study.

The observations support a model of Dexter (1988), that proposes larger aggregates to be built of interlaced even smaller aggregates down onto the level of primary particles. In this aggregate hierarchy model aggregates of a higher order do not only contain aggregates of lower order but also interjacent voids (and flaws) – so to speak smaller aggregates *exclude* voids of the higher hierarchy level. This “porosity exclusion principle” was supposed to lead to a lower stability of aggregates with high porosity (mostly large aggregates) compared to such with low porosity.

Oades and Waters (1991) fortified the hypothesis of an aggregate hierarchy (Fig. 1) in face of mechanical stress. They could show, that in one group of soil types successively enhanced mechanical stress first leads to a fracturation of larger and than finer aggregates (hierarchical behavior). Surprisingly, another group of soil types – mainly stabilized by inorganic components (Tisdall, 1996) – shows an increasing fracturation of aggregates of all size-classes with increasing mechanical stress pointing to an absence of aggregate hierarchy. In addition, the authors could show differing structural and chemical composition in differently sized aggregates: In hierarchical soils larger aggregates contain a higher content of organic matter (OM) and a higher C:N ratio than small aggregates, whereas non-hierarchical soils do not show any differences between aggregate sizes. Furthermore, electron microscopic pictures of aggregates <200 µm in diameter show cores of plant debris, whereas aggregates <90 µm contain increasingly degraded POM or voids instead. Aggregates <20 µm miss nearly any POM and have a low C:N ratio.

These results motivate a classification into macro-aggregates (>250 µm), micro-aggregates (<250 µm) and “small” microaggregates (<20 µm) (Tisdall, 1996): Smallest sub-units <0.2 µm are composed of flocculated clay particles or associations of clay particles, that are linked to humic substance by polyvalent cations. Those sub-units are aggregated in association with silt particles, partly humified bacterial debris and bacterial colonies, hyphal fragments and mineral incrustations. They contain nearly any POM but molecular organic matter of bacterial origin. Such smaller aggregate structures can be reasonably summarized to a class of “small” microaggregates <20 µm and are characterized by a high stability in face of rainfall, tillage and ultrasonication. In contrast,

microaggregates of diameters between 20 and 250 µm build up another structural class. They are composed of “small” microaggregates, that are linked and embedded by the chemical components listed above. But in addition, occlusion of small POM, which has a lower degree of decomposition than the molecular OM, increases the C:N ratio. These larger microaggregates are not stable in face of ultrasound. Within these aggregates, the grade of degradation of POM as well as water stability increase with decreasing aggregate size. The excretion of macromolecular EPS by inhabiting bacteria is assumed to play a role in whole microaggregate stabilization (Chenu and Stotzky, 2002).

In most agricultural soils, macroaggregates (>250 µm) consist of microaggregates. Although physico-chemical interactions play a linking role at the contact points of contained microaggregates, wrapping by fine roots, fungal hyphae and bacterial pseudomycelia are supposed to be important for macroaggregate stabilization (Molope et al., 1987; Six et al., 2004; Chenu and Cosentino, 2011). However, these aggregates are more water-labile than microaggregates and highly prone to mechanical stress. In contrast, non-hierarchical aggregates >250 µm react very differently: As Oades and Waters (1991) found for Oxisols, the whole macroaggregates are stable in face of high mechanical stress. This can be explained by both a dominance of mineral interactions (Oades and Waters, 1991) and a statistical lack of porosity exclusion (Dexter, 1988).

Based on data of the chemical composition of hierarchical and non-hierarchical aggregates (Oades and Waters, 1991), aggregate hierarchy gained a new level of complexity: Like formally supposed by Edwards and Bremner (1967a) and Dexter (1988), hierarchy in face of mechanical stress is attended by specific geometric and chemical properties. In consequence, flaws are not randomly distributed within the aggregate but theoretically predictable.

As, on the one hand, there is a shared understanding about aggregate structure, two fundamentally different but not contradictory theories about aggregate genesis are under consideration. In the first mechanistic model (Tisdall and Oades, 1982), aggregate formation appears ascending from associations of clay particles, organic matter and polyvalent cations, that build minor aggregates, interspersed with additional mineral components and microbial exudates. These secundary particles aggregate to microaggregates of 20 to 250 µm and finally form macroaggregates. In contrast to

microaggregates, which are mainly stabilized by physico-chemical interactions, macroaggregates stick together by biological entanglement. In the second model (Jastrow, 1996), macroaggregates of mineral and organic primary particles form around an particulate organic core. The following “consumption” of the macroaggregate, consisting of microbial decomposition and desintegration of the core and the excretion of metabolic products, lead to regions of stable physico-chemical bonds between organic and mineral particles and to the formation of more stable microaggregates within the macroaggregate. This mechanism was confirmed by Gale et al. (2000). Both the ascending and the consuming aggregate formation most likely appear together in a dynamic soil system (Six et al., 2000), as minor microaggregates could take part in macroaggregate formation around organic cores and consumed macroaggregates dissociate to microaggregates which in turn could assemble to macroaggregates by means of biotic enmeshment.

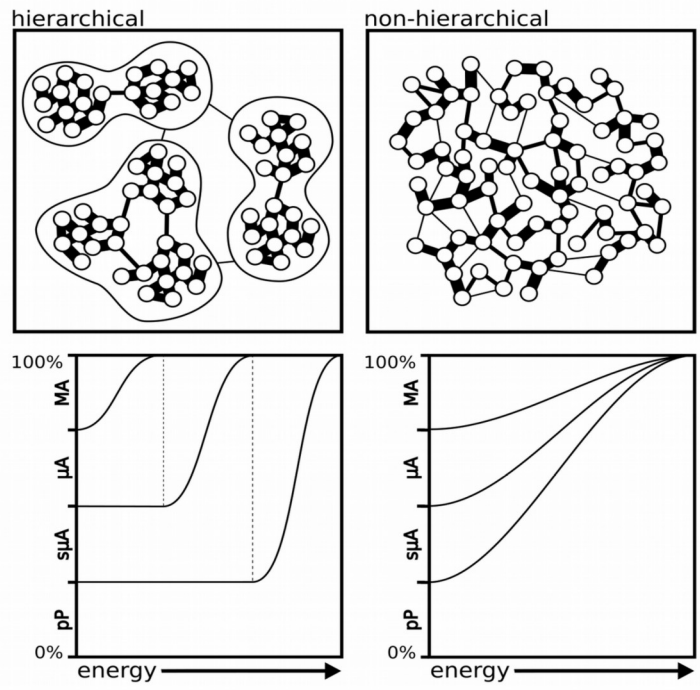

Fig. 1: Idealized binding and dispersion pattern of hierarchical and non-hierarchical soil aggregates. Circles emblematize small microaggregates (sµA), curves stand for macroaggregate (MA) borders and link thickness symbolizes binding strength between adjacent particles. In hierarchical aggregates, an increasing amount of mechanical energy input leads to stepwise disaggregation (%mass) of macroaggregates to microaggregates (µA), small microaggregates and finally primary particles (pP). In contrast, non-hierarchical aggregates show simultaneously increasing disaggregation of all aggregate size classes in face of increasing energy. (CCL by-nc-sa, Frederick Büks 2017)

1.2 Soil aggregation

1.2.1 Binding mechanisms within soil aggregates

In conclusion of the previous chapter, most soils have an aggregate hierarchy regarding destructive stress, which reflects the physico-chemical properties of the soil and hence soil performance. Soil aggregates are composed of manifold components that influence these physico-chemical properties. Silicates of the sand, silt and clay fraction, oxides and hydroxides of Fe, Al and Mn, phosphates and – in (semi)arid soils – carbonates are the major inorganic constituents (Bronick and Lal, 2005). Beside biological structures like bacterial colonies, bacterial pseudomycelia, fungal hyphae and roots (Six et al., 2004), the organic fraction within soil aggregates comprises molecular and precipitated organic components like humins, cell exudates and decomposed products as well as particulate organic matter including detritus and black carbon (Bronick and Lal, 2005; Brodowski et al., 2006a; Lützow et al., 2006). The interaction of these components is supposed to play different roles on soil aggregation depending on the hierarchical level. A current model of aggregate geometry based on the following binding mechanisms is pictured in Fig. 3.

Physico-chemical interactions

As suggested by Edwards and Bremner (1967a), aggregation of primary particles is mediated by organo-mineral interactions: Clay-humus complexes linked by polyvalent cations are supposed to play a fundamental role in the formation of smallest aggregates (<20 µm), since after oxidation of organic matter the removal of polyvalent cations leads to a total dispersion of clay soils. However, there is a broad range of physico-chemical mechanisms linking primary particles and molecules. Permanent and variable surface charge of clay minerals, metal oxides and hydroxides as well as variable charge of POM cause adsorption of charged organic and inorganic fractions. Multivalent cations with small hydrate shells like Ca^{2+}, Fe^{3+} and Al^{3+} increase the aggregate stability by coagulation of clay mineral particles, whereas low charged small cations with a large shell take effect in opposite direction. Furthermore, high charged cations mediate bindings between organic molecules and clay, especially clay-humus complexation, or further negative charged surfaces like those of metal oxides and hydroxides. Organo-metallic complexes of Fe^{3+} and Al^{3+} with DOM precipitate at low pH. Precipitates of slightly soluble minerals like Fe^{3+}

and Al^{3+} hydroxides and Ca^{2+} phosphates cause cementation of primary particles and microaggregates due to coating and increase the aggregates' shear resistance. Under (semi-)arid conditions, readily soluble minerals like calcium or magnesium carbonates show the same effect. (Bronick and Lal, 2005)

Although found to be an important agent of soil aggregation, humic substance is an extensive and vague term including a broad range of organic matter coming from highly different metabolic pathways. Among various SOM from decomposing processes, macromolecular exudates of bacteria, archaea, fungi and plants play a major role in aggregate stabilization (Traoré et al., 2000; Chenu and Cosentino, 2011). These exudates and other biological binding agents are in focus of the following sections.

Biochemical interactions I: Bacterial extracellular polymeric substances (EPS)

Slipping on stones on a river bank, complaining about mucus of a severe cold, marveling about colorful microbial mats in volcanic hot springs and being pleased about the efficacy of biological waste water treatment – we are faced to the wide abundance of bacterial biofilms (Costerton et al., 1995). Bacteria represents approximately 1/3 of the biomass in soil ecosystems (Foster, 1988). It is supposed, that most of these bacteria live on surfaces, protected by a matrix of homemade extracellular polymeric substance (EPS), making these so called biofilms an ubiquitous mode of procaryotic life (Davey and O'toole, 2000). Using histochemical staining, Foster (1988) showed intra-aggregate bacteria in soil protected within an EPS capsula. But also archaea, fungi and algae are able to produce extracellular polymers (Lewin, 1956; Rillig, 2004; Fröls, 2013) and partly live in – also syntrophic – communities with sessile bacteria (Riding, 2000; Wargo and Hogan, 2006; Stams and Plugge, 2009).

Prokaryotic biofilms consist of up to 97% water (Zhang et al., 1998; Schmitt and Flemming, 1999; Pal and Paul, 2008). The dry mass is composed of 10 to 50% cell biomass and 50-90% EPS matrix components comprising polysaccarides, extracellular DNA (eDNA), lipids, proteins, humic and low-molecular substances (Flemming and Wingender, 2010; More et al., 2014). Especially polysaccharides and eDNA have a large influence on biofilm viscosity and stability, but also proteins are known as stabilizing agent, while lipids show hydrophobic interface effects (Flemming and Wingender, 2010).

Early works describe extracellular macromolecules with focus on capsule or slime polysacchrides of bacteria (Stacey, 1947; Wilkinson, 1958). As investigated in recent works, these extracellular polysaccharides comprise 40 to 95% of the EPS dry mass (Flemming and Wingender, 2001), concentrations of about 169 to 401 µg g^{-1} dry soil with high variations between soil types (Redmile-Gordon et al., 2014) and molecular masses between $0.5x10^6$ and $2x10^6$ Da. They contribute to the viscosity of the moist extracellular matrix by entanglement and mediate attachment of EPS and cells to surfaces. This effect is enhanced by polyvalent cations (e.g. Ca^{2+}), which link polysaccharide strands by bilateral binding to hydroxy groups (-OH). (Flemming and Wingender, 2010)

Similar mechanisms underly the stabilizing effect of extracellular DNA (eDNA) on EPS matrix viscosity (Flemming and Wingender, 2010; Das et al., 2013; Das et al., 2014). Extracellular DNA appears both exudated and released from lysed cells, is not distinguishable from genomic DNA (Das et al., 2013) and has typical contents of 1 to 10% of the dry EPS matrix (More et al., 2014), molar masses of $7.75x10^4$ to $2.32x10^7$ Da (DeFlaun et al., 1987) and highly variable concentrations between 0.03 and 200 µg g^{-1} dry soil depending on soil type and extraction method (Frostegård et al., 1991; Niemeyer and Gessler, 2002; Agnelli et al., 2004; Pietramellara et al., 2009).

Extracellular proteins comprise two well known functional classes regarding biofilm viscosity and structure. (1) Structural proteins provide specific binding sites and build linkages among macromolecules (e.g. polysaccharides and eDNA) and with surfaces (Flemming and Wingender, 2010). (2) Extracellular enzymes, on the other hand, work as agents against other microorganisms, as metabolic enzymes or as instrument for restructuring the biofilm matrix e.g. when adverse chemical gradients or a lack of nutrients appeared (Donlan, 2002; Stewart and Franklin, 2008; Flemming and Wingender, 2010). As it is impossible to extract these functional classes selectively, molecular data – e.g. the average share of 60% on the EPS dry mass with extrema of 75% (Jahn et al., 1999; More et al., 2014) and concentrations in different soils of 43 to 163 µg g^{-1} dry soil (Redmile-Gordon et al., 2014) – are potentially more widespread than only for extracellular structural proteins.

Extracellular lipids are mainly biosurfactants and contribute to the attachment and adhesion of cells to surfaces (Flemming and Wingender, 2010). Actually, lipids are not

numbered among macromolecules, since their molecular mass spans 750 to 1500 Da (Abröll et al., 2008). However, lipids are major constituents of EPS dry mass (10%) (More et al., 2014), whereas concentration data in soils were not found in the actual literature.

In contrast to polyvalent cations, polysaccharides, eDNA, proteins and lipids, little is known about the function of humic substances within biofilms (Flemming and Wingender, 2010). Furthermore, the collected data of extracellular macromolecules are rare (Fig. 2), partly from non-soil ecosystems, contradictory and highly dependent on the extraction method (Redmile-Gordon et al., 2014). Therefore they only allow rough estimations about the abundance of EPS components in soil ecosystems.

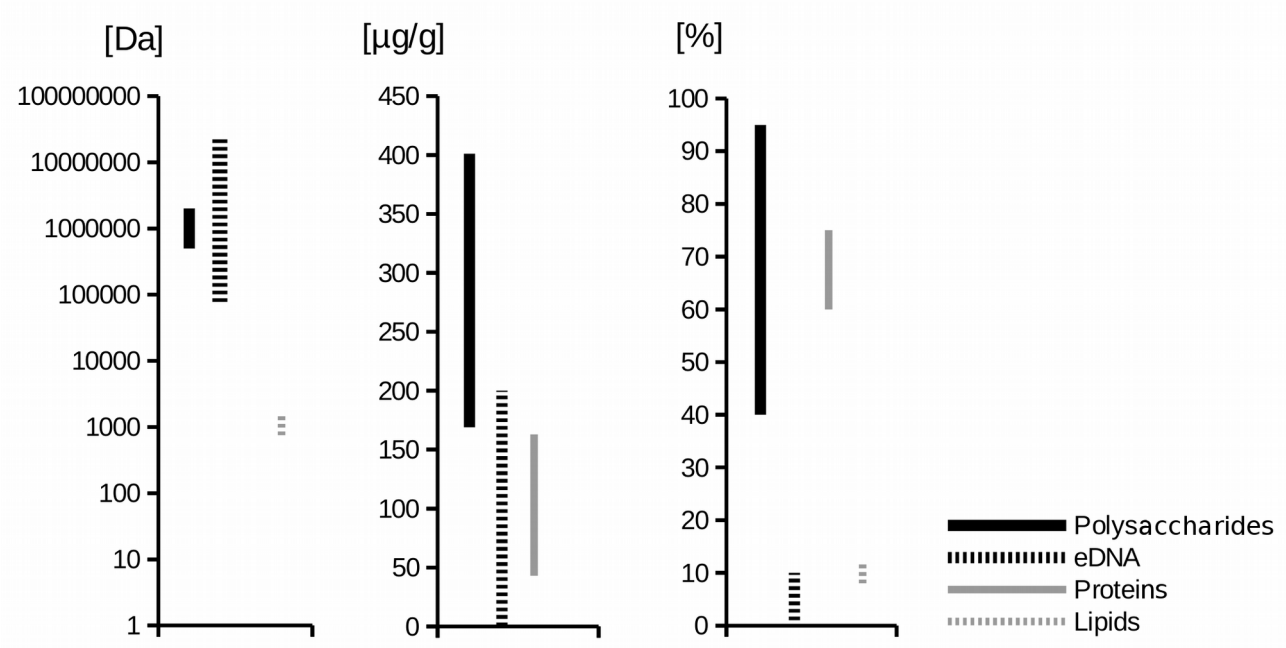

Fig. 2: Extracellular polysaccharides, DNA, proteins and lipids in soils with spans of molar masses, concentrations in soil and shares in dry EPS. Data are yet rare and have to be specified to soil type and extraction method in future research. (CCL by-nc-sa, Frederick Büks 2017)

The viscose biofilm habitat provides a bunch of protective, genetical and community services for its inhabitants. In addition to adhesion and cohesion on surfaces, which is in focus of the present work, manifold other functions are achieved by EPS capsulae. The supply of a dehydration barrier, storage for organic and inorganic nutrients and an extended extracellular space for catabolic activity, protection against disinfectants,

biocides, antibiotics, oxidation stress, UV radiation, grazing organisms and immune system cells as well as the fostered inter-cell communication, syntrophy and horizontal gene transfer are just outstanding examples (Flemming and Wingender, 2010).

Mathematical modeling and foregoing investigations showed the biofilm matrix to be an viscoelastic fluid, that is able to withstand mechanical forces (Klapper et al., 2002). Strong adhesion of biofilm-occluded bacteria on surfaces in face of mechanical stress was demonstrated by Böckelmann et al. (2003). As the growing biofilm within the narrow pore space is connected to surfaces of different particles, interconnection between soil particles most likely fosters soil aggregation. The direct mechanical contribution of biofilms to aggregate coherence was shown in laboratory trials with artificial (Czarnes et al., 2000) and native EPS (Geoghegan and Brian, 1948). However, a general and direct influence of EPS on soil aggregation and the underlying regulatory processes are controversial. For example, Martens and Frankenberger Jr (1992) and Tang et al. (2011) found aggregate stability influenced by bacterial growth, but without direct attribution to microbial polysaccharide production. Furthermore, the rheological properties of EPS strongly depend on its chemical composition, which in turn is a result of microbial composition and environmental conditions (Marty et al., 1992; Béjar et al., 1998; Steinberger and Holden, 2005; Simoes et al., 2007; Ayala-Hernández et al., 2008; Celik et al., 2008). However, in cases of direct aggregate stabilization due to EPS, fixation of small organic particles within microaggregates is conceivable. Even coarse POM might be occluded within attached microaggregates and primary particles, leading to the assumption, that effective influence of EPS on aggregate formation also have influence on POM occlusion.

EPS and bacterial habitats

Within the soil matrix bacteria appear in preferred habitats. As shown by Hissett and Gray (1976), a majority of bacteria from sandy soils is located in or on POM with similar abundances in further soils (Kerek et al., 2002). Kanazawa and Filip (1986) found, that bacteria are highly abundant in coarse organic particles and the silt and clay fraction <50 µm. In contrast, Chenu and Stotzky (2002) also reported dense bacterial growth and attachment even on sand grains, which underpins the bacterial potential to colonize surfaces of particles with various size and composition. However, Ranjard and Richaume (2001) reviewed, that the majority of soil bacteria is located in the inner part of soil

aggregates, mainly in micropores <9 µm within microaggregates <100 µm in sandy soils and within microaggregates <20 µm in clayey soils. Within these pores, single cells or microcolonies normally do not fill the whole porespace to allow gas, water and nutrient exchange (Foster, 1988). Probably as a result of mechanically induced reshaping of soil structure, also micropores <1 µm were colonized by bacteria (Foster, 1988). Also Nunan et al. (2003) analyzed porosity, bacterial population density and distribution in 30 to 40 µm thin-cuts of cropped topsoil, fallowed topsoil and subsoil of a sandy agricultural soil. The population density is reduced on the surface of inter-aggregate pores (>30 µm) and rises towards the inner aggregate with increasing organic nutrient support. In addition to Foster (1988), the authors found, that a decreasing nutrient support (cropped topsoil > fallowed topsoil > subsoil) leads to a retreat of bacteria from the aggregate's core. Higher population densities point to higher rates of growth due to increased nutrition, but are very low, leading the author to assume a general lack of biofilms within sandy arable soils. This interpretation could also be gathered from TEM pictures (Foster, 1988), but strongly conflicts to established suppositions.

Beside surface properties of soil particles and taxonomic properties of the colonizers, the spatial differentiation of bacterial densities is influenced by different factors as there are accessibility of water, gradients of oxygen and nutrients as well as grazing pressure. Being aquatic organisms, bacteria are restricted to saturated pores or water films on particle surfaces (Chenu and Stotzky, 2002). Saturation is more likely given in smaller pores within microaggregates than in draining mesopores. On the other hand, diffusion of nutrients and metabolites is decreasing with pore size diameter, leading to low nutrient supply and metabolite removal and hence decreased microbial growth rates within microaggregates compared to larger mesopores (Chenu et al., 2001). Furthermore, within small pores bacteria are protected against grazing. Predators such as large protozoa and grazers like certain soil Nematodes are restricted to accessible pore space (Wallace, 1958), whereas small predators such as amoebae, flagellates and small ciliates are able to enter the inner pore space of soil aggregates (Vargas and Hattori, 1991). In consequence, bacterial-feeding protozoa mediate a nutrient flow from the inner pore space to larger predators (Bonkowski, 2004). In addition to spatial inaccessibility and EPS encapsulation, Hattori (1970) showed strong adhesion of clay particles on *E. coli* in laboratory cultures. Thus,

bacterial cells and microcolonies can be surrounded by a coating of clay particles that hinders predation and grazing (Chenu, 1995).

In consequence, soil bacteria basically show two different ways of interaction with predators and grazers, which explain higher bacterial densities in microaggregates: (1) A life in small pores around 10 µm within soil microaggregates, protected against drought and feeding by spatial exclusion, clay-coating, EPS and taxonomic inedibility, but limited by low diffusion of nutrients and metabolic products and therefore slowly growing in established mature and stable biofilms. And (2) a life in larger pores, on surfaces of aggregates and non-aggregated particles, with sufficient nutrient supply, able to dispose metabolic waste, but highly susceptible to grazing, what results in high growth rates and young biofilms (Chenu et al., 2001).

The composition of bacterial communities in different soil compartments is often analyzed by use of ecotyping, performed with quantitative real-time PCR (qPCR). The taxonomic level of class/phylum is assumed to be sufficient to depict changes of relevant ecological processes and qualities in the soil, e.g. SOM cycling. (Von Mering et al., 2007; Fraser et al., 2009; Philippot et al., 2010; Rousk et al., 2010)

Of known bacterial phyla, Gemmatimonadetes, Actinobacteria and Verrucomicrobia are found to be mainly present in inner micro-aggregates (Kanazawa and Filip, 1986; Drążkiewicz, 1994; Ranjard and Richaume, 2001; Mummey and Stahl, 2004; Mummey et al., 2006). Other data show that Actinobacteria are also abundant or preferentially live in outer parts of soil aggregates or on coarse organic particles (Kanazawa and Filip, 1986; Drążkiewicz, 1994; Ranjard and Richaume, 2001; Mummey et al., 2006). Depending on soil type, representatives of Firmicutes and Proteobacteria (e.g. α- and γ-Proteobacteria) are suggested to prefer settling at the border between micro- and macroaggregates – just like Cyanobacteria – or within the inner microaggregates (Ranjard and Richaume, 2001; Mummey and Stahl, 2004; Mummey et al., 2006). Nitrifyers – taken as a metabolic but not a taxoniomic group – are mainly found in the <20 µm fraction, but are hardly present in macroaggregates (Lensi et al., 1995; Ranjard and Richaume, 2001), whereas Drążkiewicz (1994) also found them to be abundant in macroaggregates. In contrast, Acidobacteria are enriched within macroaggregates with decreasing abundance towards inner microaggregates. This leads to the assumption, that they are loosely attached and play a

minor role in aggregate stabilization. Data on Bacteroidetes and Chloroflexi were too sparse for evaluation (Mummey et al., 2006) and data on the distribution of other bacterial phyla (Tenericutes, Chlorobi, Fusobacteria, Nitrospirae, Spirochaetes, Synergistetes, Chrysiogenetes, Deferribacteres) as well as Archaea were not found. Furthermore, the transfer of these single-moment results to the assessment of field trials has to be done carefully, since the distribution of bacteria in soil is not static but changing with environmental conditions (Fierer et al., 2003; Griffiths et al., 2003). However, due to a lack of sufficient data about specific taxa and soil types, the relation of certain taxa to soil aggregation processes and levels of the aggregate hierarchy is not sufficiently understood.

Aggregates scaffolded by filamentous microorganisms

On the macroaggregate scale, fine roots and hyphae grow into accessible pores and thereby wrap microaggregates and primary particles. Both types of filaments produce a coating of macromolecular exudates and cell wall components, e.g. polysaccharides, that establish sticky links to the surfaces of adjacent particles. As a result, microaggregates and primary particles are interconnected to macroaggregates by a sticky string bag of roots and hyphae. The attachment of fine particles and precipitates cause an incrustation of this bag. As physico-chemical links between microaggregates are weaker than such within, this filamentous network significantly provides the formation of water-stable macroaggregates and, in turn, disturbance of fungi and roots by tillage or chemical agents, e.g. pesticides, lowers macroaggregate stability (Bossuyt et al., 2001).

As an additional mechanism, the growth-related pressure of hyphae and roots supports aggregation of soil particles by relocation and compaction. Furthermore, especially arbuscular mycorrhizal fungi (AMF) positively influence aggregation not only directly, but also provides root growth due to fungi-plant symbioses (Miller and Jastrow, 1990). In addition, fine roots and hyphae take up water and dry the adjacent soil, which enhance the mechanical stability of soil aggregates. (Tisdall, 1996)

Compared to fungi, filamentous bacteria have 10-fold smaller hyphal diameters, but also connect soil particles (Tiessen and Stewart, 1988). Actinobacteria are suggested to play a role in microaggregate formation (Mummey et al., 2006). However, their influence on aggregate stability and POM occlusion is unknown.

Biochemical interactions II: Fungal glomalin

In the last two decades, a macromolecular substance of fungal origin became a focus of research on soil structure. This substance, Glomalin related soil protein (GRSP), comprises a group of highly hydrophobic, strongly adhering and persistent glycoproteins primarily produced by AMF (Wright et al., 1996; Rillig, 2004). Originally isolated as a target of monoclonal antibodies for immunofluorescence detection of specific growing AMF hyphae on roots (Wright et al., 1996), it shaped up as an only operationally defined group of proteinaceous soil organic matter, that is coextracted with large excess of humic acids (Schindler et al., 2007). A lack of knowledge about the relation of GRSP components and functions recommends a broader denotation, e.g. glomalin related soil fraction (GRSF) (Rillig, 2004).

The GRSF is suspected to play a role in soil aggregation. In an experimental study on a variety of 37 soils across the United States and Scotland, Wright and Upadhyaya (1998) demonstrated a correlation of glomalin concentration and stability of soil aggregates, that saturates at >80% water stable aggregates (WSA). This relation was confirmed by other surveys in different climate zones (Rillig et al., 2002; Bedini et al., 2009; Hontoria et al., 2009; Spohn and Giani, 2010; Fokom et al., 2012; Wu et al., 2014). Furthermore, in a comparison of different crop rotation systems, Wright et al. (1999), Wright and Anderson (2000) and Fokom et al. (2012) found linear correlation between glomalin and aggregate stability as well as a positive effect of non-tillage management on aggregate stability compared to classical tillage. Wright et al. (2007) showed that the major part of GRSF in a non-tillage agricultural ultisol is concentrated in the macro-aggregate fraction, whereas treated soils contain most of their GRSF in the microaggregate and fine fraction. It was also demonstrated that tillage decreases GRSF concentration in macro-, microaggregates and fine material of agricultural ultisols compared with untreated soils (Wright et al., 2007). This implies a correlation depending on agricultural practice. Deviating from that, in samples of arid sandy/silty loam with stability mainly caused by high carbonate contents of average 71%, positive correlation between glomalin and aggregate stability could not be observed (Rillig et al., 2003).

These results imply that glomalin is preferentially accumulated in intact macro-aggregates and AMF glomalin productivity is somehow related to aggregate stability. That could reveal

glomalin as a proxy but not necessarily as an agent of soil aggregation. Likewise undisturbed fungal hyphae systems could produce more glomalin than those in tilled soils, while merely stabilizing soil aggregates physically by entanglement and enmeshment (Miller and Jastrow, 2000).

Results of Driver et al. (2005) showing >80% of GRSF (1.4 µg mg^{-1} mycelium) tightly bound within the hyphal cell wall confirm glomalin as not being exudated, but rather being integral part of AMF cell walls. Yet unexplored, its local function could encompass easing of hyphal surface binding by increased hydrophobicity, decreased digestibility in face of grazers or accumulation of cations (Gonzalez-Chavez et al., 2004; Driver et al., 2005).

Glomalin distribution in the soil matrix mainly appears by hyphal decay producing fragments of approximately <5 µm (Wright and Upadhyaya, 1998). With given concentrations of only 0.03 to 0.5 mg extraradical hyphae g^{-1} soil and hyphal average turnover time of 5 to 7 days in pot experiments and laboratory cultures (Friese and Allen, 1991; Staddon et al., 2003; Zhu and Miller, 2003), GRSF concentrations of 1 to 21 mg g^{-1} soil amounting 3 to 10-fold the hot water extractable soil carbohydrates can only be explained by high persistence and accumulation within the soil matrix (Wright et al., 1996; Wright and Upadhyaya, 1998; Steinberg and Rillig, 2003; Zhu and Miller, 2003).

Although interactions of hydrophobic SOC with the soil matrix are expected to enhance aggregate stability (Piccolo and Mbagwu, 1999), high aromatic and carboxyl as well as low aliphatic group concentration measured by (Schindler et al., 2007) challenge the position of glomalin as a hydrophobic substance. However, attached to hydrophobic chitin cell wall fragments it could be immobilized and somehow act as hydrophobic aggregant. Hydrophobe chitin-glomalin traces on particle surfaces could therefore support soil aggregation by decreasing soil wettability and, in consequence, decrease decomposability of organic surfaces, enhance aeration and cell attachment and provide sticking between surfaces (Wright and Upadhyaya, 1998).

Previous results provide informations about the distribution of GRSF within soil aggregates: The smaller proportion is located in AMF cell walls possibly playing a role in hyphae-soil particle interaction (Driver et al., 2005). A distinctly higher concentration is found in the soil matrix as a recalcitrant remain of hyphal turnover (Wright and Upadhyaya,

1998). Whether the latter is located around actual and former hyphal positions or evenly distributed within the meso- and macropores of macro-aggregates is still unbeknown. However, whereas GRSP is assumed to be hydrophobic and probably immobile, hyphal wall fragments could be mobile or change their position relative to aggregate surfaces by long-term reorganization of soil aggregates. In this case, an increasing equality of distribution within the macro-aggregate can be assumed with increasing sequestration time. After destructive soil treatment, GRSF is necessarily found in the micro-aggregate fraction. Should the agglutinative effect of glomalin be proved, this pattern could elucidate the high correlation of GRSF concentration and macroaggregate stability.

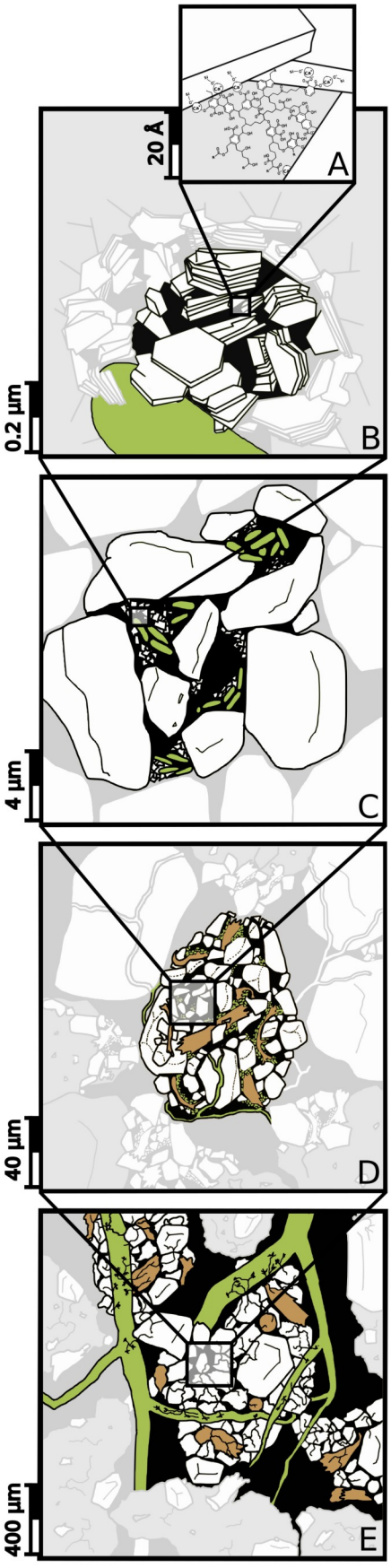

Fig. 3: A model of soil aggregate structure on different scales: Clayparticles are linked by humic substance and polyvalent cations to clay-humus complexes (A). These clay particles coagulate and can attach to bacteria (green rods) as a protective barrier (B). The interaction of coagulated clay particles, bacteria, DOM and silt-sized particles cause the formation of “small” soil aggregates (<20 µm) with habitable lower mesopore space for bacterial colonies, which are enclosed by a viscouse EPS matrix (C; DOM and EPS not shown). Microaggregates <250 µm (D) contain these “small” microaggregates, silt and sand particles as well as decomposing POM (brown). Bacteria (green rods and cocci) are inhabiting pores, whereas the aggregate's surface is colonized by filamentous fungi, that are able to connect different microaggregates and leave glomalin traces from decomposed hyphae (dotted lines). Macroaggregates >250 µm (E) are built of microaggregates and primary particles that hold together by a “sticky string bag” of fine roots (green), hyphae and their exudates. In contrast to microaggregates, pores between macroaggregates are mainly draining.

1.2.2 Carbon occlusion within soil aggregates

Nearly 1,500 Gt of organic carbon are stored in the first meter of global soils, which is twice the atmospheric carbon stock (Lal, 2008a; Stockmann et al., 2013). Owing to this enormous pool, the knowledge of decomposition and turnover rates of SOC is essential for future predictions about the atmospheric CO_2 content and SOC loss in consequence of degradation due to land use or climate change.

The turnover rates of organic matter are strongly influenced by its stability in face of chemical and biological decomposition (Schmidt et al., 2011). Although there are obvious differences in the metabolization rate of different SOM under normalized conditions (e.g. glucose versus lignin), fast decomposition of recalcitrant (Knežević et al., 2013) and persistence of easily decomposable organic matter (Sollins et al., 1996) show that degradability is not only an inherent factor of matter, but also depends on environmental conditions like the metabolic capability of the grasping microbial populations (McGuire and Treseder, 2010) or protection within the soil matrix (Six et al., 2002).

Soil aggregates contain approximately 90% of the total SOC, and up to 40% are located in microaggregates (Lützow et al., 2007). Beside inherent recalcitrance of SOM, two mechanisms are assumed to control the stabilization of SOM within the soil matrix: (1) Molecular SOM is protected against microbial decomposition by the adsorption to charged surfaces of silt and clay particles. (2) The occlusion within soil aggregates protects particulate and molecular SOM against decomposition due to physical inaccessibility as well as hindrance of nutrient and waste diffusion, which reduces microbial metabolic activity. (Six et al., 2002)

The tremendous number of feedstocks and degradative pathways causes manifold soil organic final and interstage products. However, physical pools of organic carbon - less complex than chemical classifications – comprise carbon from particulate organic matter (POM), dissolved organic matter (DOM), mineral-organic associations (MOM) as well as the minor researched fractions of co-precipitated organic matter (CPOM) and colloidal organic matter (COM). These physical carbon pools represent different functions in the soil ecosystem. Particulate organic matter mainly originates from above and belowground plant debris and further fungal and animal fragments (Blume et al., 2015). It provides

surface for microbial colonization, nutrient source and structural function in soils (Bronick and Lal, 2005). Molecular organic matter, which appears dissolved or adsorbed on surfaces, is produced by bacteria, archaea, fungi and plants and enters the soil matrix via exudation, cell break down, external input or extracellular enzymatic decomposition. It appears to be an in part mobile microbial nutrient source (Marschner and Kalbitz, 2003), but bound in mineral-organic complexes it shows reduced biodegradability and enhanced contribution to soil microaggregation (Tisdall, 1996; Six et al., 2002; Edwards and Bremner, 1967a). In addition, little is known about the appearance and behavior of colloidal organic matter like bacterial cell wall debris, which is assumed to influence hydrophobicity of soil mineral particles (Achtenhagen et al., 2015), and organic matter, which is occluded within precipitated minerals (Eusterhues et al., 2008). These last two classes are therefore excluded from further discussion in the present work.

Total SOM can be further subdivided into different C pools by means of its persistence, which are linked to physical C pools (von Lützow et al., 2008): The active pool comprises non-occluded residues, microbial biomass and other free SOM with turnover times <10 years. The intermediate pool includes imperfectly decomposed POM from plant residues and is protected by the occlusion within soil aggregates with turnover times of 10 to 100 years. Finally, the passive SOM pool mainly comprises decomposed molecular OM strongly adsorbed to mineral surfaces and charred POM, which are thus protected for a span extrapolated to >100 years.

The underlying model, that links persistence of different physical C pools and their status within the soil matrix was supposed with varying accentuation by different authors and includes different mechanisms of chemical longevity and protection (von Lützow et al., 2008; Schrumpf et al., 2013; Lehmann and Kleber, 2015): Decomposition of plant and animal residues result in a wide range of molecules with low to high molar masses as well as POM, that have different decomposabilities depending on molecular structure and surrounding microbial metabolisms, but no inherent, static recalcitrance. Stepwise degradation towards smaller biopolymers, monomers and final mineralization – with reaction constants depending on the type of matter and biochemical steps to total decomposition – leads to a steady state equilibrium between more and less persistent organic matter. The equilibrium is shifted towards longevity by the adsorption of both

particles and molecules of any size to mineral surfaces and occlusion within soil aggregates. However, this protection is enhanced with decreasing particle and molecule size, resulting in a high longevity of SOM in occluded mineral-organic associations. Following Kalbitz et al. (2005), organo-mineral association is – next to occlusion within soil aggregates – the main protection mechanism against mineralization.

The persistence of organic matter from different functional pools is linked to specific properties: High C:N ratios of 30.9±11.9 and 32.1±15.5 are related to free and occluded particulate plant debris, respectively (Wagai et al., 2009; Cerli et al., 2012), whereas a low C:N ratio of 13.5±4.6 found on the soil mineral matrix is more similar to C:N ratios around 9 related to microbial biomass (Cleveland and Liptzin, 2007; Wagai et al., 2009). In consequence, a low C:N ratio indicates OM that is metabolized by fungi, bacteria or archaea (OM of autotrophic prokaryotes excluded). Wagai et al. (2009) reviewed that the C:N ratio of POM is positively related to the particle size and microbial activity as well as negatively related to the grade of mineral coating, which underpins both an increased degree of microbial decomposition of smaller particles and the protective function of occlusion. High concentrations of carbohydrates represent both a very early state of degradation of plant and animal debris as well as extensive storage of microbial metabolites after OM decomposition (Poirier et al., 2005). This bipolarity can be solved by analysis of polymerized monosaccharides: A decrease in the ratio of xylose (a mainly plant derived sugar) to mannose (mainly microbial) indicates a microbial origin of OM (Oades, 1984). Likewise, increased aliphaticity points to increased degradation of SOM (Wagai et al., 2009).

In conclusion, type and degree of SOM protection within soil aggregates are important criteria for SOC storage and cycling. As particulate debris is the most important SOM feed, the occlusion of POM as a first step of protection in an early stage of decomposition is an important marker of turnover rates and C storage in soils.

2 Measurement of aggregate stability and C occlusion

2.1 The diversity of methods for aggregate stability measurement

Earliest measurements of soil aggregation were largely performed using standardized dry- and wet-sieving procedures (Yoder, 1936; Chepil and Bisal, 1943), which were frequently adapted for the analysis of aggregate stability (Bissonnais, 1996; Wright and Upadhyaya, 1998; Seybold and Herrick, 2001). The underlying rationale of aggregate stability measurement is to apply a distinct level of mechanical stress to a soil sample by shaking in a sieve. The stress leads to disconnection of particles within the soil aggregate, that depends on the strength of intra-aggregate binding forces. Weakly aggregated soils suffer more reduction in secondary particle size, measured as mesh aperture, than stronger aggregates. (Kemper and Rosenau, 1986)

Newer methods including water-dropping and ultrasonication share this principle of using disaggregating forces (Farres and Cousen, 1985; Edwards and Bremner, 1967a). However, both sieving and more recent methods require reference values to derive aggregate stability from the post-treatment aggregation, e.g. the pre-treatment state, a reference sample of a different or differently treated soil or the exact amount of applied energy. Some frequently used and/or promising methods shall be shortly described in the following (Table 1).

Dry-sieving in rotary sieves (Chepil and Bisal, 1943) and stacked sieves (Singh, 1952) is a simple time- and material-saving method, which is used to date (Zhang, 1994; Rajaram and Erbach, 1999). The human factor, which e.g. affects constant mechanical stress generation, can be avoided by using machined sieving, but some important disadvantages remain: The lab worker is not able to distinguish water-stable from water-labile aggregates, which form during air-drying and increase the content of coarser aggregates (Beare and Bruce, 1993). In addition, the analysis of fresh soil is restricted to lower water content to avoid luting within the sieve. Furthermore, aggregate stability negatively correlates with

water content (Francis and Cruse, 1983; Beare and Bruce, 1993). Hence, compared to air-dried aggregates fresh ones are reduced in their stability, and the reduced stability span between stable and labile aggregates could therefore reduce the resolution of dispersion measurement.

In contrast to dry-sieving, wet-sieving is conducted submerged. Although varying in mesh size, number of sieves, amount of soil as well as movement axis, range and speed of shaking (Kemper and Rosenau, 1986; Seybold and Herrick, 2001), all wet-sieving procedures base on a scheme described by Yoder (1936). However, different re-wetting methods affect the aggregate size distributions after wet-sieving (Beare and Bruce, 1993). Some dry- and wet-sieving procedures are proposed by Kemper and Rosenau (1986), Nimmo and Perkins (2002) and standardized in DIN/ISO (2002).

Alternatives to wet-sieving mainly used in early trials are the disaggregation by end-over-end shaking (Oades and Waters, 1991) as well as the elutriation of soil aggregates within a shaking tube and the further determination of aggregate sizes on the basis of sedimentation layers and sedimentation time (Baver and Rhoades, 1932). Although this method is simple, it provides dysfunctionality e.g. in face of fast-depositing particles coarser than silt-size and is blind for differences in the bulk density of different aggregates. However, it is functional for analyzing the disaggregation of microaggregates (Oades and Waters, 1991).

Another class of methods comprises water-dropping on single soil aggregates in an apparatus (Farres and Cousen, 1985) and artificial raining on soil beds (Barthes and Roose, 2002) with measurement of the dispersive effects. Thereby, the quantification of the applied energy was tried by integrating the kinetic energy of water drops with known mass and height of fall (Marshall and Quirk, 1950).

The rupture-threshold approach, applied to single soil aggregates by Perfect and Kay (1994), uses two parallel plates to provide a defined compression force to the interjacent aggregate. This method allows to derive single aggregate stability from deformation up to the point of rupture.

Strongly differing from methods involving mechanical stress is the estimation of aggregate stability by means of a substance, which strongly correlates with aggregate stability in its

concentration. Wright and Upadhyaya (1998) found a correlation to aggregate stability measured by mean weight diameter after wet-sieving for (freshly produced) immonoreactive easily extractable glomalin (IREEG) and also easily extractable glomalin (EEG) after autoclavation with 50 mM sodium citrate solution. Further experiments confirm this relation (Wright et al., 1999; Wright and Anderson, 2000; Rillig et al., 2002; Wu et al., 2014). Although this finding is not matured to a serviceable method, it has potential to be used in qualitative comparison studies. However, it is most probably restricted to soils getting their stability from organic compounds.

Table 1: Collection of frequently used and/or promising methods for aggregate stability measurement.

Method	Restrictions	Quantitative	Exemplary reference
dry-sieving (rotary sieves)	no dist. between water-labile and -stabile aggregates; restricted to lower water content; susceptible to water content; particles > silt size	no	Chepil and Bisal (1943)
dry-sieving (stacked sieves)		no	Singh (1952)
wet-sieving	particles > silt size	no	Kemper and Rosenau (1986)
elutriation	particles ≤ silt size	no	Baver and Rhoades (1932)
water-dropping	applicable to single aggregates and aggregates on surfaces	yes	Farres and Cousen (1985)
rupture-threshold approach	single or spatially separated aggregates	yes	Perfect and Kay (1994)
sonication	needs calibration per each soil; needs subsequent classification	yes	Edwards and Bremner (1967b)
glomalin	only in soils with aggregate stability dominated by organic agents	no	not yet applied

However, another currently preferred method for the mechanical disaggregation of soil aggregates is the ultrasonication.

2.2 Measuring aggregate stability by use of ultrasonication

Ultrasonication is a widely used method for the disaggregation of soil samples (Oades and Waters, 1991; Lehtinen et al., 2014; Edwards and Bremner, 1967a). In the common procedure (Kaiser and Berhe, 2014; Edwards and Bremner, 1967b), a pieco-electric

converter uses electric energy to generate axial vibration of a sonotrode, that is dipped into a flask containing a submerged soil sample. The oscillating sonotrode emits shock-waves within the aqueous medium. In front of the wave the medium is compressed, and the increased pressure causes an increased gas solubility. Behind the wave the medium relaxes below the normal pressure conditions leading to an explosive outgassing. This so called cavitation effect produces lots of exploding micro-bubbles within the soil matrix generating local pressure peaks of 200 to 500 atm accompanied by 4200 to 5000 K (Ince et al., 2001), that provoke detachment of bondings within soil aggregates. Depending on device type and settings, the vibration frequency can vary up to 10,000 kHz, but it is recommended to use low frequencies around 20-100 kHz for soil aggregate dispersion without influencing chemical composition of OM (Kaiser and Berhe, 2014).

In contrast to the methods mentioned above, ultrasonication allows semi-quantification of aggregate stability without a reference sample, if the power output of the sonotrode (P) is known. Its quantification take place by heating a known amount of water (m_{H20}) in a Dewar vessel with application of ultrasound for a certain time (t), represented by equation (1).

$$P=\left(m_{H_20}\cdot c_{H_20}+C_{Dewar}\right)\cdot\frac{\Delta T}{t}+H \quad (1)$$

The increase in temperature (ΔT) is proportional to the heating time. As the Dewar vessel is nearly thermally isolated (enthalpy flux H≈0) (North, 1976), has a heat capacity $C_{Dewar}<<(m_{H20}\cdot c_{H20})$ and the specific heat capacity of water is nearly constant between 298 and 318 K, equation (1) can be simplified to equation (2).

$$P=\left(m_{H_20}\cdot c_{H_20}\right)\cdot\frac{\Delta T}{t} \quad (2)$$

However, in case of sonifying soils, this equation only describes the energy transmission to the bulk of aqueous solution and soil, but not the ratio of heating the water, heating the soil and stressing the soil by mechanical cavitation forces. This leads to an overestimation of binding forces within the soil aggregates, when applied energy is claimed to work

completely cavitational. To reduce this overestimation, the specific dispersive power output has to be distinguished from heating energy. If the same calibration is performed with an additional amount of soil (m_s) with a specific heat capacity (c_s), the amount of dispersive power ($m_s \cdot L$) is marked by a reduction in the heating rate of the solution and described by equation (3).

$$P = \left(m_{H_20} \cdot c_{H_20} + C_{Dewar}\right) \cdot \frac{\Delta T}{t} + m_s \cdot c_s \cdot \frac{\Delta T}{t} + m_s \cdot \frac{L}{t} + H \quad (3)$$

As the specific heat capacity of the soil is also negligible ($m_s \cdot c_s << m_{H20} \cdot c_{H20}$), the equation can be simplified in accordance to equation (2) to

$$P = \left(m_{H_20} \cdot c_{H_20}\right) \cdot \frac{\Delta T}{t} + m_s \cdot \frac{L}{t} \quad (4).$$

The ΔP between equations (2) and (4) relates to the effectively used cavitational energy. Using this second calibration, a full-qualitative measurement of mechanical forces applied to the soil matrix is possible (North, 1976). However, measurement of binding forces within the soil aggregates remain semi-quantitative, as it is impossible to distinguish micro-explosions treating particle links or surfaces.

Knowing the effective cavitational power, defined amounts of energy can be applied to soil samples and the percentage of weight fraction smaller than a specific mean weight diameter (MWD) can be plotted as a function of the applied energy, whereby steeper gradients represent less stable soil aggregates (North, 1976). As cavitation bubbles have diameters of maximum 100 µm (Crum, 1995) and expand in even the smallest soil pores, ultrasonication is suitable for the disaggregation of both micro- and macroaggregates with a broad span of stabilities. The resulting size-class distribution after treatment can be determined e.g. by sieving and elutriation. In contrast to different wet-sieving methods, using this method Graf-Rosenfellner (unpublished data) showed no significant differences in disaggregation between different sonotrode types, which is known to be more effective than a sonication bath (Edwards and Bremner, 1967a).

2.3 Measurement of C occlusion

Effective methods for the measurment of SOC functional pools comprise those of physical fractionation by means of particle size or density as well as chemical extraction and decomposition methods (Lützow et al., 2007). The present work focus on ultrasonication with a subsequent density fractionation (UDF) to separate particulate non-occluded and occluded as well as mineral-organic associated SOM.

The UDF is a frequently used method to analyze these soil carbon pools (Golchin et al., 1994). For this purpose, soil samples are added to water or denser aqueous liquids to perform floating of unbound organic matter, whereby centrifugation is used to accelerate and improve the fractionation. Sodium polytungstate solution as a non-polluting, non-toxic and reusable liquid facilitating a wide range of density cut-offs (1.0 to 3.1 g cm^{-3}) is often used for this separation (Six et al., 1999). The floating matter, that is separated without mechanical destruction of aggregates, is operationally named free light fraction (fLF). The sampling of fLF is followed by ultrasonication of the remaining soil leading to a detachment, subsequent floating and separation of aggregate-occluded POM (oLF). Repeating this procedure with constant or increasing energy leads to separation of organic matter with increasing bonding strength to the mineral matrix. The OM remaining within the sedimented after separation of all particulate organic matter is named the heavy fraction (HF) and comprises mineral-associated organic matter. (Kaiser and Berhe, 2014)

The underlying method invented by Golchin et al. (1994) is used as a blue-print for diverse surveys regarding e.g. organic carbon storage and SOM turnover (Baisden et al., 2002; Crow et al., 2007), influence of land-use on carbon stocks (Tiessen and Stewart, 1983; Meyer et al., 2012), carbon indicative or functional studies (Leifeld and Kögel-Knabner, 2005; Lützow et al., 2007) or further analyses of organo-mineral associations (Basile-Doelsch et al., 2007). Those examinations often varied methodologically in the chosen density cut-off, dispersion intensity, soil/liquid ratio, immersion depth of the sonotrode's tip and other parameters and therefore lack comparability, as e.g. liquids of different densities provide largely different fractionation of POM.

As UDF often plays a major role in studies about the ecological functionality of SOM fractions, standardized parameters have to be chosen in a way, that operational fractions match the functional C pools as accurate as possible:

In a first attempt to standardize this method for a wide range of soils, Cerli et al. (2012) recommended a density cut-off of 1.6 g cm^{-3} even for the treatment of weakly aggregated soils with low C content. SPT concentration <1.6 g cm^{-3} resulted in a decrease of OM in the fLF, whereas concentrations >1.6 g cm^{-3} caused a sharp increase of the mineral content. That characterizes ρ=1.6 g cm^{-3} as the cut-off avoiding both incomplete floating of unbounded OM and contamination by OM from other functional pools, e.g. organic-mineral associations.

However, in contrast to a density cut-off, a general energetic dispersion cut-off (J ml^{-1}) for the total dispersion of soil aggregates could not be specified as it strongly depends on the soil type. This cut-off is theoretically bounded below by insufficient release of oLF and above by disruption and floating of organic-mineral associations from the HF. Kaiser and Berhe (2014) reviewed 15 studies using ultrasonication of soil aggregates in terms of energy level for total dispersion of soil aggregates and avoidance of primary particle destruction. They found destruction of POM at applied energy levels >60 J ml^{-1}, destruction of sand-sized primary particles at >710 J ml^{-1} and of coarse silt-size particles at >1500 J ml^{-1}, whereas clay-sized primary particles gain damage at energy levels >12.000 J ml^{-1}. The over-application of ultrasound not only causes rupture of mineral and organic matter, but also chemical transformation of OM due to very local heat and pressure peaks (Ince et al., 2001). These peaks result in $^{\bullet}$OH and $^{\bullet}$H reactions leading to a decrease in amount, molar mass and aliphaticity of OM. This mechanism mainly influences more volatile OM, whereas a change in chemical composition of mineral-associated organic matter was not found (Kaiser and Berhe, 2014).

Based on these findings it is recommended to prove every examined soil in pre-trials with increasing dispersion energy for the point of depletion of oLF to distinguish between occluded POM and mineral-associated OM. Only in best cases these do not overlap with the disruption of parts of the soil matrix (Cerli et al., 2012). This results in a trade-off between complete extraction of oLF-POM and the avoidance of an intermixture of functional C pools by destruction and redistribution of soil primary particles. As a reaction

to this trade-off, e.g. Kaiser and Berhe (2014) recommended a treatment to reduce those artefacts, that uses stepwise ultrasonication with a cumulated energy density of 1000 J ml^{-1} at <40°C and low frequency ultrasound (20 to 100 kHz).

Beside these standardization problems, UDF exhibit some fundamental problems regarding the identity of operational and functional C pools and the representation of the latter by binding patterns and decomposition state. In a comparative study of 36 soils with different vegetation and site characteristics, Wagai et al. (2009) found an oLF C:N ratio increased compared to the fLF, which contradicts to the theory of occlusion accompanied by biotic degradation. This is explained e.g. by the distribution of recalcitrant POM like biochar particles, spores and pollen to both the fLF and the oLF. As ultrasonication is also used to detach bacterial cells from surfaces (Böckelmann et al., 2003), it is further possible, that the C:N ratio of the oLF is an artefact, as it is increased by the removal of bacterial biomass, whereas mineral-associated OM of the HF is not affected by this method.

Furthermore, Wagai et al. (2009) suggest, that also the surface/volume ratio of the POM determines the classification as fLF or oLF: Small POM with a mineral coating could have a bulk density of >1.6 g cm^{-3} rather than larger particles with a coating of the same material and exemplary thickness. Thus, a sharp separation of both the fresh POM and the colonized plus degraded POM is not possible due to an intermixture between fLF and oLF.

Soil dispersion using UDF is also affected by SPT. As sodium acts as dispersive agent on negatively charged surfaces, dispersion efficacy depends on soil mineral composition and decrease comparability of different soil types.

Anyway, the use of 1.6 g cm^{-3} and an appropriate dispersion cut-off allows a rough separation of functional carbon pools along the bulk light and heavy fraction and a less precise separation of free and occluded light fractions. Making a complete C balance of functional pools does not only require a predetermined dispersion cut-off, but also the measurement of water/SPT solution-extractable DOM when extracting the fLF (Kaiser and Berhe, 2014). Furthermore, a standardized pre-treatment has to be used, as e.g. drying and further pre-treatment steps shift the OC content from oLF to fLF compared to field-fresh soil aggregates (Kölbl et al., 2005).

In contrast, the analysis of POM occlusive strength of one soil type after different treatments can be executed by use of an arbitrary dispersion cut-off. Differing POM release as reaction on mechanical stress can be interpreted as a change in occlusive strength, but the explanatory power about functional pools is thereby omitted. However, Cerli et al. (2012) demonstrated that the release of occluded POM caused by cavitational forces strongly depends on content, composition and binding patterns of POM as well as soil mineralogy. In consequence, the comparison of POM occlusive strength is restricted to very similar soils, if soil specific dispersion cut-offs for the total and exclusive oLF detachment are not determined or determinable.

3 Aim of this work

As most of the SOM is located in soil aggregates, its physical protection has large influence on decomposition, turnover rates and the soil carbon budget of landscapes. The present work was conducted on a plowed sandy topsoil (Su3) from a cropland near Berge (Brandenburg, Germany) and focus on POM occlusion, since this protective mechanism represents initial stages of SOM decomposition. Due to their influence on soil structure, microbial nutrition and metabolic diversity in soils, the grade and strength of POM occlusion could be a proxy for soil properties like soil fertility and soil health. The interaction of soil microorganisms and soil particles, which is an important factor of soil aggregation, is assumed to have significant influence on the occlusion of POM within soil aggregates. This overall aussumption is tested in the present thesis.

In a first experiment, the influence of bacterial EPS on the occlusive strength of POM within soil aggregates was examined. I hypothesized that POM is fixed to the mineral phase by EPS. This hypothesis was tested by a treatment of soil aggregates with increasing concentrations of EPS degrading enzymes, that should result in an additional release of POM after mechanical treatment.

The second experiment focus on the influence of grazing organisms on POM occlusion. I hypothesized, that feeding on EPS by the bacterial-feeding nematode *Acrobeloides buetschlii* would result in an additional release of occluded POM compared to a control without nematodes, when grazed bacterial biofilms and EPS remains lose their cohering function between soil particles.

In a third work, I investigated the influence of structurally different microbial populations on the POM occlusion. Based on the assumtion, that different communities provide different sets of biochemical and physical mechanisms for aggregate stabilization, we expected differing POM occlusion in two variants with strongly unequal microbial populations.

The overall aim of this work is to elucidate different aspects of the contribution of microorganisms to the POM occlusion within sandy agricultural soils (stabilization, variability with changing populations and feeding influence).

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

4.1 Abstract

The stability of soil aggregates against shearing and compressive forces as well as water caused dispersion is an integral marker of soil quality. High stability results in less compaction and erosion and has been linked to enhanced water retention, dynamic water transport and aeration regimes, increased rooting depth and protection of soil organic matter (SOM) against microbial degradation. In turn, particulate organic matter is supposed to support soil aggregate stabilization. For decades the importance of biofilm extracellular polymeric substances (EPS) regarding particulate organic matter (POM) occlusion and aggregate stability has been canonical because of its distribution, geometric structure and ability to link primary particles. However, experimental proof is still missing. This lack is mainly due to methodological reasons. Thus, the objective of this work is to develop a method of enzymatic biofilm detachment for studying the effects of EPS on POM occlusion. The method combines an enzymatic pre-treatment with different activities of α-glucosidase, β-galactosidase, DNAse and lipase with a subsequent sequential ultrasonic treatment for disaggregation and density-fractioning of soils. Particulate organic matter releases of treated samples were compared to an enzyme-free control. To test the efficacy of biofilm detachment the ratio of bacterial DNA from suspended cells and the remaining biofilm after enzymatic treatment were measured by quantitative real-time PCR. Although the enzyme treatment was not sufficient for total biofilm removal, my results indicate that EPS may attach particulate organic matter (POM) within soil aggregates. The tendency to additional POM release with increased application of enzymes was attributed to a slight loss in aggregate stability. This suggests that an effect of agricultural practices on soil microbial populations could influence POM occlusion/aggregate stability and thereby carbon cycle/soil quality.

4.2 Introduction

Soil organic matter (SOM) comprises 50% (~1,700 Gt, including peat) of the near-surface terrestrial carbon budget, compared to ~813 Gt bound in the atmosphere (Lal, 2008b). Beside carbon storage and its influence on the atmospheric CO_2 balance, manifold ecological soil functions are mediated by different SOM types like dissolved organic matter (DOM), particulate organic matter (POM), molecular organic matter of organo-mineral associations, colloidal organic matter and coprecipitated molecular organic matter (Kalbitz et al., 2000; Weng et al., 2002; Pokrovsky et al., 2005; Eusterhues et al., 2008). For example, POM is a structural component of soil aggregates, a nutrient source and provides surfaces for microbial growth (Chenu and Stotzky, 2002; Bronick and Lal, 2005). Parts of the POM are occluded within soil aggregates (Six et al., 2002). Physical isolation protects POM against microbial degradation (Six et al., 2002; Lützow et al., 2006) and maintains its ecological functions, while on the other hand this POM is thought to promote soil aggregation (Bronick and Lal, 2005). Therefore, many benefits of soil POM are linked to soil aggregate stability.

The stability of soil aggregates against shear and compression forces *(Skidmore and Powers, 1982)* as well as disaggregation caused by water (Tisdall and Oades, 1982) is an integral marker of soil quality (Bronick and Lal, 2005). Since aggregate stability implies pore stability, it results in less soil compactibility (Baumgartl and Horn, 1991; Alaoui et al., 2011) and a more dynamic water transport regime in the macropores that reduces erosion caused by surface runoff (Barthes and Roose, 2002). Other benefits in comparison to compacted soils are a higher aeration (Ball and Robertson, 1994) and lower penetration resistance (Bennie and Burger, 1988) causing increased rootability and rooting depth (Bengough and Mullins, 1990; Taylor and Brar, 1991). In addition, micropores within the aggregates enhance water retention.

The occlusion of POM within soil aggregates depends on the properties of the aggregated components. The mineral part of the solid soil matrix is composed of siliceous sand, silt and clay particles, oxides and hydroxides of Fe, Al and Mn as well as diverse minor minerals. Sticking together, pervaded and coated with multivalent cations and organic constituents (like soluble metabolic products, humic substances, black carbon and other POM) macro-aggregates (>250 µm) are formed by direct coagulation or built of micro-

aggregates (<250 µm). (Bronick and Lal, 2005; Brodowski et al., 2006b; Lützow et al., 2006)

The structure-bearing primary particles, precipitates and adsorbed molecules cohere by physico-chemical interactions between (i) permanent charge of mainly the clay mineral fraction, (ii) multivalent cations with small hydrate shells such as Ca^{2+}, Fe^{3+} and Al^{3+}, (iii) variable charges of various minerals and SOM and (IV) variable and permanent dipoles of different soil components. Also carbonates, phosphates and other microbial precipitates force up aggregation and occlusion of POM. (Jastrow and Miller, 1997; Bronick and Lal, 2005)

In addition, since a few decades biological structures like bacterial colonies, bacterial pseudomycelia, algae, fungal hyphae and their exudates (e.g. glomalin), roots as well as soil fauna are accepted as a major factor of soil aggregation (Tisdall, 1991; Oades, 1993; Wright and Upadhyaya, 1998; Brown et al., 2000; Chenu and Stotzky, 2002; Rillig, 2004; Bronick and Lal, 2005). Furthermore the role of extracellular polymeric substance (EPS) of bacterial biofilms as an adhesive between soil particles is seen to be of importance (Baldock, 2002; Ashman et al., 2009).

Physical and chemical properties of soil mineral and organic matter allow to hypothesize a simple spacial model of the inner geometry of soil aggregates, that includes biofilms as links between primary particles (Fig. 4). The biofilm itself is a viscous microenvironment mainly built up of 90 to 97% water (Zhang et al., 1998; Schmitt and Flemming, 1999; Pal and Paul, 2008). The remaining dry mass contains differing ratios of polysaccharides, extracellular DNA (eDNA), proteins and lipids besides 10 to 50% cell biomass (More et al., 2014). In contrast to 'biofilm', EPS

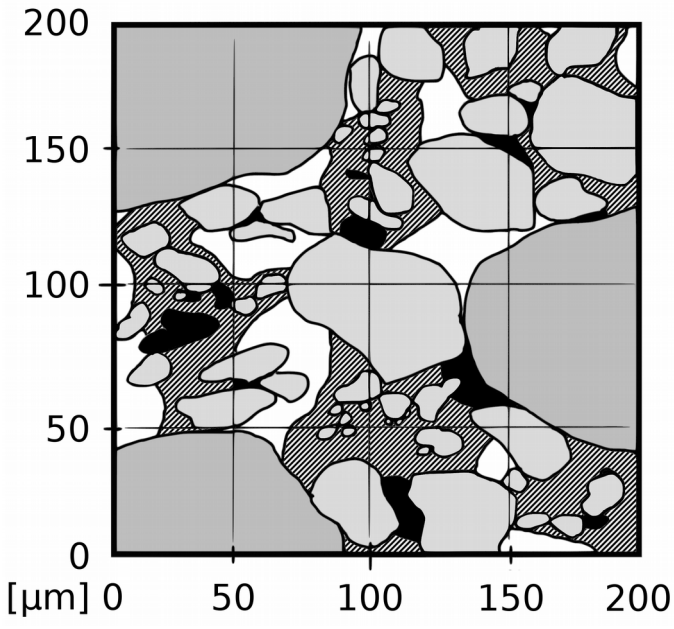

Fig. 4: Proposed model of inner soil aggregate structure including EPS: Sand and silt (both grey) and organic particles (black) stick together by physico-chemical interactions and are bridged by EPS (striped), which additionally stabilizes the soil aggregate structure and the pore space (white). (Büks and Kaupenjohann, 2016)

terms the extracellular polymeric matrix excluding cells. Extracellular polysaccharides cause the EPS structural stability by means of entanglement and Ca^{2+} bridging between molecules. So does eDNA (Das et al., 2014). Proteins function as enzymes and structural links stabilizing the polysaccharide matrix, while lipids act as biosurfactants for bacterial attachment on surfaces. (Flemming and Wingender, 2010)

The composition of EPS is highly variable depending on community composition and environmental cues (Table 2): Redmile-Gordon et al. (2014) measured a natural habitat extracellular polysaccharide concentration of 401 µg g^{-1} dry soil in grassland and 169 µg g^{-1} in fallows. Diverse single- and multi-species biofilms show a proportion of polysaccharides on dry EPS of up to 95% (Pal and Paul, 2008; More et al., 2014). Different single- and multi-species biofilms in laboratory cultures and natural soils have a dry EPS eDNA content up to 10% (More et al., 2014). For forest soils values of 1.95 up to 41.1 µg g^{-1} dry soil are known (Niemeyer and Gessler, 2002; Agnelli et al., 2004). Extracellular DNA concentration of other diverse soils ranges between 0.03 and 200 µg g^{-1} dry soil (Niemeyer and Gessler, 2002; Pietramellara et al., 2009), whereas concentrations in soils explicitly used for agriculture are unknown. Extracellular matrix protein concentration was measured at 163 µg g^{-1} dry soil in grassland and 43 µg g^{-1} dry soil in fallow (Redmile-Gordon et al., 2014), but can contribute the largest fraction of EPS dry mass, e.g. 60% (More et al., 2014), and even up to 75% in *P. putida* biofilms in laboratory cultures (Jahn et al., 1999). The typical proportion of lipids in the EPS dry-mass of different non-soil biofilms amounts up to 10% (More et al., 2014). Sparse molar mass data from different environments comprise $0.5x10^6$ to $2x10^6$ Da for polysaccharides (Flemming and Wingender, 2010), $7.75x10^4$ to $2.32x10^7$ Da for eDNA (DeFlaun et al., 1987) and 750 to 1,500 Da for lipids (Munk, 2008).

The extracellular matrix is not only exuded by soil bacteria and archaea, but also by fungi and algae. It is engineered by grazing protozoa and small metazoa as well as microbial extracellular enzymes. (Battin et al., 2007; Flemming and Wingender, 2010)

The activity of EPS degrading enzymes in natural soils spans up to two orders of magnitude: The α-glucosidase and β-galactosidase activity of various soils ranges from 0.00011 U g^{-1} to 0.0011 U g^{-1} and from 0.00017 to 0.0094 U g^{-1}, respectively (Eivazi and Tabatabai, 1988; Acosta-Martinez and Tabatabai, 2000). The lipase activity in coarse mineral soils shows values from 0.3 U g^{-1} in a sandy soil (Cooper and Morgan, 1981) to

Table 2: Concentrations and molar masses of biofilm stabilizing macromolecules (polysaccharides=PS, eDNA, lipids and proteins) in different environments.

Conc.	Proportion	Molar mass	Comment	Reference
µg (g soil)$^{-1}$	µg (100 µg EPS)$^{-1}$	Da		
PS				
169			µg g^{-1} bare fallow	Redmile-Gordon et al. (2014)
401			µg g^{-1} grassland	Redmile-Gordon et al. (2014)
	95%		% of EPS dry-mass	More et al. (2014)
	40-95%		% of EPS dry-mass	Pal and Paul (2008)
		2x10^{6}		Chenu and Roberson (1996)
		0.5-2x10^{6}		Flemming and Wingender (2010)
eDNA				
2.2-41.1			µg g^{-1} forest soil	Agnelli et al. (2004)
0.08			µg g^{-1} Luvisol	Niemeyer and Gessler (2002)
1.95			µg g^{-1} forest podzol	Niemeyer and Gessler (2002)
0.03-200			µg g^{-1} unnamed soil	Pietramellara et al. (2009)
	10%		% EPS dry-mass	More et al. (2014)
		7.75x10^{4}-2.32x10^{7}	estuarine and oceanic environments	DeFlaun et al. (1987)
Lipids				
	10%		% of EPS dry-mass	More et al. (2014)
		750-1500		Abröll et al. (2008)
Proteins				
43			µg g^{-1} bare fallow	Redmile-Gordon et al. (2014)
163			µg g^{-1} grassland	Redmile-Gordon et al. (2014)
	< 75%		% of *Ps. Putida* biofilm	Jahn et al. (1999)
	60%		% EPS dry-mass	More et al. (2014)

2.09 U g^{-1} in a Luvisol (Margesin et al., 2000) and up to 5 U g^{-1} in a Leptosol (Margesin et al., 1999). Data for eDNAse activity in soils are not available.

Not much is known about the contribution of EPS to POM occlusion and aggregate stability in relation to other aggregate stabilizing factors. That is mainly due to methodological reasons: Though e.g. Tang et al. (2011) showed a significant contribution of bacterial growth on aggregate stability, the observations could not definitely be attributed to soil microbial exopolysaccharide production. Redmile-Gordon et al. (2014) subsequently found that the techniques previously used to measure extracellular polysaccharides in soil co-extracted large quantities of 'random' soil organic matter which confounded estimates of EPS production. Owing to the widespread interest in the role of biofilms on soil fertility, the objectives of this work are (i) to design a selective method for

enzymatic biofilm detachment with minor impact on other types of aggregate bonds and (ii) to apply the method to an agricultural soil to provide indications of the influence of biofilm cohesion on POM fixation, which is expected to contribute to aggregate stability (Six et al., 2004).

The method combines a modified enzymatic pre-treatment (Böckelmann et al., 2003) with α-glucosidase, β-galactosidase, DNAse and lipase, a determination of the DNA ratio of sessile to suspended cells after enzymatic treatment and an ultrasonication of soil aggregates followed by density-fractioning and soil organic carbon (SOC) measurement (Kaiser and Berhe, 2014). The ultrasonication/density-fractionation separates SOC into three operational solid fractions: non-occluded free light fraction SOC (fLF-SOC), aggregate-embedded occluded light fraction SOC (oLF-SOC) and colloidal as well as (macro)molecular SOC, which is not detachable from mineral surfaces by the chosen fractioning method and subsumed under heavy fraction (HF-SOC) (Kaiser and Berhe, 2014).

We hypothesize that a destabilization of the EPS matrix occurs during enzymatic treatment. This should result in an increased cell detachment from aggregates. We also expect an increased fLF-SOC release from destabilized aggregates compared to the control and a shift of the oLF-SOC ratio from higher to lower binding strength (represented by ultrasonic energy levels) that is interpretable as alteration of soil aggregate stability.

4.3 Materials and methods

4.3.1 Soil properties and microbial biomass

Well aggregated silty sand (Su3) of a plowed topsoil from a cropland near Berge (Brandenburg/Germany) was air-dried and sieved to obtain a particle size of 0.63 to 2.0 mm containing mainly macro-aggregates. The aggregates have a pH_{CaCl2} of 6.9, C_{org} of 8.7 mg g^{-1} dry soil and a carbonate concentration of 0.2 mg g^{-1}.

To estimate the soil microbial biomass, first 8 x 10 g of soil aggregates have been adjusted to 70 $\%_{vol}$ soil water content and incubated for 70 h at 20°C in the dark to attain basal

respiration. Then, based on DIN EN ISO 14240-2 half of the samples were fumigated with ethanol-free chloroform in an evacuated desiccator for 24 h, whereas the other half remained untreated. Afterwards chloroform was removed and both halves were extracted with 40 ml of 0.5 M K_2SO_4 solution by 30 min of horizontal shaking and filtered through 0.7 µm glass fiber filters. The DOC concentrations of all filtrates were measured by a TOC Analyzer (TOC-5050A, Shimadzu). 176±22 µg microbial carbon g^{-1} dry soil (C_{mic}) were derived from the difference between DOC concentrations of fumigated and non-fumigated samples multiplied by a conversion factor of 2.22 (Joergensen, 1996). Soil bacterial biomass was derived from C_{mic} as 352±44 mg kg^{-1} assuming 0.5 as a ratio of C_{mic} to total cell dry mass (Bratbak and Dundas, 1984).

4.3.2 Detachment scenarios

Four degradative enzymes were selected on the basis of soil pH and the temperature used for definition of the catalytic unit (T_{def}): α-glucosidase from *S. cerevisiae* (Sigma-Aldrich, pH_{opt} 6 to 6.5, T_{def}=37°C, product number G0660) hydrolyzes terminal α-1,4-glycosidic linkages in polysaccharides as β-galactosidase from *E. coli* (Sigma-Aldrich, pH_{opt} 6 to 8, T_{def}=37°C, product number G5635) does with β-glycosidic bonds. Lipase from porcine pancreas (Sigma-Aldrich, pH_{def} 7.7, T_{def}=37°C, product number L0382) splits fatty acids from lipids via hydrolysis, but do not digest phospholipids, which are part of bacterial membranes. DNAse I from bovine pancreas (pH_{def} 5, T_{def}=25°C, product number D5025) breaks the phosphodiester linkages between nucleotides of DNA as an endonuclease. Proteases were not used because of their promiscuity and therefore incalculable influence on the other applied enzymes.

Literature shows a wide range of target concentrations related to these enzymes in different soils. As we do not know target concentrations of our soil (due to a lack of extraction methods), we considered the largest published values (Table 2) of EPS content $\left(\xi_{EPS}^{max}\right)$ and enzyme target dry mass contents $\left(\xi_{target}^{max}\right)$ from literature. Further, as bacterial dry mass $\left(\xi_{cell}^{min}\right)$ and target molar masses $\left(M_{target}^{min}\right)$ vary as well, here we choose the minimum percentage and the smallest mass, respectively. These values conduce to a “worst-case” point of view with a maximum of enzyme targets. Any other boundary

conditions such as ion activity, diffusion rates or metabolization of enzymes by soil organisms were disregarded.

Calculated by Eq. (1)

$$Unit_{target} = \frac{c_{cell} \cdot q \cdot \xi_{EPS}^{max} \cdot \xi_{target}^{max} \cdot m_{sample}}{\xi_{cell}^{min} \cdot M_{target}^{min} \cdot t} \quad (1)$$

with variables listed in Table 3 and Table 4, sufficient enzymes were provided to digest the expected EPS concentration in five scenarios: In the E1 scenario c_{cell} was given by the results of fumigation-extraction. In the E2 scenario a bacterial dry mass of 500 g m^{-2} in the upper 30 cm is considered, which is assumed to be the maximum for middle and northern European soils (Brauns, 1968). Supposing a soil bulk density of 1.4 g cm^{-3}, a c_{cell} of 1190.5 µg g^{-1} dry soil is given. Although the soil bulk density of the soil aggregate samples is ~1.15 g cm^{-3}, we decided to use the soil bulk density of the original soil, which is in the normal range of sandy silk soil (~1.40 g cm^{-3}) (Chaudhari et al., 2013). This is due to the fact that biofilm populations are mentioned to be mainly located in soil aggregates (Nunan et al., 2003) and accords to the “worst-case”-approach. The E3 scenario uses a 100-fold excess (q=100, Table 4) of the enzyme activities applied in the E2 scenario, whereas the E4 scenario contained the 2,820-fold, which is slightly higher than activities used in Böckelmann et al. (2003). Enzyme-free samples (E0) were used as a control.

Table 3: Variables, that are used for the calculation of enzyme units needed for biofilm target decomposition and scenario parameters, [a] More et al., 2014; [b] Pal and Paul, 2008; [c] Flemming and Wingender, 2010; [d] Abröll and Munk, 2008; [e] DeFlaun et al., 1987.

c_{cell}	[µg g^{-1}]	bacterial dry mass per g dry soil
q	[-]	enzyme concentration multiplier
ξ_{EPS}^{max}	[-]	maximum ratio of EPS dry mass per total biofilm dry mass ($\xi_{EPS}^{max} = 0.9^{[a]}$)
ξ_{target}^{max}	[-]	maximum ratio of enzyme target per EPS dry mass ($\xi_{polysaccharides}^{max} = 0.95^{[b]}$, $\xi_{lipids}^{max} = 0.1^{[a]}$ and $\xi_{eDNA}^{max} = 0.1^{[a]}$)
m_{sample}	[g]	sample mass
ξ_{cell}^{min}	[-]	minimum ratio of bacterial dry mass per total biofilm dry mass ($\xi_{cell}^{min} = 0.1^{[a]}$)
M_{target}^{min}	[µg µmol^{-1}]	minimum molar mass of enzyme target ($M_{polysaccharides}^{min} = 0.5\,x\,10^{6[c]}$, $M_{polysaccharides}^{min} = 700^{[d]}$, $M_{eDNA}^{min} = 7.75\,x\,10^{4[e]}$)
t	[min]	incubation time

Table 4: Specific scenario parameters of the variants E0, E1, E2, E3 and E4.

		E0	E1	E2	E3	E4
c_{cell}	[µg g^{-1} dry soil]	352	352	1191	1191	1191
q	[-]	1	1	1	100	2,820
$U^{max}_{alpha-glucosidase}$	[U g^{-1} dry soil]	0.00000	0.00010	0.00034	0.03393	0.95683
	[µg g^{-1} dry soil]	0.00000	0.00080	0.00272	0.27144	7.65464
$U^{max}_{beta-galactosidase}$	[U g^{-1} dry soil]	0.00000	0.00010	0.00034	0.03393	0.95683
	[µg g^{-1} dry soil]	0.00000	0.00020	0.00068	0.06786	1.91366
U^{max}_{lipids}	[U g^{-1} dry soil]	0.00000	0.00754	0.02551	2.55102	71.93876
	[µg g^{-1} dry soil]	0.00000	0.00038	0.00126	0.12551	3.59694
U^{max}_{eDNA}	[U g^{-1} dry soil]	0.00000	0.00007	0.00023	0.02304	0.64973
	[µg g^{-1} dry soil]	0.00000	0.00004	0.00012	0.01152	0.32487

4.3.3 Release of POM carbon

Fifteen g of air-dried soil aggregates were incubated in 5 replicates per scenario with 3.4 ml of highly concentrated artificial rainwater (ARW: 0.2 mM NH_4NO_3, 0.3 mM $MgSO_4x7H_2O$, 0.5 mM $CaCl_2$ x $2H_2O$, 0.5 mM Na_2SO_4, 15 mM KCl, pH 5.7) for 3 days at 20°C in the dark to establish basal respiration and avoid slaking in the following preparation steps. After incubation 2.5 ml of ARW containing enzymatic units according to Table 4 were added to the samples. By means of a following incubation at 37°C, enzymes were let to work near their catalytic optimum for 1 h, which is proven to be sufficient for biofilm degradation (Böckelmann et al., 2003). After this enzymatic pretreatment, 67.2 ml of 1.67 g cm^{-3} dense sodium polytungstate (SPT) solution were added resulting in a density cut-off of 1.6 g cm^{-3}, and samples were stored for 30 min to allow SPT diffusion into the aggregates. Then samples were centrifuged for 26 min with 3,569 G. Sodium polytungstate solution with floating fLF was filtered through an 1.5 µm pore size glass fibre filter to capture LF particles. Afterwards following Golchin et al. (1994) aggregate samples were consecutively disaggregated in four steps by application of each 50 J ml^{-1} of ultrasonic energy (Branson© Sonifier 250) for 1 min 15 sec. The energy output was determined by measuring the heating rate of water inside a dewar vessel (Schmidt et al., 1999). Every treatment cycle consisted of ultrasonication, centrifugation for 26 min with 3,569 G and filtering of SPT solution through an 1.5 µm pore size glass fibre filter to capture the LF. Afterwards the LFs and the remaining soil matrix ('sediment', consisting of oLF bonded >150 J ml^{-1} and the HF) were frozen, lyophilized, ground and dried at 105°C.

Total amount of fraction carbon was determined using an Elementar Vario EL III CNS Analyzer and the absence of carbonates was proved, respectively.

4.3.4 Release of bacterial DNA

The release of bacterial cells into the solution was estimated by use of DNA extraction using a FastDNA™ SPIN Kit for Soil and quantitative real-time PCR.

Therefore 45 µl of ARW were added directly to 0.1 g of air-dried aggregates. The samples were sterilely incubated in duplicate at 20°C for 3 days in the dark in a closed FastPrep Lysing Matrix E tube during run to basal respiration. Then 30 µl of ARW containing enzymatic units according to Table 4 were distributed equally to the aggregates' surfaces. The samples were incubated for 1 h at 37°C in a heating block, cooled down on ice to decrease enzyme activity and washed three times in 1 ml of ARW not by shaking but gently rotating along the tube's longitudinal axis to separate detached and planktonic cells from the soil matrix. Supernatants of all three washing steps were removed carefully with a pipette, pooled and centrifuged at 13.000 G for 15 min at 4°C. Then the supernatant was discarded, the pallet resuspended in 200 µl ARW and transfered to another FastPrep Lysing Matrix E tube. Both soil and washing ARW samples were extracted and purified at 4°C following the FastDNA™ SPIN Kit for Soil manual. All DNA samples were stored at -20°C for further use. A direct subsampling from the aggregate stability experiment was rejected due to its destructive capability regarding aggregates. Temperature, substrate, pH and water content of the DNA experiment were similar to the incubation of samples for the measurement of aggregate stability. Further differences (e.g. soil volume) were disregarded.

Amplification of 10-fold diluted DNA samples was performed using a C1000 Touch Thermal Cycler (BioRad). According to the reference for SG qPCR Master Mix (Roboklon) thermocycling comprised an initial denaturation at 95°C for 10 min as well as 55 cycles of 15 sec of denaturation at 95°C, 20 sec of annealing at 49°C and 30 sec of elongation at 72°C. The reaction mix contained 1 µl PCR-H_2O, 12.5 µl SG qPCR MasterMix, each 0.75 µl of a 20 µmol l^{-1} solution of the universal bacterial primers 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 341r (5‘-CTGCTGCCTCCCGTAGG-3‘) (Muyzer et al., 1993; Marchesi et al., 1998) and 10 µl template DNA. *Escherichia coli* 16s

DNA solution containing 10,000 copies μl^{-1} was used as qPCR standard in steps of tenfold diluted concentration from 10^6 to 10^2 copies μl^{-1}.

4.3.5 Statistics

For evaluation of the light fraction SOC (LF-SOC) release, mean values as well as standard deviations were calculated. Parallels of each variant were positively tested to provide normal distribution and evidence of variance homogeneity (Shapiro Wilk test, Levene test, both $p>0.05$, data not shown). One way analysis of variance (ANOVA) was applied followed by Tukey test to clarify significant ($p<0.05$) differences in LF-SOC release between variants of each energy level. Results of bacterial DNA release were presented as duplicates.

4.4 Results

4.4.1 Release of POM carbon

The relative LF carbon release from soil aggregate samples after different enzymatic treatments is shown in Fig. 5. The proportionate C of each captured fraction is defined as $C_{frac}\, C_{\Sigma}^{-1}$, in which C_{frac} is the release of LF-SOC per energy level or – in case of the sediment – the organic carbon remaining in the soil matrix. C_{Σ} is the total SOC of all separated LFs and the sediment. Averaging all treatments, around 79% of C_{Σ} remain in the sediment, whereas the bulk of LF-SOC is released as weakly bound oLF (50 J ml^{-1}) and fLF. Only around 4.5% of C_{Σ} is detached at 100 J ml^{-1} and 150 J ml^{-1}.

None of the enzymatic treatments altered the quantity of fLF-SOC released in the absence of sonication (0 J ml^{-1}). In contrast, visible differences to the control were shown for E1 (decrease, $p=0.34$) and E4 (increase, $p=0.42$) at mild sonication (50 J ml^{-1}), whereas E2 ($p=1.00$) and E3 ($p=1.00$) are very similar to the control. The difference between E1 and E4 was statistically significant ($p=0.01$) as indicated by the Tukey test, and the addition of the highest enzyme concentration (E4) caused the release of about 63% more oLF-SOC than occurred with the addition of the lowest concentration (E1). Released LF-SOC at 100 and 150 J ml^{-1} is not different among treatments. Only the E2 scenario shows any

tendency of increased oLF-SOC release at 100 J ml^{-1} compared to the other treatments (p=0.07 compared to E3).

The sediment represents the SOC remaining unextractable at ≤150 J ml^{-1} and accordingly shows a trend to decrease with increasing enzyme activity. In relation to the control, nearly the whole alteration in the oLF-SOC releases of E1 and E4 at 50 J ml^{-1} as well as E2 at 100 J ml^{-1} comes from the sediment fraction, but hardly from the other LFs. However, opposite reallocation of SOC between fractions due to converse physico-chemical effects can only be observed in sum. Therefore alterations must be considered as net C transfer between stability fractions.

Cumulating LF-SOC releases of all energy levels, E1 shows a reduction by 16% compared to the control (3.3% of C_Σ), whereas E4 was increased by 10% (2.2% of C_Σ). The strongest enzymatic treatment (E4) caused the release of about 58% (0.49 mg/g dry soil) more cumulated LF-SOC than occurred with scenario E1.

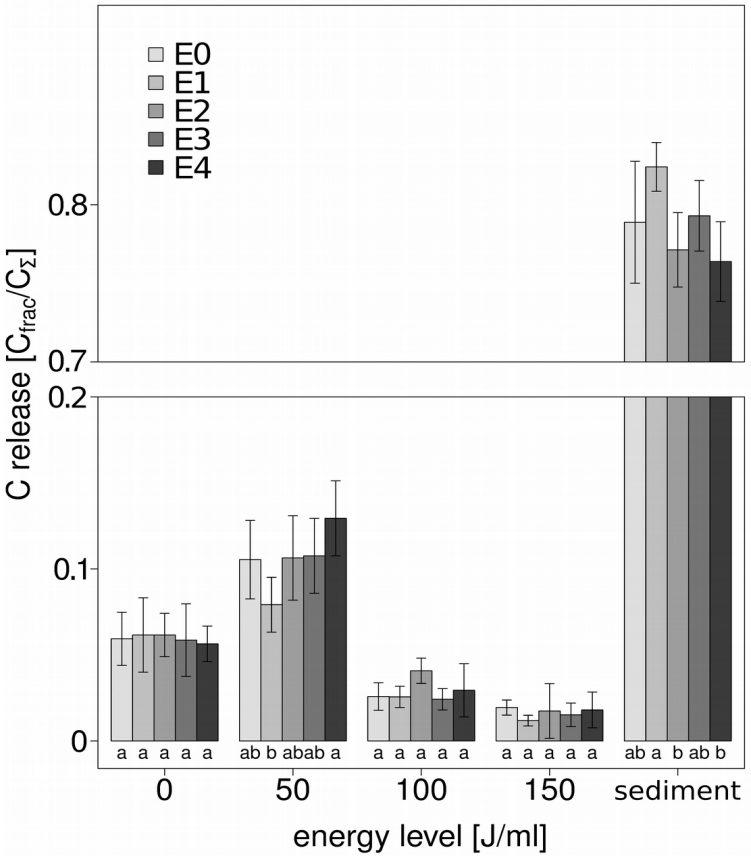

Fig. 5: Relative POC release after treatments (E0, E1, E2, E3, E4) at different energy levels (0, 50, 100, 150 J ml^{-1}, sediment), illustrated by Tukey test characters (a, ab, b). Data are shown as mean values and standard deviations (n=5). (Büks and Kaupenjohann, 2016)

4.4.2 Release of bacterial DNA

The relative DNA release after enzymatic treatment, as pictured with the treatments E0, E1 and E4 in Fig. 6, is defined as the ratio of extracted DNA from suspended bacterial cells (DNA_{susp}) to the sum of DNA extracted from suspended and sessile bacterial cells and the remaining EPS (DNA_{Σ}) multiplied by 100. While there was no difference in relative DNA release in the wash of control and low enzyme additions, treatment E4 caused an increase to more than double the DNA content of either E0 or E1, which amounts to 5.6% of total DNA. This increase is caused by both an increase in released bacterial DNA from suspended bacterial cells and a decrease in eDNA remaining on washed soil particles.

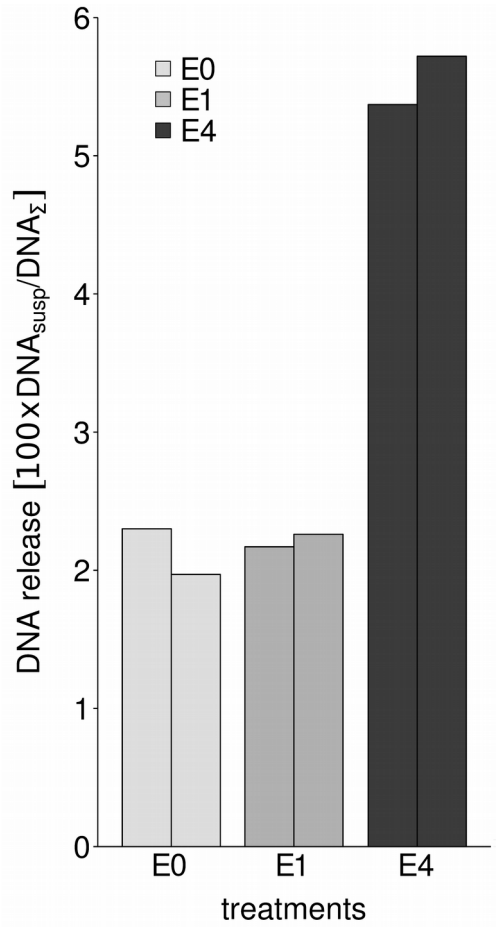

Fig. 6: Relative bacterial DNA release from soil aggregates after treatments E0, E1, and E4 defined as 100x ratio of bacterial DNA from suspended cells (DNA_{susp}) to total bacterial DNA from suspended cells, sessile cells (DNA_{Σ}) and the EPS remaining upon the soil matrix. (Büks and Kaupenjohann, 2016)

4.5 Discussion

We found that increasing the quantity of enzymes applied to aggregates led to increased release of LF-SOC when aggregates were sonicated. This detachment is explained by the following mechanism: The enzyme mix flows into the unsaturated pore space. From there α-glucosidase, β-galactosidase, DNAse and lipase diffuse into the biofilm matrix, where structural components like polysaccharides, eDNA and lipids are digested as approved for diverse enzymes and enzyme targets in ecological and medical studies (Böckelmann et al., 2003; Walker et al., 2007). We propose a simple spatial model to explain the observed findings: The biofilm bridges gaps between organic and mineral primary particles, connects them in addition to other physico-chemical bondings and builds a restructured pore system inside the aggregate (Fig. 4). As macromolecular biofilm components yield EPS as a viscoelastic structure (Sutherland, 2001), their digestion causes a loss in EPS viscosity and thereby should reduce forces involved in the occlusion of POM. The effect is expected to grow with increasing enzyme activity until the whole EPS matrix is dispersed.

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

In accordance with the model, measured oLF-SOC releases indicate a trend for increased POM release with increasing enzyme addition (Fig. 5). The E4 scenario shows that relative oLF-SOC release increased by 63% (5% of C_{Σ}) compared to E1 at 50 J ml^{-1}, but its release is similar to the mean of the other treatments at 0 J ml^{-1}, 100 J ml^{-1} and 150 J ml^{-1}. Noticeable deviations of E1 and E4 from the control do not match the usual significance criteria ($p<0.05$). However, the increase of the relative oLF-SOC release in the E4 scenario compared to the control is predominantly related to an equally lower C content of the sediment but no decrease in the 100 J ml^{-1} and 150 J ml^{-1} fractions. That points to a strong (oLF >150 J ml^{-1}) intra-aggregate fixation of POM due to enzyme targets, which is weakened by enzymatic treatment.

The relation of LF-SOC release with enzymatic biofilm digestion is supported by the comparison of bacterial DNA releases between the treatments (Fig. 6). This indicates that applied enzymes are targeting biofilm components and release bacterial cells: The E4 scenario shows EPS digestion and additional cell release leading to a doubled relative DNA release compared with the control and E1. However, considering that most of the soil bacteria are expected to live in biofilms (Davey and O'toole, 2000), the total DNA release of only 5.6% in the E4 scenario is too low for total biofilm digestion. Hence, biofilm detachment caused by E4 is still likely to be incomplete and the increased oLF-SOC release of E4 only results from a partial soil biofilm detachment. We conclude a slight influence of enzymatic treatment on the occlusion of POM at enzyme concentrations exceeding natural concentrations. This conforms to results of Böckelmann et al. (2003), which indicate that a treatment with enzyme concentrations of near that of E4 is sufficient to destabilize biofilms within 1 hour.

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

The trend for increased POM release with increasing enzyme addition was only broken by the control treatment. Probably this could be explained by pre-incubation of soil aggregates given 0.2 mM NH_4NO_3 and further addition of NH_4NO_3 with enzyme application: Redmile-Gordon et al. (2015) proposed that low C/N ratios of substrates available to soil microorganisms reduce cell specific EPS production rates, and may trigger microbial consumption of EPS to acquire C for cell-growth, which could weaken the biofilm. The observations leading to this proposed dynamic were also found by addition of NH_4NO_3. In the present study, NH_4NO_3 was applied with all treatments including the control (which also received no C from enzyme provision). The lowest C/N ratio in the control soils may itself have sustained EPS consumption and repressed reconstruction of the EPS, contributing to the higher than expected release of POM from the control soil with sonication at 50 J ml^{-1} and the break in the trend for increasing POM release with increasing enzyme addition.

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

Under certain conditions POM carbon release is indicative for soil aggregate stability. Generally, aggregate stability is characterized by determining the reduction in aggregate size after application of mechanical force. The commonly used methods are dry and wet sieving. However, the destruction of soil aggregates by ultrasonication has an advantage over these methods, which is the quantification of the applied energy (North, 1976). It is used for studying reduction of aggregate size (Imeson and Vis, 1984) as well as detachment of occluded POM carbon (Golchin et al., 1994). Kaiser and Berhe (2014) reviewed 15 studies using ultrasonication of soil aggregates in consideration of its destructiveness to the soil mineral matrix and occluded POM. They found destruction of POM at applied energy levels >60 J ml^{-1}, destruction of sand-sized primary particles at >710 J ml^{-1} and of smaller mineral particles at even higher energy levels. We used this method of gentle POM detachment from soil aggregates to measure the oLF-SOC release as a result of mechanical force and linked it to aggregate stability. Since Cerli et al. (2012) have shown that the release of free and occluded light fractions strongly depends on soil properties like mineralogy, POM content, composition and distribution, this method is

restricted to comparison of soils being similar in these properties. Having regard to this restriction, the trend for increase of oLF-SOC release over increasing enzyme additions demonstrates an alteration of soil aggregate stability.

Although our results give a slight evidence for the influence of biofilms on aggregate stability, they have to be recognized with restrictions to full quantifiability: (1) The enzyme concentration hypothetically needed to disperse the whole soil sample EPS matrix depends on diverse boundary conditions like the concentration of enzyme targets, environmental conditions such as pH, temperature as well as ion activity and delay factors such as low diffusion, kinetic influence or metabolization of enzymes by soil organisms. (2) Underlying enzyme kinetics were measured by the producer using pure targets for unit definition, while biofilm targets are much more diverse and soil matrix could interfere. (3) Alternative enzyme targets might be reasonably assumed within the complex chemism of the soil matrix. Released organic cytoplasm molecules of lysed cells can be excluded to be an additional enzyme target due to their low concentration. On the other hand, enzyme specificity to EPS targets in face of the organic soil matrix is unbeknown. (4) The decrease of extracted POM mass due to biofilm erasement from surfaces is suggested to be low, but could cause underestimation of POM release especially in scenario E4. In contrast, a direct contribution of enzyme C to the POM carbon release can be refused. Even in case of complete adsorption to the POM of only one fraction, the highest enzyme concentration (E4) would result in additional 13.5 µg enzyme g^{-1} dry soil being <0.4% of the smallest extracted POM fraction (Table 4). (5) Regarding DNA release measurement as well, data are semi-quantitative, since quantification of the detachment effect is limited by a potential adherence of detached cells to soil particles after washing (Absolom et al., 1983; Li and Logan, 2004). Thus, cell release could be underestimated as biofilm detachment increases.

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

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

4.6 Conclusions

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

4.7 Acknowledgements

This project was financially supported by the Leibnitz-Gemeinschaft (SAW Pact for Research, SAW-2012-ATB-3). We also are grateful to Prof. Dr. Ulrich Szewzyk and the helpful staff of the Chair of Environmental Microbiology (Department of Environmental Technology, TU Berlin) for the unbureaucratic possibility to use their laboratories. In addition our thanks go out to Lara Schneider. Her bachelor thesis helped us to get an overview in the early phase of this experiment. And thanx both to Dennis Prieß for initiating a good idea during a very good Taiji training and to Tom Grassmann for his help to handle R coding.

5 POM occlusion within sandy soil macroaggregates is not affected by feeding and motion of the nematode *Acrobeloides buetschlii*

5.1 Abstract

To protect in a harsh environment, bacteria gather in a viscose biofilm, which also forms a food source for the soil fauna such as bacterial-feeding nematodes. In addition to its protective and nutritive function, biofilm is supposed to provide the aggregation of soil particles, which is attended by the occlusion of particulate organic matter (POM). In the present work we hypothesized, that grazing of the nematode *A. buetschlii* on bacteria in a sandy agricultural soil affects the occlusion of POM. Soil aggregate samples from a cropland near Berge (Germany) were inoculated with on average 370 individuals g^{-1} of *A. buetschlii* and incubated for 14 days. The population development was monitored and the POM occlusion as well as the amount and pattern of phospholipid fatty acids (PLFAs) were measured at day 0, 1 and 14. Although the population of *A. buetschlii* remained stable across the experimental period, neither a changing microbial biomass and composition nor a variation of POM release was observed compared to a nematode-free control. As about 41% of the mesopores within the aggregate sample did not provide enough space for nematode migration, this suggests an inaccessibility of intra-aggregate biofilms and therefore protection against faunal grazing. These spacially protected microaggregates likely have a key role in POM occlusion.

5.2 Introduction

Biofilms represent a bacterial strategy to withstand ecological stressors, e.g. toxics and antibiotics, radiation, drought and grazing pressure (Flemming and Wingender, 2010). They consist of 90 to 97% water (Zhang et al., 1998; Schmitt and Flemming, 1999; Pal and Paul, 2008), differing ratios of polysaccharides, extracellular DNA (eDNA), proteins and lipids as well as microbial cells, which accounts for 10 to 50% of the biofilm dry mass (More et al., 2014). The viscous biofilm matrix not only functions as protective clothing for microorganisms living within, it is also a genetic cross-over hotspot and collective digestive system, which plays a role in nutrient cycling and soil aggregation. (Baldock, 2002; Ashman et al., 2009; Flemming and Wingender, 2010)

As soil bacteria are known to colonize particulate organic matter (POM), their biofilms are suggested to play a role in POM occlusion due to soil aggregate formation (Jastrow and Miller, 1997; Baldock, 2002; Chenu and Stotzky, 2002). Compared to unaggregated soils, well aggregated soils show an increased water holding capacity in micropores, drainage, aeration and rootability as well as less compactibility and erodability (Baumgartl and Horn, 1991; Taylor and Brar, 1991; Ball and Robertson, 1994; Barthes and Roose, 2002; Alaoui et al., 2011). These are parameters that affect soil fertility. Consequently, influences of agricultural praxis or natural soil processes on soil biofilms are important in sustainable land use.

Grazing soil biota, e.g. protozoa and nematodes, are assumed to affect the bacterial community and the soil nutrient cycling (Ingham et al., 1985; Bonkowski, 2004). Nematode grazing maintains bacterial populations in a youthful state and thereby enhances decomposition activity (Neher, 2010). It is therefore conceivable, that soil biofilm structure is engineered by these organisms. As part of the microfauna, the phylum Nematoda is an ecologically important branch containing >25,000 species (Zhang, 2013) in freshwater, marine, endobiontic and soil habitats. Nematodes are ubiquitary abundant even in stressed or disturbed soils (Neher, 2010) and show different feeding habits as there are plant parasites, fungal feeders, predators, unicellular eukaryote feeders as well as bacterial grazers (Yeates et al., 1993). Due to these diverse trophic interactions nematodes hold a central position in both bottom-up and top-down controlled food webs (Ferris, 2010; Yeates, 2010).

A well examined, cosmopolitic, robust and easily cultivable representative, *Acrobeloides buetschlii*, is used in this study (Nicholas, 1962; Frey, 1971). This opportunistic species is common in many soils and its ecology is well examined in studies of e.g. forest, subarctic and agricultural soils (Ruess, 1995a; Korthals et al., 1996; Ruess et al., 2002; Kästner and Germershausen, 2014). Female adults, predominant in soils, have a length between 300 and 500 µm and a diameter of 12.5 to 41.7 µm (Bongers, 1994). In pure culture *A. buetschlii* showed its highest population growth rate at 26.6°C and a slightly reduced growth rate at an incubation temperature of 20°C (Venette and Ferris, 1997). Its metabolic rate is the highest at 35°C (8.5 ng CO_2 μg^{-1} Nematode h^{-1}) and has a second local optimum (7 ng CO_2 μg^{-1} Nematode h^{-1}) at 20°C (Ferris et al., 1995). 20°C also lead to a generation time of 13 to 14 days (Nicholas, 1962). As other nematodes are known to use chemotaxis when foraging for food, *A. buetschlii* is also assumed to migrate this way (Zuckerman and Jansson, 1984). The nematode develops well at a soil pH of 6 (Frey, 1971; Korthals et al., 1996) and 60% field capacity in sandy soils (Ackermann et al., 2016). Nematodes swim in water-filled pores and water films on particle surfaces (Juarez et al., 2010) and migrate with 52 to 718 µm sec^{-1} as measured for various taxa (Gray and Lissmann, 1964; Wallace, 1968). *A. buetschlii* was shown to move randomly in the soil without bacteria present, and cover distances of at least 6.6 cm within 8 days (Ackermann et al., 2016). With a bacterial trigger *A. buetschlii* moves towards the resource, which may be located even at several centimeter distance.

By feeding on soil bacteria, digesting and excreting easily available nutrients e.g. NH_4^+, *A. buetschlii* functions as an interlink in soil elemental cycling and thereby strongly affects bacterial growth (Freckman, 1988). Given that the bulk of soil bacteria is living in biofilms (Davey and O'toole, 2000), it seems obvious, that bacterial feeders, whose feeding habit is influenced by preference and food accessibility, influence this small-scale habitat (De Mesel et al., 2004) and thus biofilm related POM occlusion within soil aggregates. Furthermore, Ghanbari et al. (2012) showed a maximum level of mechanical force generation for *C. elegans* of about 61.94 µN. As also *A. buetschlii* releases mechanical forces to its surrounding, it possibly could manipulate soil structure, e.g. by displacing primary particles within soil aggregates.

Based on this trophic and mechanical potential, we hypothesized that a dense population of *A. buetschlii* given on a habitable, nematode-free soil would affect microbial community

and POM release. Expected changes in the occlusion of POM within soil aggregates are attributed to a combined impact of both, mechanical displacement of soil particles and biofilm grazing, and can be negative (destabilization) or positive (adaptive reaction of the biofilm community). To prove our hypothesis, soil aggregates from an agricultural site near Berge (Germany) were incubated for 14 days with *A. buetschlii* at densities similar to common field populations of bacterial-feeding nematodes. The POM occlusion was determined after 0, 1 and 14 days by ultrasonic treatment of soil aggregates, density-fractioning and measurement of released particulate light fraction carbon (POC) following Golchin et al. (1994) and compared to a nematode-free control. In addition, the microbial biomass and community composition was determined by analysis of soil phospholipid fatty acids (PLFAs) at each sampling date, and the nematode population was counted. Separately, also the soil pore size distribution was measured by mercury intrusion to check the accessibility of the intra-aggregate pore space for nematodes.

5.3 Materials and methods

5.3.1 Soil sample

Soil aggregates with a size of 630 to 2,000 µm were collected from an air-dried sandy topsoil (Su3) (Sponagel et al., 2005) of an agricultural site near Berge (Brandenburg, Germany). The resulting sample had a pH_{CaCl2} of 6.9, C_{org}=8.7 g kg^{-1}, C_{min}=200 mg kg^{-1} and C_{mic}=352 mg kg^{-1}.

5.3.2 Basal respiration

Following Nordgren (1988), 20 g of air-dried soil aggregates were incubated in 9-fold replication at 20°C for 72 hours in a respiration device ($CarbO_2Bot$, PRW Electronics) to determine dry baseline CO_2 emission. Afterwards the 9 replicates were divided into triplicates and three different water contents were set (50, 70 and 80%$_{vol}$ field capacity; addition of sterile tap water: 3.2 ml, 4.5 ml and 5.2 ml, respectively). Incubation was conducted at 20°C for 95 hours to detect the water content of highest soil respiration and the point of beginning basal respiration.

5.3.3 Preparation of the inoculum

Breeding of *A. buetschlii* was performed on colonies of the fungus *Chaetomium globosum*. Sterile incubation of *C. globosum* took place on potato dextrose agar (39 g PDA, 1 l dest. Water, sterilized at 121°C for 20 min) for 4 weeks. Afterwards, Petri dishes with *C. globosum* were infected with *A. buetschlii* and sterilely incubated at 18°C in the dark until dense population. A modified Baermann's funnel extraction (Ruess, 1995b) was applied for 3 days at 20°C to segregate living Nematodes from the agar plate and collect them in 45 ml flasks. Subsequently a washing procedure was applied to clean nematodes from protozoa and bacteria: The Nematode suspension was centrifuged for 8 min at 700 rpm and 8°C in a Thermo Scientific Heraeus Multifuge 3SR+ and the supernatant was removed. The nematode pallet was resuspended in sterile tap water and washed again. Then the received pallet was resuspended in 3 ml 0.01% $HgCl_2$ solution, exposed for 3 min, subsequently washed another 2 times and resuspended in sterile tap water. The Nematode concentration was determined by brightfield microscopic counting (100x magnification) in 125 µl suspension. Afterwards the suspension was adjusted to 5,500 individuals ml^{-1} by addition of sterile tap water.

5.3.4 Nematode population development

18x15 g of dry soil aggregates were filled in 250 ml PE-bottles, adjusted to 70% field capacity with sterile tap water (3.4 ml) and incubated for 4 days at 20°C in the dark to reach basal respiration. Afterwards the evaporated water was refilled and half of the samples were inoculated with 1 ml of *A.buetschlii* suspension containing 5,500 individuals ml^{-1} (Nem), which results in an density of 370 individuals g^{-1} dry soil aggregates. The water volume added via nematodes thereby set soil aggregates on 90% field capacity. The other half, used as control (Con), got 1 ml of sterile tap water instead. Each 3 replicates per variant where incubated for 1, 6 and 14 days at 20°C in the dark. Afterwards living Nematodes were extracted from the soil following Ruess (1995b) and fixated with 4% formaldehyde solution. Collected Nematodes were counted using brightfield microscopy with 100x magnification.

5.3.5 Measurement of POC release

25 soil aggregate samples à 15 g were moistened with sterile tap water to get 70% field capacity (3.4 ml) and incubated at 20°C for 4 days in the dark. Afterwards, evaporated water was refilled. Nematode samples were incubated in 5-fold replication for 1 day (Nem1) and 14 days (Nem14) with addition of 1 ml of the above inoculum. Three further controls without Nematodes (Con0, Con1 and Con14) were established comparably. After incubation and refill to 90% field capacity, POC release was estimated by successive ultrasonication, density fractioning and C/N-analysis (Kaiser and Berhe, 2014): In consideration of the contained amount of water, 70.6 ml of 1.64 g cm^{-3} dense sodium polytungstate solution (SPT) were added to the sample for the adjustment of 1.6 g cm^{-3}. Afterwards, the samples rested for 30 min to enable SPT distribution within the pore space. Thereafter, the free light fraction (fLF) was captured using centrifugation at 3,569 G for 26 min and filtering of supernatant trough a glass fibre filter with 1.5 µm pore size. For the following ultrasonic treatment, energy output of the ultrasonication device (Branson© Sonifier 250) was derived from the heating rate of water inside a dewar vessel (North, 1976). The remaining soil was refilled to 75 ml 1.6 g cm^{-3} dense SPT, treated with 50 J ml^{-1} and again centrifuged and filtered to capture the weakly bond occluded light fraction (oLF_{50}). After further refill, soil samples were ultrasonicated twice with each additional 50 J ml^{-1} and again centrifuged and fractionated (oLF_{100} and oLF_{150}). Light fractions (LF) and the remaining sediment (heavy fraction HF plus strongly bond POM) were froze at 20°C, lyophilized, ground, dried at 105°C and analyzed for organic C concentration by use of an Elementar Vario EL III CNS Analyzer. As the soil aggregates do not contain carbonates (data not shown) and dissolved organic matter (DOM) was removed during density fractionation, collected soil organic carbon (SOC) can be interpreted as POC.

5.3.6 Phospholipid fatty acid (PLFA) extraction and analysis

Similar to the measurement of POM release, 5 variants (Con0, Con1, Con14, Nem1 and Nem14) with 5 replications were prepared. After incubation, samples were directly stored at -20°C for later PLFA analysis.

Extraction of PLFAs from soil aggregates was performed following Frostegård et al. (1993). Briefly, 2 to 3 g of soil substrate (wet weight) were extracted with Bligh/Dyer

solvent (chloroform:methanol:citrate buffer ratio of 1:2:0.8, ph 4). Lipids were fractionated into neutral lipids, glycolipids and PLFAs on a silica column (HF BOND ELUT – SI, Varian Inc.) by elution with chloroform, acetone and methanol, respectively. PLFAs were subjected to mild alkaline methanolysis, resulting in fatty acid methyl esters (FAMEs), which were extracted with hexane-chloroform. Methylnonadecanoate (19:0) was used as internal standard. FAMEs were dissolved in isooctane and stored at -20ºC until analysis.

PLFAs were analyzed by gas chromatography using an Agilent 7890 gas chromatograph (GC) and flame ionization detector (FID) equipped with an HP Ultra 2 capillary column (25 m x 0.2 mm i.d., film thickness 0.33 µm) and a computer associated software (Sherlock Pattern Recognition Software, MIDI®). The system was operated in split mode (1:40) with hydrogen as carrier gas. The oven temperature program started with 170°C and increased by 28°C min^{-1} to 288°C, followed by 60°C min^{-1} to 310°C (hold time 1.3 min). FAMEs were identified on the basis of their retention times in comparison to a standard mixture. Correct identification, i.e. chain length and saturation, was verified by GC-mass spectrometry (GC-MS). Representative samples were analysed with an Agilent Series 7890A GC connected to a Mass Selective Detector (Agilent 7000 Triplequadrupole) equipped with HP5MS capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm), operated in splitless mode with helium as carrier gas. Oven temperature program sated at 40°C and increased by 46°C min^{-1} to 200°C, followed by 5°C min^{-1} to 238°C, 120°C min^{-1} to 295°C and 2°C min^{-1} to 300°C, held for 2 min. A mass range of 40 to 400 m z^{-1} was monitored in scan mode.

The microbial community was assigned using the PLFA 18:2ω6 for saprotrophic fungi, and i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, 17:0 10-meth, cy19:0, 16:1ω7, 17:1ω8 and 18:1ω7 for bacteria according to Frostegård et al. (1993), Frostegård and Bååth (1996) and Zelles (1999). The total amount of PLFAs represents a mean for microbial biomass (Zelles, 1999). For more details see Table 5.

Table 5: List of group specific PLFAs used in the present study.

Target group	PLFA	References
Actinobacteria	10-meth16:0	Kroppenstedt (1985); Vestal and White (1989); Mirza et al. (1991)
	10-meth-17:0	
	10-meth-18:0	
gram(+) Eubacteria	14:0 iso	O'Leary and Wilkinson (1988); Vestal and White (1989); Zelles (1997); Zelles (1999)
	15:0 anteiso	
	15:0 iso	
	16:0 iso	
	17:0 anteiso	
	17:0 iso	
gram(-) Eubacteria	17:0 cyclo	O'Leary and Wilkinson (1988); Zelles (1997); Zelles (1999)
	19:0 cyclo	
Eubacteria	15:1 ω9c iso	Bowman (2015)
	16:1 ω9c iso	Bowman (2015)
	16:1 ω5c	Nichols et al. (1986); Zelles (1997)
	16:1 ω7c	Guckert et al. (1991); Zelles (1999)
	16:1 ω9c	Zelles (1997)
	16:1 ω11c	Zelles (1997)
	17:1 ω7c anteiso	Bowman (2015)
	17:1 ω9c anteiso	Bowman (2015)
	17:1 ω8c	Kaneda (1991); Bühring et al. (2014)
	18:1 ω7c	Zelles (1999)
Fungi	18:2 ω6c	Federle (1986); Frostegård and Bååth (1996); Stahl and Klug (1996); Zelles (1999)
Plants	22:0	Zelles (1999); Ruess et al. (2007)
	24:0	Zelles (1999); Ruess et al. (2007)
Animals	20:3 ω6c	Hutzell and Krusberg (1982); Ringelberg et al. (1997); Watts (2009); Buyer et al. (2010)
	20:4 ω6c	Lechevalier and Lechevalier (1988); Stanley and Nelson (1993); Chen et al. (2001)
Miscellaneous origin	14:0	Balasooriya et al. (2014); Lange et al. (2014)
	15:0	
	16:0	
	17:0	
	18:0	
	20:0	
	23:0	
	18:1 ω5c	Hutzell and Krusberg (1982); O'Leary and Wilkinson (1988); Zelles (1997)
	18:1 ω9c	Vestal and White (1989); Zelles (1999); Bååth (2003); Ruess et al. (2007)

5.3.7 Mercury intrusion

The measurement of the pore size distribution within the soil aggregate sample was performed using mercury intrusion (Porosimeter 2000 WS, Carlo Erba Instruments). The soil aggregate sample (0.51 g) was air-dried for 24 hours at 40°C and measured using a maximum test pressure of 200 MPa and a pressure decrease of 3.6 MPa min^{-1} at 26.1°C (find additional data in the supplements). Data are given for pore sizes between 0.005 and

50 µm representing the non-draining intra-aggregate pore space, which is filled with water at field capacity (Blume et al., 2015).

5.3.8 Statistics

Data of Nematode density, POC release and PLFA composition analyzed with Shapiro and Wilk's test as well as Levene's test are assumed to be normal distributed (Shapiro and Wilk, 1965) and have homogeneity of variance (Lim and Loh, 1996), respectively. Basal respiration was expressed as simple moving average with a span of 4 adjacent sampling times. The characteristics of the triplicate with the largest ratio of CO_2 emission to soil water content was chosen for later incubation. A repeated measurement design (two-factorial ANOVA, $p \leq 0.05$) was used to test for differences between Nematode and control samples in both nematode density and PLFA composition (von Ende, 2001). Data of POM release were analyzed using one-way analysis of variance (ANOVA, $p \leq 0.05$) followed by Tukey's test (Christensen, 1996).

5.4 Results

5.4.1 Soil respiration

As dry soils showed nearly no CO_2 emission, wetting resulted in a respiration maximum around 6 µg CO_2 g^{-1} h^{-1} after 17 hours and a basal respiration of about 2.7 µg CO_2 g^{-1} h^{-1} after 60 hours in all samples. In the first 20 hours after wetting, samples of 50% water holding capacity exhibit a lower basal respiration compared to samples of 70% and 80%, which had quiet similar CO_2 emissions. (Fig. 7)

On the basis of these data, a water holding capacity of 70% was chosen for pre-incubation in all experiments, for it provides highest bacterial metabolic activity and sufficient air capacity after application of Nematodes.

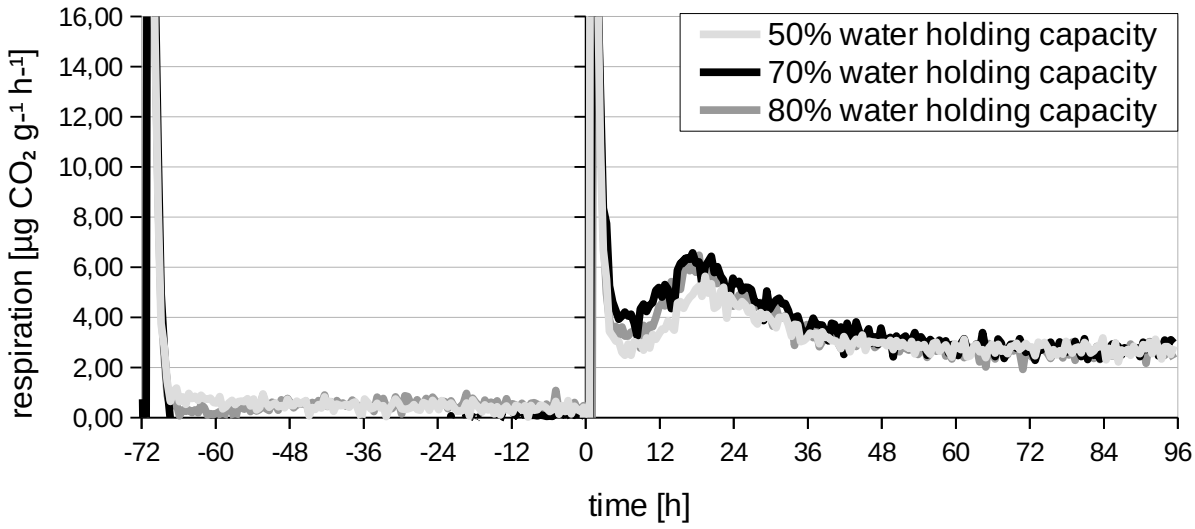

Fig. 7: Soil respiration of dry samples (day -72 to 0) and samples with 50%, 70% and 80% (day 0 to 72) water holding capacity. Data are presented as simple moving average. Peaks at -72 and 0 h are caused by opening the device and have to be ignored.

5.4.2 Population development

On average 367 nematodes g^{-1} dry soil were added to each inoculated sample. Nematode counting after 1, 6 and 14 days points to a stable population with numbers between 187 and 239 nematodes g^{-1} soil (Fig. 8). In control soils few endogenous nematodes were recorded with densities below 1 individual g^{-1} dry soil until day 6, which slightly increased to 5 and 18 individuals g^{-1} dry soil at day 14 in two of the six replicate samples. Overall, control samples comprised very few to no nematodes, whereas inoculated aggregate samples function as habitat for a significantly higher and nearly stable nematode population ($p=0.0026$) containing a large number of young nematodes at day 14 (not counted).

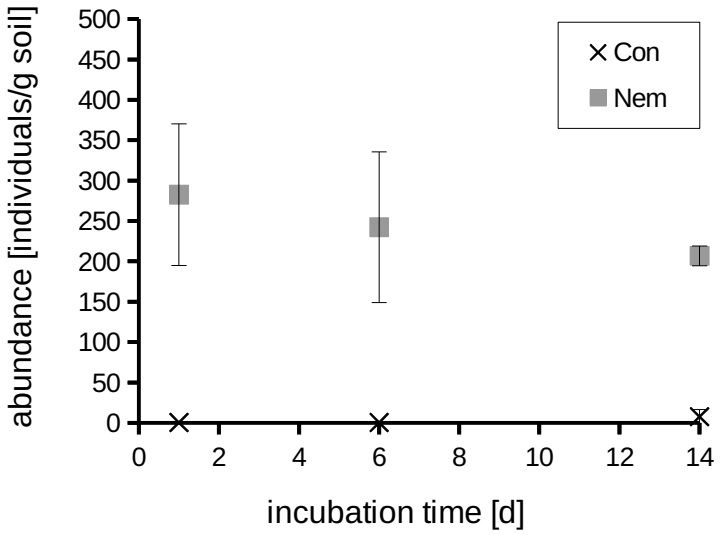

Fig. 8: Population development of nematodes (individuals g^{-1} dry soil) in 9 soil aggregate samples with (■) and 9 without (x) amendment of *Acrobeloides buetschlii* after 1, 6 and 14 days of incubation at 20°C.

5.4.3 POC release

A comparison of the POC release – defined as ratio of the organic carbon release per energy level (C_{frac}) to the sum of organic carbon of all light fractions and the sediment (C_{Σ}) – shows a high similarity between the variants (Fig. 9): Tukey's test does not show significant differences for 0, 50 and 100 J ml^{-1}, neither between sampling dates of one variant nor between variants for a fixed point in time. However, at day 14 the oLF_{150} release of the Nematode samples is reduced compared to day 1, amounting to 1.6% of C_{Σ} or 0.13 mg C g^{-1}, respectively. The average relative POC release amounts to 5.9% in the fLF, 9.4% in the oLF_{50}, 2.8% in the oLF_{100} and 1.9% in the oLF_{150}, whereas 80% of the POC remain in the soil matrix. The absolute POC releases in mg POC g^{-1} dry soil show very similar relations and significances (see supplements).

The cumulative POC release of both treatments shows a tendency (p=0.08 for Nem and p=0.06 for Con) to decrease from day 0 (2.0 mg C g^{-1}) to day 1 (1.8 C g^{-1}) and further to day 14 (1.6 C g^{-1}) after stepwise application of 3x50 J ml^{-1}.

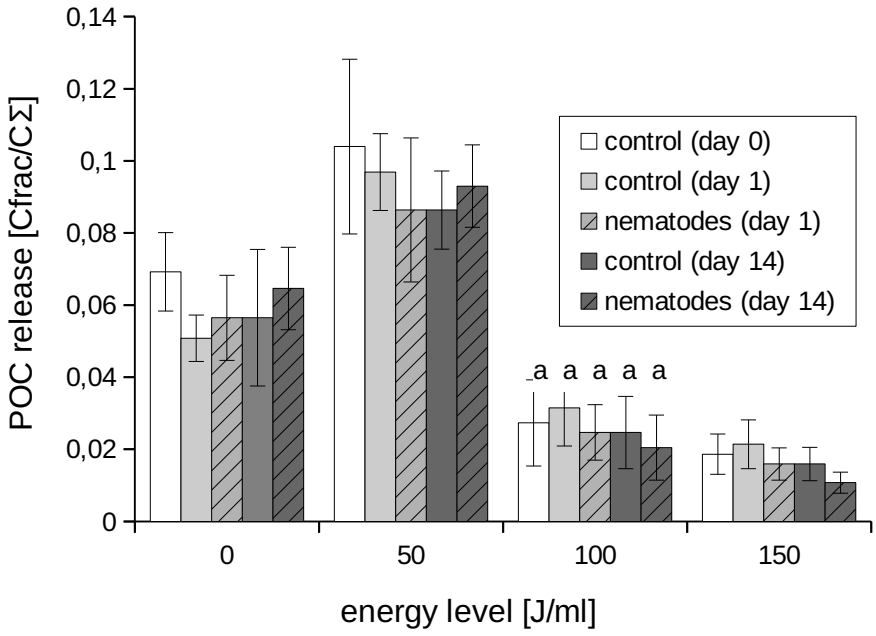

Fig. 9: Relative POC release (mean values with standard deviations) of samples with *A. buetschlii* and controls at different incubation times (0, 1 and 14 days) and applied ultrasonciation levels (0, 50, 100 and 150 J ml^{-1}). Significances are illustrated by Tukey test characters a, b and c.

5.4.4 PLFA analysis

The total amount of PLFAs, as measure for microbial biomass, ranged from 28.0 to 42.5 nmol g^{-1} dry soil. No significant differences between samples colonized by *A. buetschlii* and control samples were detected, and also no significant variation of microbial biomass over time (Fig. 10). Both inoculated and control samples have total PLFA concentrations of 17.4 to 28.3 nmol g^{-1} for Eubacteria, 6.0 to 9.0 nmol g^{-1} for gram positive bacteria, 1.7 to 4.7 nmol g^{-1} for gram negative bacteria, 2.3 to 4.6 for Actinobacteria and 0.6 to 2.1 nmol g^{-1} for fungi, but do also not show different concentrations neither within the phylogenetic groups nor regarding single PLFAs. The fungal population is surprisingly low. From the 35 assessed individual PLFAs, only 20:4ω6c, a marker for soil animals, significantly increases with the application of nematodes (p=0.05 at day 14).

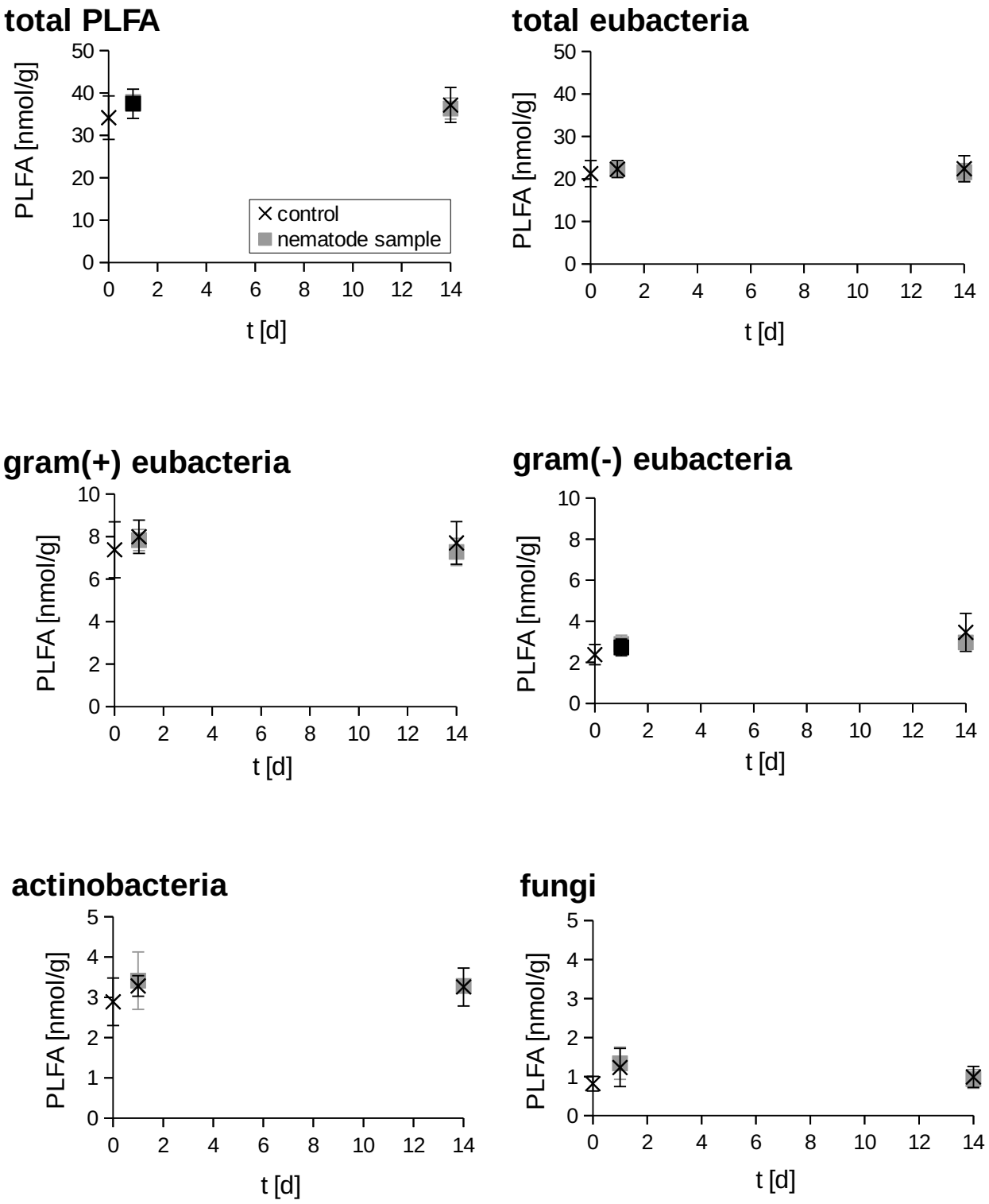

Fig. 10: Amount of group specific PLFAs in soil aggregate samples with (■) and without amendment of *A. buetschlii* (x) at day 0, 1 and 14.

5.4.5 Mercury intrusion

The pore size distribution within the range of 0.005 to 50 µm is plotted in Fig. 11. At 90% field capacity, pores <40 µm are filled with water. If the water content drops below 63%, only pores of an equivalent diameter of 12.5 µm are completely filled with water, which corresponds to the smallest known diameter of adult female *A. buetschlii*.

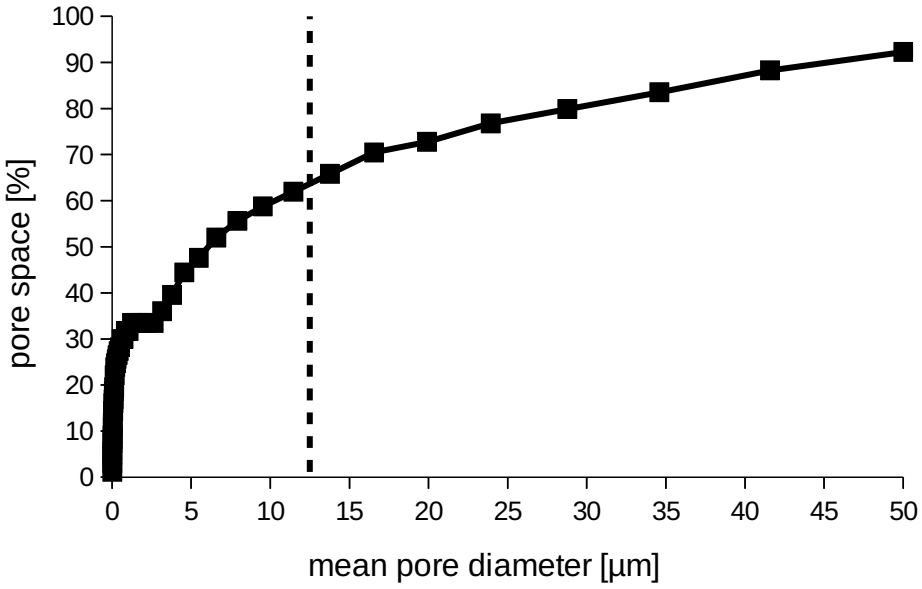

Fig. 11: Cumulative pore space diagram of macro-aggregates from a sandy agricultural soil. The dotted line marks the body diameter of adult *A. buetschlii*.

5.5 Discussion

The experimental setting featured environmental conditions that match the requirements of soil-born grazing nematodes: Cultivation temperature and pH conform to literature and are related to metabolic optima of *A. buetschlii* (Frey, 1971; Korthals et al., 1996; Venette and Ferris, 1997). The measured nematode-free soil basal respiration of about 2.7 µg CO_2 g^{-1} h^{-1} exceeds data reported from a regional-scale survey in Northeastern Germany by the 2-fold (Wirth, 1999), with corresponding values in microbial carbon (C_{mic}=352±44 mg kg^{-1} soil). Furthermore, the bacterial PLFA amount with an average 22.7 nmol g^{-1} dry soil aggregates during the experimental period conforms to literature

values (Bååth and Anderson, 2003). This implies a normal microbial activity and abundance provided by the experimental soil substrate. As in addition sandy agricultural soils in temperate regions are proved to be a habitat for *A. buetschlii* (Korthals et al., 1996), an adequate bacterial food source can be reasonably assumed. This is supported by the stable *A. buetschlii* population, which amounts ~1.4 times the Cephalobidae density of a comparable agricultural soil (Scharroba et al., 2012). Nematode densities of further soils from field and laboratory experiments show this density as above-avarage (Yeates and Brid, 1994; Dmowska and Ilieva, 1995; Bouwman et al., 1996; Yeates et al., 1999; Kästner and Germershausen, 2014). Additionally, the increased proportion of juvenile nematodes occurring in the inoculated samples of day 14 (data not shown) underpin the existence of a feasible environment to support nematode development and accords to generation times found by Nicholas (1962). Furthermore, Ackermann et al. (2016) reported a chemotactic perception of food sources by *A. buetschlii* within 6.6 cm. This points to perceptibility of food sources at every point within the PET flask and a potential migration of *A. buetschlii* within the whole soil sample. The soil moisture was adjusted to high values that most probably provide connectivity between habitable pores. Sufficient oxygen supply is expected, as anaerobic conditions appear as a shift in abundance towards anaerobic bacterial taxa, e.g. represented by vaccenic type fatty acids (e.g. ω7 type) (Zelles, 1999). Measured PLFAs do not show such a shift, pointing to constant aerobic conditions during the whole incubation period.

However, no influence of the inoculum on POM occlusion and PLFA composition was observed: The tendency of both variants to increase POM occlusion over time suggests a rebuilding of aggregate structure after slaking induced by the initial wetting (Beare and Bruce, 1993), but the occlusion of POM is not influenced by the application of nematodes. This might be due to minor influence of nematode grazing on the abundance and composition of the microbial community: The bacterial groups, represented by cumulated amounts of specific PLFA markers, do not show any differences between nematode samples and the control. This suggests no shift within major bacterial groups, e.g. gram positive forms or acidobacteria. Also 34 of 35 individual PLFAs do not show significant differences of amount and proportion between the variants. Only 20:4ω6c, a marker for soil animals (Ruess and Chamberlain, 2010), is significantly enriched after nematode application. This PLFA was most likely derived from the inoculated nematodes, yet

Protozoa developing in the soil may be an additional source. The PLFA 16:1ω5c showed a tendency to decline in the presence of nematodes, suggesting nematode feeding on bacteria. However, as 16:1ω5c is a general bacterial marker (Ngosong et al., 2012), no specific group as nematode prey can be assigned. In sum, the limited shift between aggregate soil with/without nematodes indicates only a small influence of grazing on the soil microbial community.

Different reasons can be considered for the lack in hypothesized grazing effects:

1) The Nematodes do not feed upon the given microbial community. Although this is not directly tested, refusing food seems implausible in the present study due to proper habitat quality and observed nematode reproduction. Moreover, *A. buetschlii* is an opportunistic bacterial feeder with a broad feeding range (Nicholas, 1962; Bird and Ryder, 1993; Venette and Ferris, 1998).

2) Extracellular polymeric substance is known to be a hindrance against grazing. However, whether this barrier is highly protective against certain grazers leading to preservation of nearly the whole biofilm population or incompletely effective depends on various factors such as biofilm architecture, toxicity or signaling and cannot be estimated in the present study. (Höckelmann et al., 2004; Matz et al., 2004; Weitere et al., 2005)

3) Biofilms might be ineffectual for POM occlusion in sandy soils. The influence of biofilms on aggregate formation is assumed to be weaker in sandy than in clayey soils, as mainly silt, clay and small organic particles are bound by the biofilm extracellular matrix (EPS) and protection of bacteria against grazers is enhanced by a fine pored, hardly accessible habitat (Chenu, 1995; Chenu and Stotzky, 2002; Six et al., 2004). However, work on the same sandy soil indicates a slight influence of EPS on the occlusion of POM (Büks and Kaupenjohann, 2016).

4) Grazing Nematodes might be restricted to zones, whose bacterial population do not contribute to the POM occlusion: At field capacity, soil pores <50 µm are water-saturated. In the present study the soil water content reaches up to 90% field capacity, which allows free swimming of Nematodes within the non-draining pore space <40 µm as well as gliding even in thin water films within larger pores (Wallace, 1968). However, the motility of *A. buetschlii* is restricted to pores larger than its body diameter, here 12.5 µm equal to ~37% of the non-draining pore space of the present soil. Further ~22% of the non-draining pore space have diameters <0.3 µm and are therefore not colonized by bacteria (Foster, 1988),

whereas all larger pore size classes, aggregate surfaces and the inter-aggregate space are inhabited by bacterial communities. As a result, 41% of the non-draining pore space comprise pores between 0.3 and 12.5 µm, that contain bacteria, which are spatially protected against nematode grazing. This is in accordance with data of Ranjard and Richaume (2001), who found that in sandy soils the majority of the bacterial population is located in the inner part of soil aggregates, mainly in micropores <9 µm within aggregates <100 µm, and also matches data of Winding et al. (1997). This undisturbed bacterial community might be the agent for soil aggregation: Following a comprehensive review of Tisdall (1996), the hierarchical structure of soil aggregates base upon “minor” microaggregates (<20 µm), which are built of clay-humus-complexes associated with silt particles, partly humified bacterial debris and exudates, mineral incrustations and even on this level bacterial colonies. Larger microaggregates (<250 µm) consist of these elements and further POM. Considering the function of bacterial macromolecular exudates on soil aggregation (Chenu and Stotzky, 2002), spatially protected intra-aggregate bacteria could play a role in POM occlusion, whereas grazed bacteria in large pores are subject to a higher turnover rate and not able to establish a permanent structure for sticking primary particles. Referring to the similar POM release in both treatment variants, also the mechanical force generation of *A. buetschlii* does not suffice to overcome physico-chemical bonds and to move/separate soil particles to reach new grazing sites within microaggregates.

We propose to explain the missing effect of *A. buetschlii* on POM occlusion in the following way. *A. buetschlii* most probably feed on EPS within the accessible pore space, but is not able to reach bacteria in smaller mesopores. On its grazing sites, feeding is non-selective and maintains a growing bacterial population resulting in an equilibrium and hence constant PLFA amounts comparable to the control (Ingham et al., 1985). The bacterial community of smaller mesopores, which is supposed to provide POM occlusion within soil aggregates, remain protected against grazing. In consequence, *A. buetschlii* is not able to influence the POM occlusion. However, deeper investigations are necessary to prove this explanation. Long-term effects on soil structure, e.g. due to acclimatization of *A. buetschlii* or mineral N exudation (Ferris et al., 1997), were excluded from this work. Further experiments with different soils and nematode taxa as well as longer incubation time are necessary.

5.6 Conclusions

We hypothesized a grazing influence of an excess population of *A. buetschlii* on soil PLFA composition concomitant with a change of POM occlusion. This hypothesis has to be refused, as a large, stable and fertile population of *A. buetschlii* only marginally affected the soil PLFA composition and had no influence on POM occlusion in aggregates of a sandy agricultural soil. This is explained by a majority of inadequate pore diameters hindering *A. buetschlii* to access the finer pore space of microaggregates. However, the bacterial communities in those microaggregates are assumed to contribute to aggregation and POM occlusion.

5.7 Acknowledgements

This project was financially supported by the Leibnitz-Gemeinschaft (SAW Pact for Research, SAW-2012-ATB-3). Our thanks go out to the Department of Building Materials and Construction Chemistry (TU Berlin) for the possibility to use their Mercury Intrusion Porosimeter and to it's helpful staff.

6 Two different microbial communities did not cause differences in occlusion of particulate organic matter in a sandy agricultural soil

6.1 Abstract

Apart from physico-chemical interactions between soil components, microbial life is assumed to be an important factor of soil structure forming processes. Bacterial exudates, the entanglement by fungal hypae and bacterial pseudomycelia as well as fungal glomalin are supposed to provide the occlusion of particulate organic matter (POM) through aggregation of soil particles.

This work investigates the resilience of POM occlusion in face of different microbial communities under controlled environmental conditions. We hypothesized that the formation of different communities would cause different grades of POM occlusion. For this purpose samples of a sterile sandy agricultural soil were incubated for 76 days in bioreactors. Particles of pyrochar from pine wood were added as POM analogue. One variant was inoculated with a native soil extract, whereas the control was infected by airborne microbes. A second control soil remained non-incubated. During the incubation, soil samples were taken for taxon-specific qPCR to determine the abundance of Eubacteria, Fungi, Archaea, Acidobacteria, Actinobacteria, α-Proteobacteria and β-Proteobacteria. After the incubation soil aggregates (100 to 2000 µm) were collected by sieving and disaggregated using ultrasound to subject the released POM to an analysis of organic carbon (OC).

Our results show, that the eubacterial DNA of both incubated variants reached a similar concentration after 51 days. However, the structural composition of the two communities was completely different. The soil-born variant was dominated by Acidobacteria, Actinobacteria and an additional fungal population, whereas the air-born variant mainly contained β-Proteobacteria. Both variants showed a strong occlusion of POM into aggregates during the incubation. Yet, despite the different population structure, there were only marginal differences in the release of POM along with the successive destruction of soil aggregates by ultrasonication. This leads to the tentative assumption that POM occlusion in agricultural soils could be resilient in face of changing microbial communities.

6.2 Introduction

Microbial communities play an irreplaceable role in soil ecosystems. Due to their metabolic diversity and abundance, especially bacteria and Fungi have considerable influence on mineral and organic matter transformation (Torsvik and Øvreås, 2002; Gianfreda and Rao, 2004; Uroz et al., 2009; Madigan et al., 2015) and often represent the first element in manifold faunal food webs. They also release a broad variety of molecules involved in nutritional or functional cell-plant symbioses supporting plant growth and health (Pühler et al., 2004; Van Der Heijden et al., 2008).

This work focus on a further ecological function of microbial communities: There is evidence, that the soil microbial community takes part in soil aggregate formation, which is supposed to be positively related to the occlusion of particulate organic matter (POM) within soil aggregates. The grade of occlusion influences the carbon cycle, as occluded POM is superior protected against microbial degradation compared to free POM and mutually promotes development of stable macroaggregates. (Jastrow and Miller, 1997; Bronick and Lal, 2005; Brodowski et al., 2006a; Lützow et al., 2006)

The physico-chemical mechanisms underlying aggregate formation comprise interactions between permanent and variable charges of silicates, (hydr)oxides of Fe, Al and Mn, phosphates, carbonates, DOM and POM, which are meditated by multivalent cations with small hydrate shells (e.g. Ca^{2+}, Fe^{3+} and Al^{3+}), and also hydrophobic interactions (Bronick and Lal, 2005). Fine roots form a physical stabilizing network in and around soil macroaggregates and release cementing root exudates (Bronick and Lal, 2005). The microbial influence is supposed to be achieved by the following mechanisms:

(1) Hyphal Fungi and possibly Actinobacteria as well as filamentous colonies of Cyanobacteria wrap and pervade soil aggregates and increase their mechanical strength (Chenu and Cosentino, 2011). Length, strength, surface adherence and geometry of the mycelia determine the contribution to the bulk stability (Chenu and Cosentino, 2011). When disturbed e.g. by tillage, mycelia were found to be less contributive to the formation of water stable aggregates than intact ones (Beare et al., 1997). Whereas fungal hyphae are assumed to mainly stabilize macroaggregates by formation of a sticky string bag (Gupta and Germida, 1988; Miller and Jastrow, 2000), actinobacterial pseudomycelia were

found both within and around soil microaggregates (Kanazawa and Filip, 1986; Ranjard and Richaume, 2001; Mummey and Stahl, 2004).

(2) Microbial exudates and debris adsorb to soil particles and alter their surface properties, e.g. increase the hydrophobicity which decrease water-caused dispersion of soil aggregates (Chenu and Cosentino, 2011; Achtenhagen et al., 2015).

(3) Microbial biomineralization could cement or block soil particles at their contact regions (Bronick and Lal, 2005). However, little is known about the influence on POM occlusion.

(4) Arbuscular Mycorrhizal Fungi (AMF) are able to produce a proteinaceous substance opperationally defined as Glomalin Related Soil Fraction (GRSF) or – shortly – glomalin (Wright et al., 1996; Rillig, 2004). It appears in large quantities in various soils (Wright and Upadhyaya, 1998), but is most probably not an exudate, since Driver et al. (2005) showed that >80% of the soil glomalin are strongly bond within hyphal cell walls even after harsh extraction. Soil aggregates rich in glomalin showed a high mechanical stability. However, the frequently found correlation between soil aggregate stability and glomalin concentration (Rillig et al., 2002; Bedini et al., 2009; Hontoria et al., 2009; Spohn and Giani, 2010; Fokom et al., 2012; Wu et al., 2014) does not necessarily imply glomalin as an agent of soil aggregation, as for example undisturbed AMF populations could produce a lot of glomalin while in effect aggregate soil particles by wrapping. Therefore the influence of glomalin concentration on POM occlusion is hypothetical.

(5) In contrast to Fungi, the bulk of bacteria is assumed to encapsulate within a viscose matrix of extracellular polymeric substance (EPS) as a reaction to diverse ecological stressors (Roberson and Firestone, 1992; Davey and O'toole, 2000; Mah and O'Toole, 2001; Weitere et al., 2005; Chang et al., 2007; Flemming and Wingender, 2010; Ozturk and Aslim, 2010). This biofilm contains in average 90% water (Zhang et al., 1998; Schmitt and Flemming, 1999; Pal and Paul, 2008). Only 10% to 50% of the remaining dry mass are microbial biomass, whereas the bulk mainly consists of extracellular macromolecules like polysaccharides, extracellular DNA, proteins, lipids and humic substance (Flemming and Wingender, 2010; More et al., 2014). As a result of the ubiquity (Davey and O'toole, 2000), mechanical strength (viscosity) (Möhle et al., 2007; Flemming and Wingender, 2010), structure (Van Loosdrecht et al., 2002) and distribution across the soil aggregate (Nunan et al., 2003), biofilms are supposed to be an important factor of soil aggregation (Baldock, 2002). However, the viscosity of EPS is affected by its molecular composition

(Ayala-Hernández et al., 2008), which strongly depends on species and environmental conditions: For example, different single-species biofilms cultivated under similar conditions have a strongly differing EPS composition (Béjar et al., 1998; Steinberger and Holden, 2005; Celik et al., 2008). But also similar single-species biofilms show differently composed EPS under varying environmental conditions as demonstrated for *Pseudomonas aeruginosa* (Marty et al., 1992; Ayala-Hernández et al., 2008). Little is known about the capability of different bacterial taxa to produce EPS. For example, *Rhizibia* species are considered to be strong EPS producers within the phylum of α-Proteobacteria (Rinaudi and Giordano, 2010), and the genetic ability to produce large amounts of high-molecular polysaccharides and proteins was found in different Acidobacteria (Ward et al., 2009). However, these sparse data do not allow predictions about the potential of specific microbial communities to take part in POM occlusion.

The five above specified mechanisms are all supposed to affect POM occlusion, and all of them are obviously influenced by the composition of the soil microbial community. The aim of this work is to test the resilience of POM occlusion in face of the development of two fundamentally different microbial communities in a sandy agricultural soil. In this case study, a gamma-sterilized sandy soil with pyrogenic biochar amendment from pine wood was inoculated in two variants with microbial and sterile soil extract and incubated for 76 days in a bioreactor at field capacity. The second variant was routinely exposed to room air during sampling to initiate the development of an air-born bacterial population. We chose this inoculation, to receive two complex populations that have no potential to converge their taxonomic abundances, as Delmont et al. (2014) recently found that the development of microbial communities is controlled by physical-chemical properties of soils rather than the initial population: E.g. a population taken from a forest soil was given on a sterile grassland soil and there developed like the original grassland population. The biochar was used as a POM analogue, but also represents an upcoming class of soil amendments (Lehmann and Joseph, 2015). During incubation the DNA of Eubacteria, Fungi, Archaea, Acidobacteria, Actinobacteria, α- and β-Proteobacteria in soil samples from both variants was quantified using taxon-specific qPCR. After incubation, soil aggregates of a size between 0.1 and 2 mm were separated by sieving. Following the method of Golchin et al. (1994), aggregates were treated with ultrasound, and the release of intra-aggregate particulate organic carbon (POC) was quantified by use of POM density fractionation and

carbon analysis. The amount of released POC depends on the destruction of soil aggregates, which is a function of applied energy, and gives information about the binding strength of POM within the aggregates.

We hypothesized that the establishment of different microbial communities will lead to a different occlusion of POM. A lower occlusion strength is attended by an increased POC release when applying a specific amount of mechanical stress to soil aggregates under further similar conditions.

6.3 Materials and methods

6.3.1 Preparation of soil and soil extracts

Air-dried soil from a sandy A-horizon (Su3) of an agricultural experimental site in Berge (Germany) was sieved to <2 mm particle size and mechanically disaggregated in a mortar to create an macroaggregate-free soil sample with C_{org}=8.7 mg g^{-1} dry soil. The soil sample was amended with 5%$_{vol}$ of pyrogenic biochar (pine wood, PYREG® GmbH, Dörth/Germany) with a particle size <0.1 mm (71% <40 µm, see supplements) and homogenized by end-over end shaking. Subsequently, the biochar-soil-mixture was sterilized with 40.000 Gy using a Cobalt-60 γ-radiation source and an exposure time of 2 weeks following McNamara et al. (2003). The resulting soil had a pH of 7.1 in 0.01 M $CaCl_2$ solution, a four times increased C_{org} concentration of 36.2 mg g^{-1} and a grain gross density of 2.54 g cm^{-3}.

In addition, 1200 g of untreated fresh soil were extracted with 1560 ml of 10-fold diluted modified R2A broth (0.1 g l^{-1} NH_4NO_3, 0.05 g l^{-1} yeast extract, 0.05 g l^{-1} soy peptone, 0.05 g l^{-1} casamino acids, 0.05 g l^{-1} glucose, 0.05 g l^{-1} soluble starch, 0.03 g l^{-1} K_2HPO_4, 0.0024 g l^{-1} $MgSO_4$, pH 7.2±0.2, autoclaved at 121°C for 20 min) (Atlas, 2010) by end-over end shaking for 3 h. The extract was filtered twice through two layers of laboratory tissue paper and afterwards split into two halves. One half was autoclaved at 120°C for 20 min, whereas the other half remained untreated to provide an inoculum with a soil-born microbial population.

6.3.2 Incubation and sampling

Under sterile conditions, two triplicates of each 300 g sterile soil were filled into pF-bioreactors (Fig. 12) and packed to get a bulk density of 1.36 g cm^{-3}. When closed and connected to a hydrostatic head, the reactors provide constant matrix potential, similar evaporation rates and sterile air supply for soil microbial containment experiments. In the present study, the headspace was continually replaced with a flow rate of 0.4 l min^{-1} by room air filtered with an 0.2 µm membrane filter. The hydrostatic head was 120 cm (pF 2.08) and thus provided a soil water content of about 35.0%$_{vol}$ and a soil air content of 11.5%$_{vol}$. The water content of 35%$_{vol}$ equates to 77 ml soil solution. For example, giving 100 ml soil extract to the dry sample, hence 23 ml are subsequently removed by the hydrostatic head when water tension is adjusted. The adjustment of soil water content was tested in pre-trials with addition of 100 ml of tap water to 300 g of dry soil sample – here the impounded water was rejected within 15 min and the adjustment to 37%$_{vol}$ soil water content at pF=2 took place within 4 days (data not shown). These characteristics were also assumed for the main experiment.

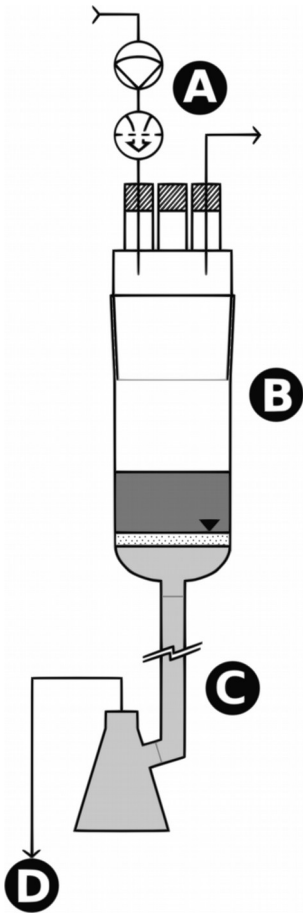

Fig. 12: pF-bioreactor with its components A) air supply composed of diaphragm pump and membrane filter, B) filter column with soil sample (dark grey) and filter plate (dotted), C) hydrostatic head (pale grey) and D) liquid waste container.

The first triplicate (SP_{soil}) was inoculated with each 100 ml of the non-autoclaved inoculum to reestablish the native microbial population. The sterilized inoculate was given to the second triplicate to start with an abiotic environment, that is susceptible for infection by air-born microorganisms (SP_{air}) when exposed to unsterile air. Soil extract exceeding the adjusted soil water content was removed by the hydrostatic head and discarded.

The soil columns were incubated for a total of 76 days. During the incubation, a stress factor setting was established that includes warm-humid conditions from day 1 to 24, warm and drying-out conditions between day 25 and 50 as well as cold-humid conditions from day 51 to 76. This setting is supposed to promote EPS production and fungal growth

(Roberson and Firestone, 1992; Di Bonaventura et al., 2008; Borowik and Wyszkowska, 2016). Therefore, incubation took place at room temperature between 24.5°C and 32.5°C until day 50 and at 8°C from day 51 to 76. Hanging water columns were disconnected at day 24 and reconnected after addition of 100 ml of 10-fold diluted modified R2A broth at day 50.

Soil sampling for DNA analysis was performed with sterile plastic pipes used as sampling rings. About 500 mg composite sample compounded of soil from 3 evenly distributed sampling points was taken from each column 18 and 29 h as well as 3, 5, 16, 49, 51 and 76 days after inoculation. The samples were filled in 2 ml reaction tubes and stored at -20°C for later DNA extraction and quantification. During each sampling the bioreactors with air-born cultures were exposed for 15 min to the unsterile room air to enforce infection, whereas the soil-born variant was sampled in a cleanbench. After each sampling, both variants were reconnected to sterile air supply.

After day 76, the soil was removed from the reactors and air-dried for 2 weeks in a laminar flow hood. A pH of 6.8±0.3 was measured for all variants. Afterwards soil aggregates between 0.1 and 2.0 mm in diameter were used for analysis of POM occlusion. In addition, a non-incubated third triplicate ($SP_{control}$) was analyzed in the same way.

6.3.3 DNA extraction and qPCR

DNA was extracted from 370 mg dry soil equivalent by use of a NucleoSpin® Soil Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren/Germany) following the manual instructions. DNA sample purity, represented by 260/230 nm and 260/280 nm extinction ratios, was determined with a NanoDrop1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and assessed as free of contamination (NanoDrop, 2008).

For quantification of different phylogenetic classes (Acidobacteria, Actinobacteria, α- and β-Proteobacteria) and domains (Archaea, Eubacteria, Fungi), a quantitative real-time PCR with specific primer pairs (Table 6) was performed using a QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies, Grand Island, NY/USA). The reaction mix per sample contained 4 µl of 5x HOT FIREPol® EvaGreen® HRM Mix ROX (Solis Biodyne, Tartu/Estonia), each 0.25 µl of the proper 10 pM fwd and rev primer solution (biomers.net, Ulm, Germany; Table 7), 14.5 µl of PCR-H_2O and 1 µl of template DNA solution.

Amplification of DNA templates was executed having an initial denaturation at 95°C for 15 min followed by 40 thermocycles consisting of a denaturation at 95°C for 15 sec, annealing for 20 sec at primer-specific temperatures listed in Table 6 and elongation at 72°C for 30 sec. PCR was checked for consistency by melting curve analysis implemented in the QuantStudio™ 12K Flex Real-Time PCR System. Extracted DNA from standard organisms named in Table 6 was used as DNA standard for the relevant taxa, whereas DNA of non-target organisms from soil samples in return functioned as negative control. Sample-DNA dilution ranged between 1:1 and 1:100 in steps of 1:10.

Table 6: Target classes and domains, appropriate primer pairs, annealing temperatures (AT) and standard organisms for qPCR. (AWI=Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research; DSM=German Collection of Microorganisms and Cell Cultures; ZALF=Leibniz Center for Agricultural Landscape Research)

Target organism	Primer pair	AT	Standard organism (origin)
Archaea	Ar109f / Ar915r	57°C	*Methanosarcina mazei* (AWI)
Acidobacteria	Acido31 / Eub518	50°C	*Acidobacterium capsulatum* (DSM11244)
Actinobacteria	Actino235 / Eub518	60°C	*Streptomyces avermitis* (DSM46492)
α-Proteobacteria	Eub338 / Alf685	60°C	*Agrobacterium tumefaciens* pGV2260 (ZALF)
β-Proteobacteria	Eub338 / Bet680	60°C	*Burkholderia phymatum* (DSM17167)
Eubacteria	Eub338 / Eub518	53°C	*Pseudomonas putida F1* (ZALF)
Fungi	ITS1f / 5.8s	52°C	*Verticillium dahliae* EP806 (ZALF)

Table 7: Applied primer sequences for class- and domain-specific qPCR.

Primer	Primer sequence	Reference
5.8s	5'–CGCTGCGTTCTTCATCG–3'	Fierer et al. (2005)
Acido31	5'–GATCCTGGCTCAGAATC–3'	Fierer et al. (2005)
Actino235	5'–CGCGGCCTATCAGCTTGTTG–3'	Stach et al. (2003)
Alf685	5'–TCTACGRATTTCACCYCTAC–3'	Lane (1991)
Ar109f	5'–ACKGCTCAGTAACACGT–3'	Lueders and Friedrich (2003)
Ar915r	5'–GTGCTCCCCCGCCAATTCCT–3'	Lueders and Friedrich (2003)
Bet680	5'–TCACTGCTACACGYG–3'	Overmann et al. (1999)
Eub338	5'–ACTCCTACGGGAGGCAGCAG–3'	Lane (1991)
Eub518	5'–ATTACCGCGGCTGCTGG–3'	Muyzer et al. (1993)
ITS1f	5'–TCCGTAGGTGAACCTGCGG–3'	Fierer et al. (2005)

6.3.4 Disaggregation of soil aggregates and quantification of POC

Successive destruction of soil aggregates by ultrasonication was used to release occluded POM from its bonding sites (Kaiser and Berhe, 2014). Therefore, in a first step, 75 ml of 1.6 g cm^{-3} dense sodium polytungstate solution (SPT) were added to 15 g of air-dried SP_{soil}, SP_{air} and $SP_{control}$ soil samples. After 30 min of SPT infiltration into the soil matrix and centrifugation at 3,569 G for 26 min, the floating free light fraction (fLF) comprising non-occluded POM was collected by filtering the SPT solution through an 1.5 µm pore size glass fibre filter. In a following step, the remaining soil was filled up to 75 ml SPT solution and ultrasonicated with 50 J ml^{-1} using a sonotrode (Branson© Sonifier 250) to destroy weaker aggregate bonds and release occluded POM. After centrifugation, the floating occluded light fraction (oLF_{50}) was collected. For this purpose, the energy output of the sonotrode was determined by measuring the heating rate of water inside a dewar vessel (Schmidt et al., 1999). Then again the SPT solution was filled up to 75 ml and the sample was treated with an additional energy of 450 J ml^{-1}. After centrifugation, the floating occluded light fraction (oLF_{500}) and the “sediment”, which contains stronger bound POM as well as molecular OM adsorbed to the mineral matrix, were separated and all separated light fractions (LFs) and sediment samples were frozen at -20°C, lyophilized, ground and analyzed for organic carbon concentration using an Elementar Vario EL III CNS Analyzer. As dissolved organic matter (DOM) were leached by SPT solution during the first step of density fractionation, extracted light fraction OC is interpreted as light fraction POC.

6.3.5 Statistical analyses

The statistical analysis of microbial populations and POC release comprised the calculation of mean values, standard deviations and analysis of variance ($p<0.05$). After application of the Shapiro-Wilk test (Shapiro and Wilk, 1965) and Levene test (Lim and Loh, 1996) samples were assumed to be normally distributed and to have variance homogeneity. Total bacterial populations were assumed to be similar in a sample, if the absolute difference between the DNA mean values of both variants is smaller than the averaged standard deviation. A repeated measurement design (two-factorial ANOVA) was used to test for significant differences of class, domain and total DNA concentrations and shares between SP_{soil} and SP_{air} within the final period (von Ende, 2001). Particulate organic

matter releases of SP_{soil}, SP_{air} and $SP_{control}$ were analyzed using one way ANOVA followed by Tukey's test (Christensen, 1996).

6.4 Results

6.4.1 Microbial population analysis

The DNA extracted from both incubated variants shows qualitative differences in the composition of eubacterial populations and further quantitative differences in the fungal population. It is expressed as ng DNA per mg dry soil (ng mg^{-1}) and includes intra- and extracellular DNA. (Fig. 13)

The sum of total measured DNA (DNA_{tot}=DNA_{EUB}+DNA_{FUNG}+DNA_{ARCH}) in SP_{air} averages 2 ng mg^{-1} until day 6, increases to 13.6 ng mg^{-1} at day 49 and decreases again to 6.8 ng mg^{-1} until day 76. In contrast, SP_{soil} quickly increases from 2.4 ng mg^{-1} at the beginning to 19.6 ng mg^{-1} at day 6 and then decreases to 11.4 ng mg^{-1}. Between day 51 and 76 (final period) both variants show a parallel development, but a significant difference in DNA abundance (p=0.049), which is mainly due to fungal DNA. However, both variants have similar total eubacterial populations (DNA_{EUB}, amplified with Eub338/Eub518 primer pair) within the final period with growth curves similar to DNA_{tot}. From day 49 to day 76 the population densities of both variants converge. Within the final period their difference fall below the threshold for similarity.

Fungi (DNA_{FUNG}) show nearly no growth in SP_{air} and remain at DNA concentrations below 0.2 ng mg^{-1}, whereas the fungal population of SP_{soil} grows from 1.11 ng mg^{-1} at day 0 to 5.6 at day 49 and then decreases to 4.7 ng mg^{-1}. Fungal populations of SP_{soil} and SP_{air} differ significantly within the final periode (p=0.001). In contrast, the amount of archaeal DNA (DNA_{ARCH}) remains <0,002 ng mg^{-1} in both variants and does not show a significant difference.

Some eubacterial classes show significant differences between the variants. The amount of acidobacterial DNA differs significantly within the final period (p=0.003). While SP_{air} does not exceed values of 0.3 ng mg^{-1}, the DNA concentration in SP_{soil} increases from

0.4 ng mg^{-1} to values between 2.19 and 3.2 ng mg^{-1}. Actinobacteria in SP_{air} exhibit a nearly constant DNA concentration <0.5 ng mg^{-1}. In contrast, the SP_{soil} population quickly rises to 1.7 ng mg^{-1} at day 6 and then decreases to 1.0 ng mg^{-1} at day 76. Although SP_{soil} shows an in tendency higher population then SP_{air}, differences of both variants within the final period are not significant (p=0.067). The concentration of α-proteobacterial DNA in SP_{soil} quickly rises from 0.1 ng mg^{-1} to 1.0 within 6 days and then decreases continuously to 0.4 ng mg^{-1}, whereas SP_{air} does not exceed 0.2 ng mg^{-1}. Within the final period there are no significant differences between the variants (p=0.237). Among the examined eubacterial classes, only β-Proteobacteria show a significantly higher population in SP_{air} than in SP_{soil}: Until day 16 the DNA concentration in SP_{air} remains smaller than 0.1 ng mg^{-1}, but increases to 5.9 ng mg^{-1} at the end. In contrast, SP_{soil} quickly increases to 2.8 ng mg^{-1} at day 6 and then stabilizes at around 0.9 ng mg^{-1}.

The DNA of eubacterial taxa as a percentage of the total eubacterial DNA (Table 8) shows a dominance of Acidobacteria in SP_{soil} reaching shares of 32.7% (day 51) and 36.8% (day 76), whereas values in SP_{air} stay below 0.9% (p=0.002). Actinobacteria show a 3-fold higher percentage of around 14.6% in SP_{soil} compared to SP_{air} within the final period (p=0.057). In SP_{air} and SP_{soil}, α-Proteobacteria show percentages of around 2.4% and 5.2%, respectively, and therefore do not represent a dominant class (p=0.27). In strong contrast, β-Proteobacteria hold increasing percentages of 79.8% and 88.1% in SP_{air} compared to 8.8% and 12.3% in SP_{soil} (p=0.023). Cumulation shows that these classes cover 88.9% to 96.6% in SP_{air}, mainly dominated by β-Proteobacteria, and 60.9% to 69.1% in SP_{soil}, that is dominated by Acidobacteria, Actinobacteria and also Fungi. In both variants these classes hold an increasing percentage of the total DNA over time.

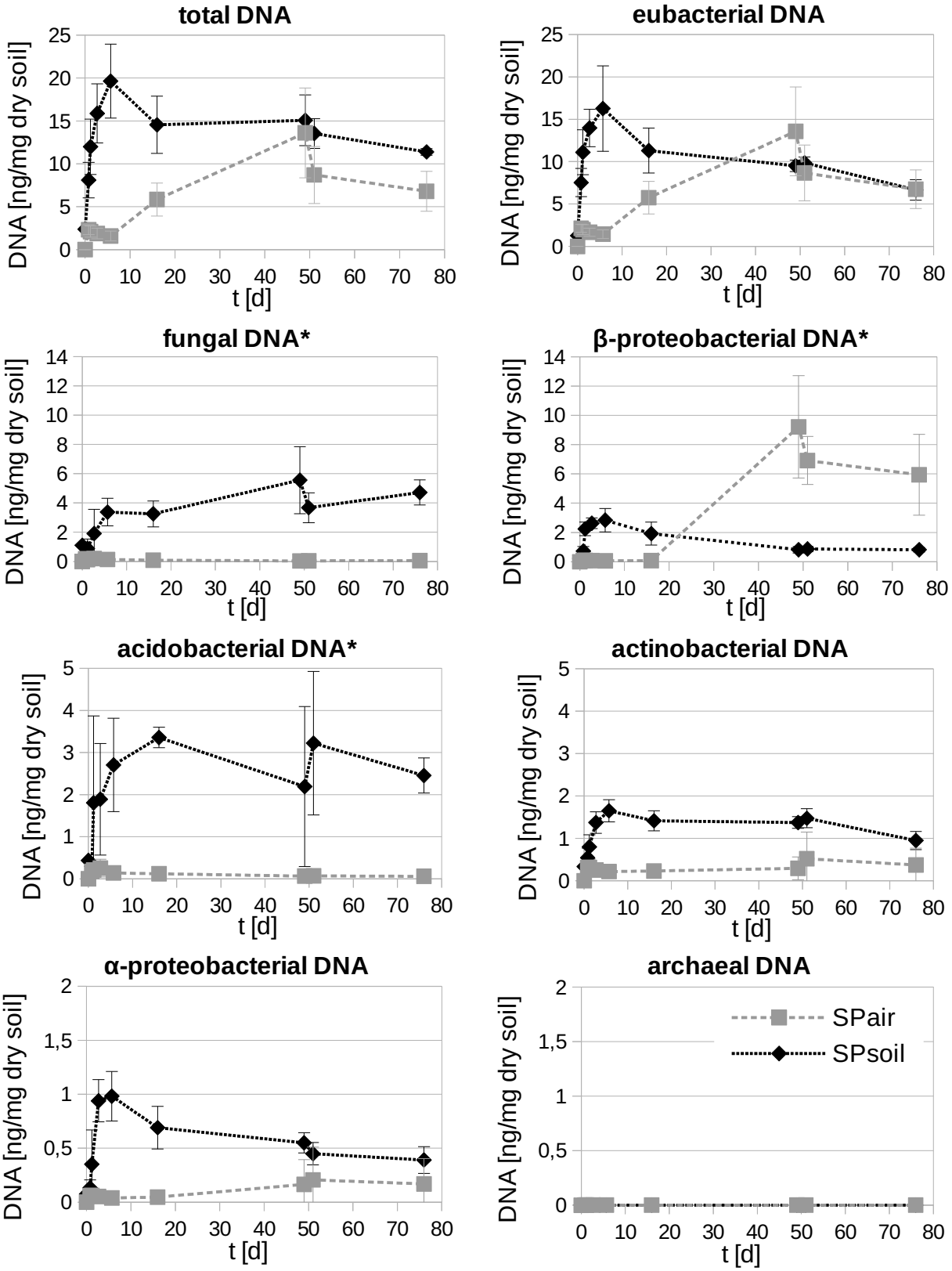

Fig. 13: DNA concentrations of phylogenetic classes and domains in soil with natural inoculate (SP_{soil}) and air-born infection (SP_{air}) (values in ng DNA per mg dry soil; * marks samples with $p<0.05$; n=3)

Table 8: Measured eubacterial class DNA of SP_{air} and SP_{soil} in relation (%) to the total eubacterial DNA at days 49, 51 and 76. Within the final period (day 51 to 76) the total eubacterial population is assumed to be similar between both variants. P-values are given for comparison of shares within the final period. (n=3)

Eubacterial class	**SP_{air}**		**SP_{soil}**		
at day	**51**	**76**	**51**	**76**	**p-value**
Acidobacteria	0.79	0.86	32.69	36.77	0.002
Actinobacteria	5.97	5.51	14.94	14.23	0.057
α-Proteobacteria	2.37	2.51	4.55	5.85	0.270
β-Proteobacteria	79.75	88.10	8.83	12.27	0.023
sum	*88.88*	*96.57*	*60.88*	*69.12*	

6.4.2 POC release

The relative light fraction POC release C_{rel} is defined as the ratio of the POC release at the respective energy level (C_{frac}) to the cumulative POC release of all collected light fractions plus the sediment (C_{tot}), expressed by $C_{re}=C_{frac} \cdot C_{tot}^{-1}$.

SP_{soil} and SP_{air} do not differ in their relative fLF release, which is around 4.6% of the C_{tot}. In contrast, the fLF release of $SP_{control}$ amounts to 44.7% (Fig. 14). SP_{soil} releases 2.4% of the C_{tot} within the oLF_{50}, whereas $SP_{control}$ releases 10.3% (p=0.051). SP_{air} lies in between releasing 6.3% without a significant difference to both. At 500 J ml^{-1}, all variants release similar percentages of C_{tot}. The POC release of SP_{soil} and SP_{air} is similar to the amount released at 50 J ml^{-1}, whereas $SP_{control}$ is reduced to 1.3%. SP_{air} shows a tendency to exceed SP_{soil} and $SP_{control}$.

The carbon content of each sediment corresponds to the sum of the respective light fraction POC release and amounts to 92.3% (29.9 mg g^{-1}) in SP_{soil}, 83.9% (26.5 mg g^{-1}) in SP_{air} and 43.8% (15.8 mg g^{-1}) in $SP_{control}$. Thus, only $SP_{control}$ shows a significantly reduced carbon content remaining in the soil matrix. In consequence, the C-release from SP_{soil} and SP_{air} does not differ significantly in any fraction (although SP_{air} shows a tendency to release more POC than SP_{soil} in both occluded light fractions). In contrast, $SP_{control}$ loses nearly half of its C_{tot} in the fLF and additional 10% after application of 50 J ml^{-1}.

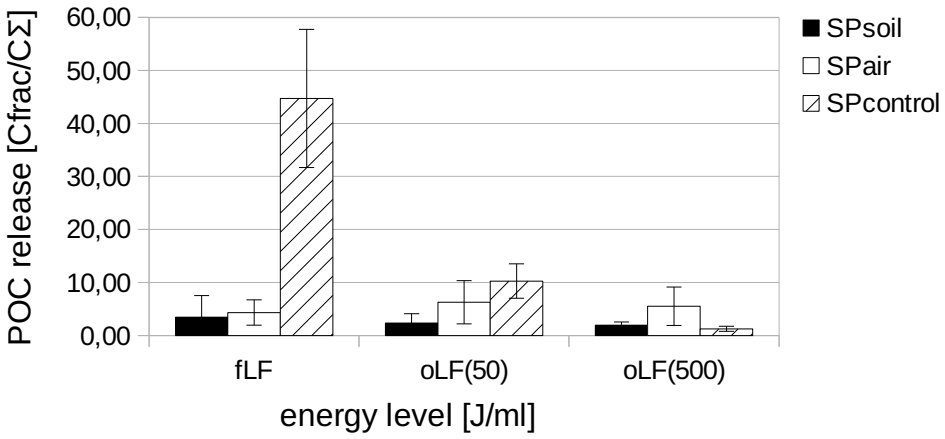

Fig. 14: Relative POC release of the variants SP_{soil}, SP_{air}, $SP_{control}$ at different energy levels (0, 50, 500 J ml^{-1}). The highest carbon release is associated with the lowest occlusive strength of POM at the respective energy level. (n=3)

6.5 Discussion

The total eubacterial DNA in SP_{soil} und SP_{air} converge between day 6 and day 49 and match the condition for similarity between day 51 and day 76 (the final period). Also the observed eubacterial classes in both variants seem to be established until day 51 and show a stable or slightly decreasing population development within the final period (Fig. 8). This development lead to a cumulative percentage of Acidobacteria, Actinobacteria, α-Proteobacteria and β-Proteobacteria on total eubacterial DNA, that increases from 88.9% to 96.6% in SP_{air} and from 60.9% to 69.1% in SP_{soil}. It can be seen that this bundle of eubacterial classes holds the majority in both variants and becomes increasingly dominant. For these three reasons, the effect of named eubacterial as well as fungal and archaeal populations on POM occlusion is discussed based on the final period.

Although there is a similar total eubacterial DNA amount, the population structure is strongly varying between the variants: Acidobacteria and β-Proteobacteria show a significant and Actinobacteria an in tendency but not significant difference between variants, whereas α-Proteobacteria, which have low abundances (<6%) in both variants, did not develop differently. Beside Eubacteria, a fungal population developed in SP_{soil},

whose DNA spans 27.2% to 41.4% of the total measured population (DNA_{tot}), whereas only very small amounts of fungal DNA were found in SP_{air} samples. Hence, ecosystems of both variants were dominated by strongly different microbial classes: During the final period Acidobacteria, Actinobacteria and Fungi together hold 61.9% to 71.3% of the total measured DNA in SP_{soil}. In contrast, SP_{air} is strongly dominated by β-Proteobacteria, which provide 79.4% to 87.3% of the total measured DNA. We conclude, that both variants differ in their community structure within the final period. Following our hypothesis, this implies a different POM occlusion in SP_{soil} and SP_{air}.

A strong occlusion of POM during incubation becomes apparent comparing the incubated variants with $SP_{control}$: The carbon content in the fLFs of SP_{soil} and SP_{air} decreased, while increased in the sediment. However, contrary to our hypothesis SP_{soil} and SP_{air} do not show a significant ($p<0.05$) difference of POM occlusion in any fraction, although SP_{soil} has a tendency to release less POC. Even considering a relation of microbial development and POM occlusion in single parallels, no correlation of the growth of a specific taxon and POM occlusion was observed (data not shown). The occlusion in both variants is extensive: Total occluded POC amounts to ~30 mg g^{-1} dry soil in both variants and therefore exceed occlusion in comparable soils by four-fold (Büks and Kaupenjohann, 2016). Our POM mainly consists of pyrochar particles <20 µm. Since Kaiser and Berhe (2014) reviewed, that microaggregates <63 µm are stable in face of ultrasonication levels >500 J ml^{-1}, an occlusion within very stable microaggregates of the sediment is expected. The main biological agent for this occlusion is most likely bacterial EPS (Six et al., 2004). Thus, in the present study POM occlusion exceeds that of a native soil, but is most probably not affected by the community composition.

However, triplicates usually do not provide sufficient test power to avoid type 1 and 2 errors. Therefore the convention of $p<0.05$ only gives a weak statement. If instead discussing the in tendency increased POM occlusion in SP_{soil} as a fact, fungal glomalin and archaeal EPS can be refused as relevant mechanisms: As AMFs are obligatory symbionts of plant roots (Bago and Bécard, 2002), remains of glomalin might exist in the soil sample as a remain from the field, but neither are expected to differ between the variants nor could be enriched by fungal growth. Also archaeal EPS (Fröls, 2013) could be excluded, since Archaea hardly exist in both variants. Low-molecular weight exudates and biomineralization could play a role in physico-chemical POM occlusion, but chemical

diversity and unknown effect levels do not allow an estimation of their influence in the present study.

Fungi are highly abundant in SP_{soil}. Therefore, wrapping of macroaggregates by fungal hyphae is expected to enhance POM occlusion. In contrast, Actinobacteria, which are assumed to have the capability to form microaggregates, show only slight differences between the variants and are therefore not supposed to contribute to the occlusion of POM. (Aspiras et al., 1971; Gasperi-Mago and Troeh, 1979; Tisdall, 1991; Bossuyt et al., 2001)

As the broad molecular diversity of EPS (Leigh and Coplin, 1992; Votselko et al., 1993; Allison, 1998; Al-Halbouni et al., 2009; Flemming and Wingender, 2010; Ras et al., 2011) develops in dependency of species and environmental factors and affects viscosity, it seems self-evident that two different complex multi-species biofilms should show different binding strength of POM within soil aggregates. However, even assuming no influence of other microbial binding mechanisms, the bacterial community composition seems to be less relevant in the present study. What are the explanations?

First, relicts of the original EPS could endured drying, mechanical dispersion, γ-sterilization and recolonization along the whole soil treatment and form a background load, which overlays the effect of the newly built EPS on POM occlusion. This explanation for similar POM occlusion of SP_{soil} and SP_{air} seems improbable due to γ-degradation and metabolization (Kitamikado et al., 1990; Wasikiewicz et al., 2005), but cannot finally be ruled out in this work. More likely, the different microbial development in both incubated variants (1) causes only a little difference in EPS molecular composition, that is not sufficient to affect POM occlusion in large extent, or (2) the span of possible molecular EPS compositions has in general no significant influence on the mechanical characteristics of EPS. Furthermore, (3) despite a broad acceptance of EPS as agents of soil aggregation, its influence could be of minor importance under certain conditions (e.g. in sandy soils). (4) Probably, but also not tested, similar POM occlusions in both variants can be caused by a multi-species balancing mechanisms, in which a loss of coherence due to the dominance of one group of taxa is compensated by another group.

Our results only give a first insight to the relation of microbial community composition and POM occlusion. A more quantitative analysis would require more replicate samples, manifold microbial communities and probably soils from different land use. This was

beyond the scope of the present study. Our findings show that soil-microbial ecosystems with vastly different community structures can develop a nearly similar grade of POM occlusion. This implies that soil ecosystems could be able to compensate the influence of population shifts on POM occlusion.

6.6 Conclusions

Our incubation experiment demonstrated the possibility to breed stable soil aggregates in the laboratory within 3 month. However, our hypothesis was not supported by the data. After 76 days of incubation, two variants of the same sandy agricultural soil (Su3) established a similar total eubacterial abundance, but different community structures – one strongly dominated by β-Proteobacteria, the other one by Acidobacteria, Actinobacteria and Fungi. Structural differences between these microbial communities did not cause significant differences in the occlusion of POM. This leads to the tentative assumption that POM occlusion in agricultural soils could be resilient in face of changing microbial communities. Nonetheless, a population shift can affect e.g. soil metabolic characteristics. Therefore, the state of the soil microbial community should remain in focus of agricultural practice.

6.7 Acknowledgements

This project was financially supported by the Leibnitz-Gemeinschaft (SAW Pact for Research, SAW-2012-ATB-3). We are also grateful to our students Kathrein Fischer, Christine Hellerström, Annabelle Kallähne, Paula Nitsch, Susann-Elisabeth Schütze, Anne Timm and Karolin Woitke for pre-trials and soil preparation.

7 Synthesis and conclusions

7.1 Single experiments

The present experiments show only little or even no significant effects of microbial binding factors on the POM occlusion in a sandy agricultural soil.

Enzymatic treatment. The treatment of aggregate samples with a highly concentrated enzymatic solution shows no significantly ($p<0.05$) enhanced POM release compared to the control. But, there is a tendency for the reduction of POM occlusive strength. This POM detachment is attributed to the enzymatic digestion of bacterial EPS. However, the concomitant low additional release of DNA from intact bacterial cells confirms an incomplete detachment and/or that the majority of cells within aggregates is not fixed by EPS, but e.g. by occlusion. Following Ranjard and Richaume (2001), a majority of soil bacteria is located in pores <9 µm in microaggregates <100 µm. Also Monrozier et al. (1991) found 39 to 60% of the microbial biomass associated with microaggregates <30 µm. This could explain the lack of POM release by the inaccessibility of bacterial EPS for diffusing enzyme molecules: After pre-incubation, smaller mesopores are already filled with soil solution, so that – in contrast to macropores and larger mesopores – enzymes do not reach via convection, but only diffusively. In consequence, enzyme migration into small mesopores is hindered, whereas larger pores are easily accessible for enzymes and susceptible for instant enzymatic digestion of EPS components. By comparison, enzymatic plus mechanical treatment led to accessibility of inner aggregate EPS and a significantly higher cell release compared to pure mechanical and my enzymatic detachment (Böckelmann et al., 2003).

In conclusion, the enzymatic treatment of extracellular polymers within the larger meso- and macropore space has little effect on POM occlusion. This indicates little influence of EPS on POM occlusive strength within the aggregate-hierarchical level of macroaggregates and matches current assumtions (Chenu and Stotzky, 2002). The slight additional POM release after application of the highest enzyme concentration mainly originates from strongly bound POM (>150 J ml^{-1}) of the sediment fraction and points to the destabilization of stable macroaggregates (Kaiser and Berhe, 2014). However, the

effect on small mesopores could not be determinded due to little diffusive accessibility and additionally depends on the amount of POM occluded in these aggregate regions.

Nematode grazing. The missing influence of a stable, fertile and grazing population of the bacterial-feeding Nematode *Acrobeloides buetschlii* on the composition and abundance of the bacterial community as well as on POM release conflicts with the initial hypothesis, that grazing on bacterial biofilms and EPS would reduce the strength of POM bonds with mineral particles. Like in the former experiment, this lack of effect might be caused by the inaccessibility of the finer mesopore space. Restricted by its body diameter, *A. buetschlii* is not able to enter small mesopores. In consequence, grazing is restricted to larger pores, where the influence of bacterial biofilms and EPS on POM occlusive strength is assumed to be insignificant (Chenu and Stotzky, 2002). As all samples showed a natural bacterial population density derived from the PLFA concentrations, the results underpin the idea of small mesopores as relevant retreat habitat for soil bacteria. However, the results give no information if micropore-protected bacterial colonies are a relevant factor of aggregate formation and POM occlusion within microaggregates. In addition, this experiment gives no information about long-term influences of bacterial-feeding on POM occlusion, e.g. by nematodal excretion and microbial population shifts.

Microbial communities. After incubation with two different microbial populations the inoculated variants showed a significant increase of occluded biochar particles (POM analogue), which is, however, not affected by the composition of the microbial community. Tendencies of decreased POM release in the soil-born variant can be explained with the high abundance of fungi, which are assumed to be an important biological factor of macroaggregate formation. This, on the other hand, points to a negligible influence of the bacterial community composition on POM occlusion in any pore size class, especially within microaggregates, that are inaccessible for fungal hyphae (Chenu and Stotzky, 2002).

7.2 Statistical restrictions

Due to the little number of replicates within each variant, the analysis of these data suffer from restrictions regarding the perceptibility of significance of the observed results: Originally p-values were intended to support the estimation of statistical significance by ranking given results on a scale of probability for the rejection of an actually true null hypothesis (H_0) (Fisher, 1925). Later p-values were conventionalized by means of accepting $p<0.05$ and $p<0.01$ as sharp threshold values to distinguish between insignificance, significance and strong significance, respectively (Biau et al., 2010). Beside the obvious disutility of accepting data with – for instance – $p=0.049$ as statistically insignificant and such of $p=0.051$ as significant, solely use of p-values hold insufficiencies not only in the analysis of variants with little parallels. Nonsignificance never means a lack of difference between variants (Goodman, 2008): For example, high variance between parallels can result in $p>0.05$, although compared means are largely different and the effect size is large (Zhu, 2016). Higher validity of statistical analyses can be reached by use of further statistical markers. Whereas, on one hand, the p-value indicates the type-I error, the probability of erroneously accepting the null hypothesis (also known as type-II error) is given by the test power (1-β) (Biau et al., 2010). The test power increases with decreasing variance within each variant as well as increasing $|\mu_A-\mu_B|$ (the difference between the means of the compared variants) and number of samples. Furthermore, even in cases of few parallels, the effect size give information about the importance of the difference between the compared variants in face of their amount and and give substantial information about the relevance of found data even in case of $p>0.05$ (Cohen, 1988; Zhu, 2016).

In the present thesis, the released POM fractions of the enzymatic treatment and microbial community experiments show a broad variance within samples of each variant. Differences between the variants of these experiments are not significant ($p>0.05$), but show a high effect size comparing E0 and E4 ($d=0.98$) as well as SP_{soil} and SP_{air} ($d=1.25$) at 50 J ml^{-1} applying Cohens d-test for single comparison (Cohen, 1988). The test power for one way analysis of variance (ANOVA) was not calculated, but is most probably low due to few parallels, high variance and small differences between the means of the variants. In conclusion, the present results show tendencies with increased probability of

type-I errors. Hence, on the statistical level this work represents the standard problem of environmental research: little money, little time, little parallels, little explanatory power by applied statistics.

7.3 General conclusion

In soils there is no fundamental hindrance for bacteria or fungi to colonize any surface, which is not in a pore smaller than the cell's size or fully covert by other particles. Therefore, biofilms, EPS residues and further microbial binding factors are expected to appear both in smaller mesopores and on particle surfaces within larger pores and could therefore potentially be effective in both micro- and macroaggregates.

The distribution of bacterial biomass between these smaller and larger pore size ranges most probably depends on factors such as soil texture, chemical and biological composition, that affect the appearance of niches, predators and nutrient supply. Sandy soils contain 30±10% of pores >10 µm (Blume et al., 2015), which allow free motion of nematodes within the soil pore volume, whereas finer texture hinders nematode motion and less likely provide a network of accessible pores for unhindered grazing (Wallace, 1968). The microbial biomass of a sandy soil is therefore prone to grazing and assumed to be concentrated in small protective mesopores.

Soil respiration, PLFA concentrations and DNA amounts point to a microbial population density, which is in the normal range of sandy soils. As biofilms and EPS residues of larger pores are accessible and susceptible to enzymatic and trophic treatments, an influence on POM occlusive strength should be measurable, if these substances have influence on POM occlusion within the named pore size range. By reason that this is not the case, my results can be interpreted in two ways:

(1) The enzymatic detachment, grazing and population changes in the upper mesopore scale and beyond are of minor relevance for the strength of POM occlusion. The part of the bacterial population, which is important for soil aggregation and POM occlusion, might live in smaller mesopores <10 µm of microaggregates, where microbial binding factors

play a relevant role for aggregation, as assumed by different authors (Tisdall, 1994; Six et al., 2004; Bronick and Lal, 2005).

(2) Within the probed sandy soil biofilms, EPS, bacterial exudates and residues have no influence at all neither in smaller mesopores nor in the pore space above. This interpretation conflicts with the established model but matches data of Foster (1988), who did not find any biofilms within sandy soils.

However, the influence of microbial binding factors in smaller mesopores remained non-proven in the present work due to methodological reasons and have to be part of future research.

The third experiment furthermore shows the minor influence of microbial aggregation factors resulting from different populations within the whole pore size range. This most likely results from little differences in the rheological properties of EPSs with different composition as well as other binding factors and do not affect POM occlusive strength.

Different soils – varying in content, composition, distribution and physico-chemical properties of their OM and mineral phase – most likely do not release the same amount or share of OM when treated with the same mechanical stress. Cerli et al. (2012) addressed this problem in their comprehensive work about separation of operational OM fractions. However, in the present study aggregate samples do not differ in their chemical composition, while the applied treatments are used to selectively alter the influence of the microbial community on physico-chemical binding properties. For that reason, the POM occlusive strength is interpretable as proportional to aggregate stability when restricted to soils with the same structural abilities. In consequence, enzymatic and trophic treatment of biofilms/EPS as well as the two different microbial populations are assumed to have no influence on aggregate stability.

The results of the present thesis underpin the future focus on microaggregates. Precise differentiation of specific *intra*-microaggregate POM from other occluded POM and mineral-associated OM is a challenging task, some authors estimated this fraction to comprise 10 to 30% of the total POM in different soils (Besnard et al., 1996; O'Brien and Jastrow, 2013) and up to 90% of the occluded POM in aggregates (Six et al., 2000). Given this high proportion, to understand the contribution of microbial communities to the intra-microaggregate occlusion of POM with all its ecological functions is an important task for the research and practice on soil fertility in agriculture.

7.4 Transferability to other soils

Silty sand (Su3) from the topsoil of the agricultural test site was used in all three experiments. The samples showed a high sand and a low clay content (~72% and ~4%, respectively), a low organic carbon content (~0.9%, with exception of the microbial community experiment) and a low microbial carbon content of about 176 mg C kg^{-1} soil, which is on the bottom of C_{mic} values for agricultural soils (Blume et al., 2015). Compared to soils with larger silt, clay and OM content, the soil samples contain less than half of the microbial population density (Anderson and Domsch, 1989; Anderson and Domsch, 2010), hence with lower capability of EPS production. Microbial biomass correlates to the organic matter and the clay content of soils (Schnürer et al., 1985; Anderson and Domsch, 1989) and benefits from the amount of protective niches (Chenu et al., 2001), which is increased in soils with finer texture. Therefore it could be argued, that microbial binding factors have minor effect on aggregate stability and POM occlusion in soils, which are poor in clay and OM. Assuming higher occlusive strength of POM in soil types with higher content of those aggregation agents, future investigation on agricultural topsoils such as from Cambisols, Luvisols or Chernozems might be proper to get more general insight into the function of microbial communities in POM occlusion (Zech et al., 2014).

7.5 Future research

Aggregated soils with increased silt and clay content show a shift of the pore size distribution towards smaller pores (Blume et al., 2015) leading to enhance the effect of diffusion hindrance of macromolecules. In consequence, both the evaluation of the results from the enzymatic experiment on the basis of statistically significant re-measurements and the analyses of further soil types for micro- and macroaggregate occlusion of POM require a preceding test procedure to optimize the application time of enzymes. The object is to reach a preferably complete diffusion of enzymes into the whole pore space >0.3 µm without significant reduction of POM mass by enzymatic digestion of organic surfaces.

Just like the diffusion behavior of macromolecules, the development of the microbial community strongly depends on the soil type (Bossio et al., 1998; Buyer et al., 2002). However, there is a lack of comprehensive studies both on the composition and distribution of bacterial, archaeal and fungal phyla within aggregates of different soils. Ecotyping on microbial phyla, their metabolic and physical properties (such as EPS excretion, production of hydrophobic substances, entangling abilities and biomineralization), their localization within the aggregate structure (inside microaggregates, between microaggregates, on macroaggregate surfaces and outside of aggregates) and knowledge about specific microbial community compositions at different surfaces (POM, mineral surfaces, pyrochar, microplastic) could give insights in the influence of microorganisms on the binding of soil particles, the aggregation processes and the occlusion of POM.

In addition, grazing organisms might influence the potential of microbial phyla in accessible pore space by depleting target populations and hold them in equilibrated permanent growth. Results of the present work showed, that even small Nematodes like *A. buetschlii* might be unable to influence bacterial populations within pores of 0.3 to 12.5 µm in diameter. Following Griffiths et al. (1999), smaller protozoal grazers are able to reach bacterial populations within microaggregates and could be used for further investigation on the relation of biofilm/EPS grazing and aggregation of soil particles. Furthermore, selective grazing of different protozoal taxa might give information about special roles of bacterial, fungal and archaeal prays on the stabilization of aggregates.

At the instrumental level, the water content has large influence on aggregate stability and in consequence on POM occlusive strength. Extensive analyses of various samples often require storage of air-dried soil samples. To avoid slaking and further mechanical damage by fast re-wetting, a device is needed to slowly increase the water content of soil aggregates to a constant value (e.g. via filter plates) and directly perform ultrasonication/density fractionation without destructive transfer to other tubes. This gentle treatment is assumed to reduce variability of POM releases caused by undesired mechanical disruption.

Future knowledge about the role of microorganisms in soil aggregate stabilization will contribute to predictions about related soil fertility factors as a consequence of e.g. land-use changes, application of biocides, climatic or seasonal changes. Future agriculturalists

might be enabled to optimize their food production allowing for microbial communities and their effects on the soil-plant system.

References

Abröll, C., Kurth, T., Langer, T., Munk, K.and Nethe-Jaenchen, R.: Biochemie-Zellbiologie, Georg Thieme Verlag, 2008.

Absolom, D. R., Lamberti, F. V., Policova, Z., Zingg, W., van Oss, C. J. and Neumann, A.: Surface thermodynamics of bacterial adhesion, Appl. Environ. Microbiol., 46, 90--97, 1983.

Achtenhagen, J., Goebel, M.-O., Miltner, A., Woche, S. K. and Kästner, M.: Bacterial impact on the wetting properties of soil minerals, Biogeochemistry, 122, 269--280, 2015.

Ackermann, M., Prill, P. and Ruess, L.: Disentangling nematode-bacteria interactions using a modular soil model system and biochemical markers, Nematology, 18, 403--415, 2016.

Acosta-Martinez, V. and Tabatabai, M.: Enzyme activities in a limed agricultural soil, Biology and Fertility of soils, 31, 85--91, 2000.

Agnelli, A., Ascher, J., Corti, G., Ceccherini, M. T., Nannipieri, P. and Pietramellara, G.: Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA, Soil Biology and Biochemistry, 36, 859--868, 2004.

Alaoui, A., Lipiec, J. and Gerke, H.: A review of the changes in the soil pore system due to soil deformation: A hydrodynamic perspective, Soil and Tillage Research, 115-116, 1--15, 2011.

Al-Halbouni, D., Dott, W. and Hollender, J.: Occurrence and composition of extracellular lipids and polysaccharides in a full-scale membrane bioreactor, water research, 43, 97--106, 2009.

Allison, D. G.: Exopolysaccharide production in bacterial biofilms, Biofilm Journal, 3, 1998.

Anderson, T.-H. and Domsch, K. H.: Ratios of microbial biomass carbon to total organic carbon in arable soils, Soil biology and biochemistry, 21, 471--479, 1989.

Anderson, T.-H. and Domsch, K. H.: Soil microbial biomass: the eco-physiological approach, Soil Biology and Biochemistry, 42, 2039--2043, 2010.

Ashman, M., Hallett, P., Brookes, P. and Allen, J.: Evaluating soil stabilisation by biological processes using step-wise aggregate fractionation, Soil and Tillage Research, 102, 209--215, 2009.

Aspiras, R., Allen, O., Harris, R. and Chesters, G.: Aggregate stabilization by filamentous microorganisms., Soil Science, 112, 282--284, 1971.

Atlas, R. M.: Handbook of microbiological media, CRC press, 2010.

Ayala-Hernández, I., Hassan, A., Goff, H., de Orduña, R. M. and Corredig, M.: Production, isolation and characterization of exopolysaccharides produced by Lactococcus lactis subsp. cremoris JFR1 and their interaction with milk proteins: Effect of pH and media composition, International dairy journal, 18, 1109--1118, 2008.

Bago, B. & Bécard, G. 2002. Bases of the obligate biotrophy of arbuscular mycorrhizal fungi. *In: Mycorrhizal Technology in Agriculture,* 33--48, Springer

Baisden, W., Amundson, R., Cook, A. and Brenner, D.: Turnover and storage of C and N in five density fractions from California annual grassland surface soils, Global Biogeochemical Cycles, 16, 2002.

Baldock, J.: Interactions of organic materials and microorganisms with minerals in the stabilization of soil structure, in: Interactions between Soil Particles and Microorganisms - Impact on the Terrestrial Ecosystem, 84--129, John Wiley&Sons, Ltd: Chichester, West Sussex, UK, 2002.

Ball, B. and Robertson, E.: Effects of uniaxial compaction on aeration and structure of ploughed or direct drilled soils, Soil and Tillage research, 31, 135--148, 1994.

Barthes, B. and Roose, E.: Aggregate stability as an indicator of soil susceptibility to runoff and erosion; validation at several levels, Catena, 47, 133--149, 2002.

Basile-Doelsch, I., Amundson, R., Stone, W., Borschneck, D., Bottero, J.-Y., Moustier, S., Masin, F. and Colin, F.: Mineral control of carbon pools in a volcanic soil horizon, Geoderma, 137, 477--489, 2007.

Battin, T. J., Sloan, W. T., Kjelleberg, S., Daims, H., Head, I. M., Curtis, T. P. and Eberl, L.: Microbial landscapes: new paths to biofilm research, Nat. Rev. Microbiol., 5, 76--81, 2007.

Baumgartl, T. and Horn, R.: Effect of aggregate stability on soil compaction, Soil and Tillage Research, 19, 203--213, 1991.

Baver, L. and Rhoades, H.: Aggregate analysis as an aid in the study of soil structure relationships, Journal of the American Society of Agronomy, 24, 920-930, 1932.

Beare, M., Hu, S., Coleman, D. and Hendrix, P.: Influences of mycelial fungi on soil aggregation and organic matter storage in conventional and no-tillage soils, Applied Soil Ecology, 5, 211--219, 1997.

Beare, M. H. and Bruce, R. R.: A comparison of methods for measuring water-stable aggregates: implications for determining environmental effects on soil structure, Geoderma, 56, 87--104, 1993.

Bedini, S., Pellegrino, E., Avio, L., Pellegrini, S., Bazzoffi, P., Argese, E. and Giovannetti, M.: Changes in soil aggregation and glomalin-related soil protein content as affected by the arbuscular mycorrhizal fungal species Glomus mosseae and Glomus intraradices, Soil Biology and Biochemistry, 41, 1491--1496, 2009.

Béjar, V., Llamas, I., Calvo, C. and Quesada, E.: Characterization of exopolysaccharides produced by 19 halophilic strains of the species Halomonas eurihalina, Journal of biotechnology, 61, 135--141, 1998.

Bengough, A. and Mullins, C.: Mechanical impedance to root growth: a review of experimental techniques and root growth responses, Journal of soil science, 41, 341--358, 1990.

Bennie, A. and Burger, R. d. T.: Penetration resistance of fine sandy apedal soils as affected by relative bulk density, water content and texture, South African Journal of Plant and Soil, 5, 5--10, 1988.

Besnard, E., Chenu, C., Balesdent, J., Puget, P. and Arrouays, D.: Fate of particulate organic matter in soil aggregates during cultivation, European Journal of Soil Science, 47, 495--503, 1996.

Biau, D. J., Jolles, B. M. and Porcher, R.: P value and the theory of hypothesis testing: an explanation for new researchers, Clinical Orthopaedics and Related Researchtextregistered, 468, 885--892, 2010.

Bissonnais, Y. L.: Aggregate stability and assessment of soil crustability and erodibility: I. Theory and methodology, European Journal of soil science, 47, 425--437, 1996.

Blume, H., Brümmer, G., Fleige, H., Horn, R., Kandeler, E., Kögel-Knabner, I., Kretzschmar, R., Stahr, K.and Wilke, B.: Scheffer/Schachtschabel Soil Science, Springer Berlin Heidelberg, 2015.

Böckelmann, U., Szewzyk, U. and Grohmann, E.: A new enzymatic method for the detachment of particle associated soil bacteria, J. Microbiol. Methods, 55, 201--211, 2003.

Bongers, T.: De Nematoden van Nederland Koninklejke Nedelandse Natuuhistorische Vereniging, Stichting Uitgeverij van de Koninklijke Natuurhistorische Vereniging Utrecht, 1994.

Bonkowski, M.: Protozoa and plant growth: the microbial loop in soil revisited, New Phytologist, 162, 617--631, 2004.

Borowik, A. and Wyszkowska, J.: Impact of temperature on the biological properties of soil, International Agrophysics, 30, 1--8, 2016.

Bossio, D. A., Scow, K. M., Gunapala, N. and Graham, K.: Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles, Microbial ecology, 36, 1--12, 1998.

Bossuyt, H., Denef, K., Six, J., Frey, S., Merckx, R. and Paustian, K.: Influence of microbial populations and residue quality on aggregate stability, Applied Soil Ecology, 16, 195--208, 2001.

Bratbak, G. and Dundas, I.: Bacterial dry matter content and biomass estimations, Appl. Environ. Microbiol., 48, 755--757, 1984.

Braunack, M., Hewitt, J. and Dexter, A.: Brittle fracture of soil aggregates and the compaction of aggregate beds, Journal of Soil Science, 30, 653--667, 1979.

Brauns, A.: Praktische Bodenbiologie, G. Fischer Verlag: Stuttgart, Germany, 1--470, 1968.

Brodowski, S., John, B., Flessa, H. and Amelung, W.: Aggregate-occluded black carbon in soil, European Journal of Soil Science, 57, 539--546, 2006.

Brodowski, S., John, B., Flessa, H. and Amelung, W.: Aggregate-occluded black carbon in soil, European Journal of Soil Science, 57, 539--546, 2006.

Bronick, C. J. and Lal, R.: Soil structure and management: a review, Geoderma, 124, 3--22, 2005.

Brown, G. G., Barois, I. and Lavelle, P.: Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains, European Journal of Soil Biology, 36, 177--198, 2000.

Büks, F. and Kaupenjohann, M.: Enzymatic biofilm digestion in soil aggregates facilitates the release of particulate organic matter by sonication, SOIL Journal, 2, 499-509, 2016.

Burns, R. G., DeForest, J. L., Marxsen, J., Sinsabaugh, R. L., Stromberger, M. E., Wallenstein, M. D., Weintraub, M. N. and Zoppini, A.: Soil enzymes in a changing environment: current knowledge and future directions, Soil Biology and Biochemistry, 58, 216--234, 2013.

Buyer, J. S., Roberts, D. P. and Russek-Cohen, E.: Soil and plant effects on microbial community structure, Canadian Journal of Microbiology, 48, 955--964, 2002.

Celik, G. Y., Aslim, B. and Beyatli, Y.: Characterization and production of the exopolysaccharide (EPS) from Pseudomonas aeruginosa G1 and Pseudomonas putida G12 strains, Carbohydrate polymers, 73, 178--182, 2008.

Cerli, C., Celi, L., Kalbitz, K., Guggenberger, G. and Kaiser, K.: Separation of light and heavy organic matter fractions in soil—Testing for proper density cut-off and dispersion level, Geoderma, 170, 403--416, 2012.

Chang, W.-S., van de Mortel, M., Nielsen, L., de Guzman, G. N., Li, X. and Halverson, L. J.: Alginate production by Pseudomonas putida creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions, Journal of bacteriology, 189, 8290--8299, 2007.

Chaudhari, P. R., Ahire, D. V., Ahire, V. D., Chkravarty, M. and Maity, S.: Soil bulk density as related to soil texture, organic matter content and available total nutrients of Coimbatore soil, International Journal of Scientific and Research Publications, 3, 1--8, 2013.

Chenu, C. 1995. Extracellular polysaccharides: an interface between microorganisms and soil constituents. *In: Environmental Impact of Soil Component Interactions - Natural and Anthropogenic Organics,* 217--233, CRC Press, London

Chenu, C. and Cosentino, D.: Microbial regulation of soil structural dynamics, The architecture and biology of soils: life in inner space, 37--70, 2011.

Chenu, C., Hassink, J. and Bloem, J.: Short-term changes in the spatial distribution of microorganisms in soil aggregates as affected by glucose addition, Biology and Fertility of Soils, 34, 349--356, 2001.

Chenu, C. and Roberson, E.: Diffusion of glucose in microbial extracellular polysaccharide as affected by water potential, Soil Biology and Biochemistry, 28, 877--884, 1996.

Chenu, C. and Stotzky, G.: Interactions between microorganisms and soil particles: an overview, in: Interactions between Soil Particles and Microorganisms: Impact on the Terrestrial Ecosystem, 1--40, IUPAC Series on Analytical and Physical Chemistry of Environmental Systems, 2002.

Chepil, W. and Bisal, F.: A Rotary Sieve Method for Determining the Size Distribution of Soil Clods., Soil Science, 56, 95--100, 1943.

Christensen, O.: An Index of Friability of Soils., Soil Science, 29, 119--136, 1930.

Christensen, R. 1996. One-way ANOVA. *In: Plane Answers to Complex Questions,* 79--93, Springer

Cleveland, C. C. and Liptzin, D.: C: N: P stoichiometry in soil: is there a “Redfield ratio” for the microbial biomass?, Biogeochemistry, 85, 235--252, 2007.

Cohen, J.: Statistical power analysis for the behavioral sciences Lawrence Earlbaum Associates, Hillsdale, NJ, 20--26, 1988.

Cooper, A. and Morgan, H.: Improved fluorometric method to assay for soil lipase activity, Soil biology and biochemistry, 13, 307--311, 1981.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. and Lappin-Scott, H. M.: Microbial biofilms, Annual Reviews in Microbiology, 49, 711--745, 1995.

Crow, S. E., Swanston, C. W., Lajtha, K., Brooks, J. R. and Keirstead, H.: Density fractionation of forest soils: methodological questions and interpretation of incubation results and turnover time in an ecosystem context, Biogeochemistry, 85, 69--90, 2007.

Crum, L. A.: Comments on the evolving field of sonochemistry by a cavitation physicist, Ultrasonics Sonochemistry, 2, S147--S152, 1995.

Czarnes, S., Hallett, P., Bengough, A. and Young, I.: Root-and microbial-derived mucilages affect soil structure and water transport, European Journal of Soil Science, 51, 435--443, 2000.

Das, T., Sehar, S., Koop, L., Wong, Y. K., Ahmed, S., Siddiqui, K. S. and Manefield, M.: Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation, PloS one, 9, 2014.

Das, T., Sehar, S. and Manefield, M.: The roles of extracellular DNA in the structural integrity of extracellular polymeric substance and bacterial biofilm development, Environmental microbiology reports, 5, 778--786, 2013.

Davey, M. E. and O'toole, G. A.: Microbial biofilms: from ecology to molecular genetics, Microbiol. Mol. Biol. Rev., 64, 847--867, 2000.

De Mesel, I., Derycke, S., Moens, T., Van der Gucht, K., Vincx, M. and Swings, J.: Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study, Environmental Microbiology, 6, 733--744, 2004.

DeFlaun, M. F., Paul, J. H. and Jeffrey, W. H.: Distribution and molecular weight of dissolved DNA in subtropical estuarine and oceanic environments, Marine Ecology-Progress Series, 38, 65-73, 1987.

Delmont, T. O., Francioli, D., Jacquesson, S., Laoudi, S., Mathieu, A., Nesme, J., Ceccherini, M. T., Nannipieri, P., Simonet, P. and Vogel, T. M.: Microbial community development and unseen diversity recovery in inoculated sterile soil, Biology and Fertility of Soils, 50, 1069--1076, 2014.

Dexter, A.: Advances in characterization of soil structure, Soil and tillage research, 11, 199--238, 1988.

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'orio, V., Vergara, A., Conter, M. and Ianieri, A.: Influence of temperature on biofilm formation by Listeria monocytogenes on

various food-contact surfaces: relationship with motility and cell surface hydrophobicity, Journal of applied microbiology, 104, 1552--1561, 2008.

DIN/ISO: 11277 Soil quality—determination of particle size distribution in mineral soil material—method by sieving and sedimentation, DIN Deutsches Institut für Normung e.V., Beuth, 2002.

Donlan, R. M.: Biofilms: microbial life on surfaces, Emerg Infect Dis, 8, 2002.

Drążkiewicz, M.: Distribution of microorganisms in soil aggregates: effect of aggregate size, Folia microbiologica, 39, 276--282, 1994.

Driver, J. D., Holben, W. E. and Rillig, M. C.: Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi, Soil Biology and Biochemistry, 37, 101--106, 2005.

Edwards, A. and Bremner, J.: Dispersion of Soil Particles by Sonic Vibration, Journal of Soil Science, 18, 47--63, 1967b.

Edwards, A. P. and Bremner, J.: Microaggregates in Soils, Journal of Soil Science, 18, 64--73, 1967a.

Eivazi, F. and Tabatabai, M.: Glucosidases and galactosidases in soils, Soil Biology and Biochemistry, 20, 601--606, 1988.

von Ende, C. N.: Repeated-measures analysis, Design and analysis of ecological experiments. Oxford University Press, Oxford, 134--157, 2001.

Eusterhues, K., Wagner, F. E., Häusler, W., Hanzlik, M., Knicker, H., Totsche, K. U., Kögel-Knabner, I. and Schwertmann, U.: Characterization of ferrihydrite-soil organic matter coprecipitates by X-ray diffraction and Mossbauer spectroscopy, Environmental science & technology, 42, 7891--7897, 2008.

Farres, P. and Cousen, S.: An improved method of aggregate stability measurement, Earth Surface Processes and Landforms, 10, 321--329, 1985.

Ferris, H.: Contribution of nematodes to the structure and function of the soil food web, J. Nematol, 42, 63--67, 2010.

Ferris, H., Lau, S. and Venette, R.: Population energetics of bacterial-feeding nematodes: respiration and metabolic rates based on CO_2 production, Soil Biology and Biochemistry, 27, 319--330, 1995.

Fierer, N., Jackson, J. A., Vilgalys, R. and Jackson, R. B.: Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays, Appl. Environ. Microbiol., 71, 4117--4120, 2005.

Fierer, N., Schimel, J. and Holden, P.: Influence of drying-rewetting frequency on soil bacterial community structure, Microbial ecology, 45, 63--71, 2003.

Fisher, R.: Statistical Methods For Research Workers, Cosmo Publications, 1925.

Flemming, H.-C. and Wingender, J.: Relevance of microbial extracellular polymeric substances (EPSs)-Part I: Structural and ecological aspects, Water science and technology, 43, 1--8, 2001.

Flemming, H.-C. and Wingender, J.: The biofilm matrix, Nat. Rev. Microbiol., 8, 623--633, 2010.

Fokom, R., Adamou, S., Teugwa, M., Boyogueno, A. B., Nana, W., Ngonkeu, M., Tchameni, N., Nwaga, D., Ndzomo, G. T. and Zollo, P. A.: Glomalin related soil protein, carbon, nitrogen and soil aggregate stability as affected by land use variation in the humid forest zone of south Cameroon, Soil and Tillage Research, 120, 69--75, 2012.

Foster, R.: Microenvironments of soil microorganisms, Biology and fertility of soils, 6, 189--203, 1988.

Francis, P. and Cruse, R.: Soil water matric potential effects on aggregate stability, Soil Science Society of America Journal, 47, 578--581, 1983.

Fraser, C., Alm, E. J., Polz, M. F., Spratt, B. G. and Hanage, W. P.: The bacterial species challenge: making sense of genetic and ecological diversity, Science, 323, 741--746, 2009.

Freckman, D. W.: Bacterivorous nematodes and organic-matter decomposition, Agriculture, Ecosystems & Environment, 24, 195--217, 1988.

Frey, F.: Über die Eignung von Acrobeloides buetschlii (Cephalobidae) für nematologische Laboruntersuchungen, Nematologica, 17, 474--477, 1971.

Friese, C. F. and Allen, M. F.: The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture, Mycologia, 409--418, 1991.

Fröls, S.: Archaeal biofilms: widespread and complex, Biochemical Society Transactions, 41, 393--398, 2013.

Frostegård, Å. and Bååth, E.: The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil, Biology and Fertility of Soils, 22, 59--65, 1996.

Frostegård, Å., Tunlid, A. and Bååth, E.: Microbial biomass measured as total lipid phosphate in soils of different organic content, Journal of Microbiological Methods, 14, 151--163, 1991.

Frostegård, Å., Tunlid, A. and Bååth, E.: Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals, Applied and Environmental Microbiology, 59, 3605--3617, 1993.

Gale, W., Cambardella, C. and Bailey, T.: Root-derived carbon and the formation and stabilization of aggregates, Soil Science Society of America Journal, 64, 201--207, 2000.

Gasperi-Mago, R. R. and Troeh, F. R.: Microbial effects on soil erodibility, Soil Science Society of America Journal, 43, 765--768, 1979.

Geoghegan, M. and Brian, R.: Aggregate formation in soil. 1. Influence of some bacterial polysaccharides on the binding of soil particles, Biochemical Journal, 43, 5, 1948.

Ghanbari, A., Nock, V., Johari, S., Blaikie, R., Chen, X. and Wang, W.: A micropillar-based on-chip system for continuous force measurement of C. elegans, Journal of Micromechanics and Microengineering, 22, 095009, 2012.

Gianfreda, L. and Rao, M. A.: Potential of extra cellular enzymes in remediation of polluted soils: a review, Enzyme and Microbial Technology, 35, 339--354, 2004.

Golchin, A., Oades, J., Skjemstad, J. and Clarke, P.: Study of free and occluded particulate organic matter in soils by solid state 13C CP/MAS NMR spectroscopy and scanning electron microscopy, Soil Research, 32, 285--309, 1994.

Gonzalez-Chavez, M., Carrillo-Gonzalez, R., Wright, S. and Nichols, K.: The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements, Environmental Pollution, 130, 317--323, 2004.

Goodman, S. 2008. A dirty dozen: twelve p-value misconceptions. *In: Seminars in hematology,* 45. 135--140,

Graf-Rosenfellner, M. e. a.: Ringversuchsstudie zu Ultraschalldispergierung, unpublished data.

Gray, J. and Lissmann, H. W.: The locomotion of nematodes, Journal of Experimental Biology, 41, 135--154, 1964.

Greacen, E.: Water content and soil strength, Journal of Soil Science, 11, 313--333, 1960.

Griffiths, B. S., Bonkowski, M., Dobson, G. and Caul, S.: Changes in soil microbial community structure in the presence of microbial-feeding nematodes and protozoa, Pedobiologia, 43, 297--304, 1999.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G. and Bailey, M. J.: Influence of depth and sampling time on bacterial community structure in an upland grassland soil, FEMS Microbiology Ecology, 43, 35--43, 2003.

Gupta, V. and Germida, J.: Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation, Soil Biology and Biochemistry, 20, 777--786, 1988.

Hattori, T.: Adhesion between cells of E. coli and clay particles, The Journal of General and Applied Microbiology, 16, 351--359, 1970.

Hissett, R. & Gray, T. 1976. Microsites and time changes in soil microbe ecology. *In: Symposium of the British Ecological Society,*

Hontoria, C., Velásquez, R., Benito, M., Almorox, J. and Moliner, A.: Bradford-reactive soil proteins and aggregate stability under abandoned versus tilled olive groves in a semi-arid calcisol, Soil Biology and Biochemistry, 41, 1583--1585, 2009.

Imeson, A. and Vis, M.: Assessing soil aggregate stability by water-drop impact and ultrasonic dispersion, Geoderma, 34, 185--200, 1984.

Ince, N., Tezcanli, G., Belen, R. and Apikyan, I. G.: Ultrasound as a catalyzer of aqueous reaction systems: the state of the art and environmental applications, Applied Catalysis B: Environmental, 29, 167--176, 2001.

Ingham, R. E., Trofymow, J., Ingham, E. R. and Coleman, D. C.: Interactions of bacteria, fungi, and their nematode grazers: effects on nutrient cycling and plant growth, Ecological monographs, 55, 119--140, 1985.

Jahn, A., Griebe, T. and Nielsen, P. H.: Composition of Pseudomonas putida biofilms: accumulation of protein in the biofilm matrix, Biofouling, 14, 49--57, 1999.

Jastrow, J.: Soil aggregate formation and the accrual of particulate and mineral-associated organic matter, Soil Biology and Biochemistry, 28, 665--676, 1996.

Jastrow, J. and Miller, R.: Soil aggregate stabilization and carbon sequestration: feedbacks through organomineral associations, in: Soil processes and the carbon cycle, 207--223, CRC Press Boca Raton, FL, 1997.

Joergensen, R. G.: The fumigation-extraction method to estimate soil microbial biomass: calibration of the k EC value, Soil Biology and Biochemistry, 28, 25--31, 1996.

Juarez, G., Lu, K., Sznitman, J. and Arratia, P. E.: Motility of small nematodes in wet granular media, EPL (Europhysics Letters), 92, 44002, 2010.

Kaiser, M. and Berhe, A. A.: How does sonication affect the mineral and organic constituents of soil aggregates?—A review, J. Plant Nutr. Soil Sci., 177, 479--495, 2014.

Kalbitz, K., Schwesig, D., Rethemeyer, J. and Matzner, E.: Stabilization of dissolved organic matter by sorption to the mineral soil, Soil Biology and Biochemistry, 37, 1319--1331, 2005.

Kalbitz, K., Solinger, S., Park, J.-H., Michalzik, B. and Matzner, E.: Controls on the dynamics of dissolved organic matter in soils: a review, Soil science, 165, 277--304, 2000.

Kanazawa, S. and Filip, Z.: Distribution of microorganisms, total biomass, and enzyme activities in different particles of brown soil, Microbial Ecology, 12, 205--215, 1986.

Kästner, A. and Germershausen, K.: Struktur und Abundanzdynamik der Nematodenfauna in einem Schwarzerde-Lößboden, Hercynia-Ökologie und Umwelt in Mitteleuropa, 26, 71--93, 2014.

Kemper, W. and Rosenau, R.: Aggregate stability and size distribution, Methods of Soil Analysis. Part 1. Physical and Mineralogical Methods - Agronomy Monograph no. 9 (2nd Edition), 1986.

Kerek, M., Drijber, R. A., Powers, W. L., Shearman, R. C., Gaussoin, R. E. and Streich, A. M.: Accumulation of microbial biomass within particulate organic matter of aging golf greens, Agronomy Journal, 94, 455--461, 2002.

Kitamikado, M., Yamaguchi, K., Tseng, C.-H. and Okabe, B.: Method designed to detect alginate-degrading bacteria, Applied and environmental microbiology, 56, 2939--2940, 1990.

Klapper, I., Rupp, C., Cargo, R., Purvedorj, B. and Stoodley, P.: Viscoelastic fluid description of bacterial biofilm material properties, Biotechnology and Bioengineering, 80, 289--296, 2002.

Knežević, A., Milovanović, I., Stajić, M., Lončar, N., Brčeski, I., Vukojević, J. and Ćilerdžić, J.: Lignin degradation by selected fungal species, Bioresource technology, 138, 117--123, 2013.

Kölbl, A., Leifeld, J. and Kögel-Knabner, I.: A comparison of two methods for the isolation of free and occluded particulate organic matter, Journal of Plant Nutrition and Soil Science, 168, 660--667, 2005.

Korthals, G. W., Bongers, T., Kammenga, J. E., Alexiev, A. D. and Lexmond, T. M.: Long-term effects of copper and ph on the nematode community in an agroecosystem, Environmental Toxicology and Chemistry, 15, 979--985, 1996.

Lal, R.: Black and buried carbons' impacts on soil quality and ecosystem services, Soil and Tillage Research, 99, 1--3, 2008.

Lal, R.: Sequestration of atmospheric CO_2 in global carbon pools, Energy & Environmental Science, 1, 86--100, 2008.

Lane, D.: 16S/23S rRNA sequencing, Nucleic acid techniques in bacterial systematics, 125--175, 1991.

Lehmann, J.and Joseph, S.: Biochar for environmental management: science, technology and implementation, Routledge, 2015.

Lehmann, J. and Kleber, M.: The contentious nature of soil organic matter, Nature, 528, 60--68, 2015.

Lehtinen, T., Lair, G. J., Mentler, A., Gisladóttir, G., Ragnarsdóttir, K. V. and Blum, W. E.: Soil aggregate stability in different soil orders quantified by low dispersive ultrasonic energy levels, Soil Science Society of America Journal, 78, 713--723, 2014.

Leifeld, J. and Kögel-Knabner, I.: Soil organic matter fractions as early indicators for carbon stock changes under different land-use?, Geoderma, 124, 143--155, 2005.

Leigh, J. A. and Coplin, D. L.: Exopolysaccharides in plant-bacterial interactions, Annual Reviews in Microbiology, 46, 307--346, 1992.

Lensi, R., Clays-Josserand, A. and Monrozier, L. J.: Denitrifiers and denitrifying activity in size fractions of a mollisol under permanent pasture and continuous cultivation, Soil Biology and Biochemistry, 27, 61--69, 1995.

Lewin, R. A.: Extracellular polysaccharides of green algae, Canadian Journal of Microbiology, 2, 665--672, 1956.

Li, B. and Logan, B. E.: Bacterial adhesion to glass and metal-oxide surfaces, Colloids and Surfaces B: Biointerfaces, 36, 81--90, 2004.

Lieleg, O. and Ribbeck, K.: Biological hydrogels as selective diffusion barriers, Trends in cell biology, 21, 543--551, 2011.

Lim, T.-S. and Loh, W.-Y.: A comparison of tests of equality of variances, Computational Statistics & Data Analysis, 22, 287--301, 1996.

Lueders, T. and Friedrich, M. W.: Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts, Applied and Environmental Microbiology, 69, 320--326, 2003.

von Lützow, M., Kögel-Knabner, I., Ludwig, B., Matzner, E., Flessa, H., Ekschmitt, K., Guggenberger, G., Marschner, B. and Kalbitz, K.: Stabilization mechanisms of organic matter in four temperate soils: development and application of a conceptual model, Journal of Plant Nutrition and Soil Science, 171, 111--124, 2008.

Lützow, M. v., Kögel-Knabner, I., Ekschmitt, K., Flessa, H., Guggenberger, G., Matzner, E. and Marschner, B.: SOM fractionation methods: relevance to functional pools and to stabilization mechanisms, Soil Biology and Biochemistry, 39, 2183--2207, 2007.

Lützow, M. v., Kögel-Knabner, I., Ekschmitt, K., Matzner, E., Guggenberger, G., Marschner, B. and Flessa, H.: Stabilization of organic matter in temperate soils: mechanisms and their relevance under different soil conditions--a review, European Journal of Soil Science, 57, 426--445, 2006.

Madigan, M., Martinko, J., Bender, K., Buckley, D.and Stahl, D.: Brock Biology of Microorganisms, Global Edition: UEL, Pearson Education Limited, 2015.

Mah, T.-F. C. and O'Toole, G. A.: Mechanisms of biofilm resistance to antimicrobial agents, Trends in microbiology, 9, 34--39, 2001.

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. and Wade, W. G.: Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA, Appl. Environ. Microbiol., 64, 795--799, 1998.

Margesin, R., Zimmerbauer, A. and Schinner, F.: Soil lipase activity--a useful indicator of oil biodegradation, Biotechnology Techniques, 13, 859--863, 1999.

Margesin, R., Zimmerbauer, A. and Schinner, F.: Monitoring of bioremediation by soil biological activities, Chemosphere, 40, 339--346, 2000.

Marschner, B. and Kalbitz, K.: Controls of bioavailability and biodegradability of dissolved organic matter in soils, Geoderma, 113, 211--235, 2003.

Marshall, T. and Quirk, J.: Stability of structural aggregates of dry soil, Crop and Pasture Science, 1, 266--275, 1950.

Martens, D. and Frankenberger Jr, W.: Decomposition of bacterial polymers in soil and their influence on soil structure, Biology and fertility of soils, 13, 65--73, 1992.

Martinson, D. C., Olmstead, L. and others: Crushing strength of aggregated soil materials., Proceedings. Soil Science Society of America, 1949, 14, 34--38, 1950.

Marty, N., Dournes, J.-L., Chabanon, G. and Montrozier, H.: Influence of nutrient media on the chemical composition of the exopolysaccharide from mucoid and non-mucoid Pseudomonas aeruginosa, FEMS microbiology letters, 98, 35--44, 1992.

McGuire, K. L. and Treseder, K. K.: Microbial communities and their relevance for ecosystem models: decomposition as a case study, Soil Biology and Biochemistry, 42, 529--535, 2010.

McNamara, N., Black, H., Beresford, N. and Parekh, N.: Effects of acute gamma irradiation on chemical, physical and biological properties of soils, Applied Soil Ecology, 24, 117--132, 2003.

Meyer, S., Leifeld, J., Bahn, M. and Fuhrer, J.: Land-use change in subalpine grassland soils: Effect on particulate organic carbon fractions and aggregation, Journal of Plant Nutrition and Soil Science, 175, 401--409, 2012.

Miller, R. and Jastrow, J.: Hierarchy of root and mycorrhizal fungal interactions with soil aggregation, Soil Biology and Biochemistry, 22, 579--584, 1990.

Miller, R. & Jastrow, J. 2000. Mycorrhizal fungi influence soil structure. *In: Arbuscular mycorrhizas: physiology and function,* 3--18, Springer

Möhle, R. B., Langemann, T., Haesner, M., Augustin, W., Scholl, S., Neu, T. R., Hempel, D. C. and Horn, H.: Structure and shear strength of microbial biofilms as determined with confocal laser scanning microscopy and fluid dynamic gauging using a novel rotating disc biofilm reactor, Biotechnology and bioengineering, 98, 747--755, 2007.

Molope, M., Grieve, I. and Page, E.: Contributions by fungi and bacteria to aggregate stability of cultivated soils, Journal of Soil Science, 38, 71--77, 1987.

Monrozier, L. J., Ladd, J., Fitzpatrick, R. W., Foster, R. and Rapauch, M.: Components and microbial biomass content of size fractions in soils of contrasting aggregation, Geoderma, 50, 37--62, 1991.

More, T., Yadav, J., Yan, S., Tyagi, R. and Surampalli, R.: Extracellular polymeric substances of bacteria and their potential environmental applications, Journal of environmental management, 144, 1--25, 2014.

Mummey, D., Holben, W., Six, J. and Stahl, P.: Spatial stratification of soil bacterial populations in aggregates of diverse soils, Microbial Ecology, 51, 404--411, 2006.

Mummey, D. and Stahl, P.: Analysis of soil whole-and inner-microaggregate bacterial communities, Microbial Ecology, 48, 41--50, 2004.

Munk, K.: Biochemie-Zellbiologie, Georg Thieme Verlag, Stuttgart, Germany, 1--576, 2008.

Muyzer, G., De Waal, E. C. and Uitterlinden, A. G.: Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environ. Microbiol., 59, 695--700, 1993.

NanoDrop: NanoDrop 1000 Spectrophotometer V3. 7 User’s Manual, Thermo Fisher Scientific, 1--105, 2008.

Neher, D. A.: Ecology of plant and free-living nematodes in natural and agricultural soil, Phytopathology, 48, 2010.

Nicholas, W.: A study of a species of Acrobeloides (Cephalobidae) in laboratory culture., Nemstologica, 8, 99--109, 1962.

Niemeyer, J. and Gessler, F.: Determination of free DNA in soils, J. Plant Nutr. Soil Sci., 165, 121--124, 2002.

Nimmo, J. R. and Perkins, K. S.: 2.6 Aggregate Stability and Size Distribution, Methods of soil analysis: Part, 4, 317--328, 2002.

Nordgren, A.: Apparatus for the continuous, long-term monitoring of soil respiration rate in large numbers of samples, Soil Biology and Biochemistry, 20, 955--957, 1988.

North, P.: Towards an absolute measurement of soil structural stability using ultrasound, Journal of Soil Science, 27, 451--459, 1976.

Nunan, N., Wu, K., Young, I. M., Crawford, J. W. and Ritz, K.: Spatial distribution of bacterial communities and their relationships with the micro-architecture of soil, FEMS Microbiology Ecology, 44, 203--215, 2003.

O'Brien, S. L. and Jastrow, J. D.: Physical and chemical protection in hierarchical soil aggregates regulates soil carbon and nitrogen recovery in restored perennial grasslands, Soil Biology and Biochemistry, 61, 1--13, 2013.

Oades, J.: The role of biology in the formation, stabilization and degradation of soil structure, Geoderma, 56, 377--400, 1993.

Oades, J. and Waters, A.: Aggregate hierarchy in soils, Soil Research, 29, 815--828, 1991.

Oades, J. M. 1984. Soil organic matter and structural stability: mechanisms and implications for management. *In: Biological Processes and Soil Fertility,* Tinsley, J. & Darbyshire, J. (eds.) 319--337, Springer

Or, D., Smets, B. F., Wraith, J., Dechesne, A. and Friedman, S.: Physical constraints affecting bacterial habitats and activity in unsaturated porous media--a review, Advances in Water Resources, 30, 1505--1527, 2007.

Overmann, J., Coolen, M. J. and Tuschak, C.: Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments, Arch. Microbiol., 172, 83--94, 1999.

Ozturk, S. and Aslim, B.: Modification of exopolysaccharide composition and production by three cyanobacterial isolates under salt stress, Environmental Science and Pollution Research, 17, 595--602, 2010.

Pal, A. and Paul, A.: Microbial extracellular polymeric substances: central elements in heavy metal bioremediation, Indian Journal of Microbiology, 48, 49--64, 2008.

Perfect, E. and Kay, B.: Statistical characterization of dry aggregate strength using rupture energy, Soil science society of America Journal, 58, 1804--1809, 1994.

Philippot, L., Andersson, S. G., Battin, T. J., Prosser, J. I., Schimel, J. P., Whitman, W. B. and Hallin, S.: The ecological coherence of high bacterial taxonomic ranks, Nat. Rev. Microbiol., 8, 523--529, 2010.

Piccolo, A. and Mbagwu, J. S.: Role of hydrophobic components of soil organic matter in soil aggregate stability, Soil Science Society of America Journal, 63, 1801--1810, 1999.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M., Guerri, G. and Nannipieri, P.: Extracellular DNA in soil and sediment: fate and ecological relevance, Biology and Fertility of Soils, 45, 219--235, 2009.

Poirier, N., Sohi, S. P., Gaunt, J. L., Mahieu, N., Randall, E. W., Powlson, D. S. and Evershed, R. P.: The chemical composition of measurable soil organic matter pools, Organic Geochemistry, 36, 1174--1189, 2005.

Pokrovsky, O. S., Dupré, B. and Schott, J.: Fe--Al--organic colloids control of trace elements in peat soil solutions: results of ultrafiltration and dialysis, Aquatic Geochemistry, 11, 241--278, 2005.

Pühler, A., Arlat, M., Becker, A., Göttfert, M., Morrissey, J. P. and O'Gara, F.: What can bacterial genome research teach us about bacteria--plant interactions?, Current opinion in plant biology, 7, 137--147, 2004.

Rajaram, G. and Erbach, D.: Effect of wetting and drying on soil physical properties, Journal of Terramechanics, 36, 39--49, 1999.

Ranjard, L. and Richaume, A.: Quantitative and qualitative microscale distribution of bacteria in soil, Research in microbiology, 152, 707--716, 2001.

Ras, M., Lefebvre, D., Derlon, N., Paul, E. and Girbal-Neuhauser, E.: Extracellular polymeric substances diversity of biofilms grown under contrasted environmental conditions, Water research, 45, 1529--1538, 2011.

Redmile-Gordon, M., Brookes, P., Evershed, R., Goulding, K. and Hirsch, P.: Measuring the soil-microbial interface: Extraction of extracellular polymeric substances (EPS) from soil biofilms, Soil Biology and Biochemistry, 72, 163--171, 2014.

Redmile-Gordon, M., Evershed, R., Hirsch, P., White, R. and Goulding, K.: Soil organic matter and the extracellular microbial matrix show contrasting responses to C and N availability, Soil Biology and Biochemistry, 88, 257--267, 2015.

Riding, R.: Microbial carbonates: the geological record of calcified bacterial--algal mats and biofilms, Sedimentology, 47, 179--214, 2000.

Rillig, M. C.: Arbuscular mycorrhizae, glomalin, and soil aggregation, Canadian Journal of Soil Science, 84, 355--363, 2004.

Rillig, M. C., Maestre, F. T. and Lamit, L. J.: Microsite differences in fungal hyphal length, glomalin, and soil aggregate stability in semiarid Mediterranean steppes, Soil Biology and Biochemistry, 35, 1257--1260, 2003.

Rillig, M. C., Wright, S. F. and Eviner, V. T.: The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species, Plant and Soil, 238, 325--333, 2002.

Rinaudi, L. V. and Giordano, W.: An integrated view of biofilm formation in rhizobia, FEMS microbiology letters, 304, 1--11, 2010.

Roberson, E. B. and Firestone, M. K.: Relationship between desiccation and exopolysaccharide production in a soil Pseudomonas sp, Applied and Environmental Microbiology, 58, 1284--1291, 1992.

Rosenberg, N. J.: Response of plants to the physical effects of soil compaction, Advances in Agronomy, 16, 181--196, 1964.

Rousk, J., Bååth, E., Brookes, P. C., Lauber, C. L., Lozupone, C., Caporaso, J. G., Knight, R. and Fierer, N.: Soil bacterial and fungal communities across a pH gradient in an arable soil, The ISME journal, 4, 1340--1351, 2010.

Ruess, L.: Nematode fauna in spruce forest soils: a qualitative/quantitative comparison, Nematologica, 41, 106--124, 1995.

Ruess, L.: Studies on the nematode fauna of an acid forest soil: spatial distribution and extraction, Nematologica, 41, 229--239, 1995.

Ruess, L., Schmidt, I. K., Michelsen, A. and Jonasson, S.: Responses of nematode species composition to factorial addition of carbon, fertiliser, bactericide and fungicide at two sub-arctic sites, Nematology, 4, 527--539, 2002.

Russell, J.: Report of the subcommittee on soil structure and consistency, Soil Science Society of America Journal, 9, 10--22, 1928.

Schindler, F. V., Mercer, E. J. and Rice, J. A.: Chemical characteristics of glomalin-related soil protein (GRSP) extracted from soils of varying organic matter content, Soil Biology and Biochemistry, 39, 320--329, 2007.

Schmidt, M., Rumpel, C. and Kögel-Knabner: Evaluation of an ultrasonic dispersion procedure to isolate primary organomineral complexes from soils, European Journal of Soil Science, 50, 87--94, 1999.

Schmidt, M. W., Torn, M. S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I. A., Kleber, M., Kögel-Knabner, I., Lehmann, J., Manning, D. A. and others: Persistence of soil organic matter as an ecosystem property, Nature, 478, 49--56, 2011.

Schmitt, J. and Flemming, H.-C.: Water binding in biofilms, Water science and technology, 39, 77--82, 1999.

Schnürer, J., Clarholm, M. and Rosswall, T.: Microbial biomass and activity in an agricultural soil with different organic matter contents, Soil Biology and Biochemistry, 17, 611--618, 1985.

Schrumpf, M., Kaiser, K., Guggenberger, G., Persson, T., Kögel-Knabner, I. and Schulze, E.-D.: Storage and stability of organic carbon in soils as related to depth, occlusion within aggregates, and attachment to minerals, Biogeosciences, 10, 1675--1691, 2013.

Seybold, C. and Herrick, J.: Aggregate stability kit for soil quality assessments, Catena, 44, 37--45, 2001.

Shapiro, S. S. and Wilk, M. B.: An analysis of variance test for normality (complete samples), Biometrika, 52, 591--611, 1965.

Simoes, M., Cleto, S., Pereira, M. O. and Vieira, M.: Influence of biofilm composition on the resistance to detachment, Water Science and Technology, 55, 473--480, 2007.

Singh, K.: Aggregate analysis of soils. I.—apparatus and method, Journal of the Science of Food and Agriculture, 3, 205--209, 1952.

Six, J., Bossuyt, H., Degryze, S. and Denef, K.: A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics, Soil and Tillage Research, 79, 7--31, 2004.

Six, J., Conant, R., Paul, E. A. and Paustian, K.: Stabilization mechanisms of soil organic matter: implications for C-saturation of soils, Plant Soil, 241, 155--176, 2002.

Six, J., Elliott, E. and Paustian, K.: Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture, Soil Biology and Biochemistry, 32, 2099--2103, 2000.

Six, J., Schultz, P., Jastrow, J. and Merckx, R.: Recycling of sodium polytungstate used in soil organic matter studies, Soil Biology and Biochemistry, 31, 1193--1196, 1999.

Skidmore, E. and Powers, D.: Dry soil-aggregate stability: energy-based index, Soil Sci. Soc. Am. J., 46, 1274--1279, 1982.

Sollins, P., Homann, P. and Caldwell, B. A.: Stabilization and destabilization of soil organic matter: mechanisms and controls, Geoderma, 74, 65--105, 1996.

Spohn, M. and Giani, L.: Water-stable aggregates, glomalin-related soil protein, and carbohydrates in a chronosequence of sandy hydromorphic soils, Soil Biology and Biochemistry, 42, 1505--1511, 2010.

Sponagel, H., Grottenthaler, W., Hartmann, K.-J., Hartwich, R., Janetzko, P., Joisten, H., Kühn, D., Sabel, K.-J.and Traidl, R.: Bodenkundliche Kartieranleitung [KA 5], Schweizerbart, 2005.

Stacey, M.: Macromolecules synthesised by micro-organisms, Journal of the Chemical Society (Resumed), 853--864, 1947.

Stach, J. E., Maldonado, L. A., Ward, A. C., Goodfellow, M. and Bull, A. T.: New primers for the class Actinobacteria: application to marine and terrestrial environments, Environ. Microbiol., 5, 828--841, 2003.

Staddon, P. L., Ramsey, C. B., Ostle, N., Ineson, P. and Fitter, A. H.: Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of 14C, Science, 300, 1138--1140, 2003.

Stams, A. J. and Plugge, C. M.: Electron transfer in syntrophic communities of anaerobic bacteria and archaea, Nature Reviews Microbiology, 7, 568--577, 2009.

Steinberg, P. D. and Rillig, M. C.: Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin, Soil Biology and Biochemistry, 35, 191--194, 2003.

Steinberger, R. and Holden, P.: Extracellular DNA in single-and multiple-species unsaturated biofilms, Applied and environmental microbiology, 71, 5404--5410, 2005.

Stewart, P. S.: A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms, Biotechnol. Bioeng., 59, 261--272, 1998.

Stewart, P. S. and Franklin, M. J.: Physiological heterogeneity in biofilms, Nature Reviews Microbiology, 6, 199--210, 2008.

Stockmann, U., Adams, M. A., Crawford, J. W., Field, D. J., Henakaarchchi, N., Jenkins, M., Minasny, B., McBratney, A. B., de Courcelles, V. d. R., Singh, K. and others: The knowns, known unknowns and unknowns of sequestration of soil organic carbon, Agriculture, Ecosystems & Environment, 164, 80--99, 2013.

Sutherland, I. W.: Biofilm exopolysaccharides: a strong and sticky framework, Microbiology, 147, 3--9, 2001.

Tang, J., Mo, Y., Zhang, J. and Zhang, R.: Influence of biological aggregating agents associated with microbial population on soil aggregate stability, Applied Soil Ecology, 47, 153--159, 2011.

Taylor, H. and Brar, G.: Effect of soil compaction on root development, Soil and Tillage Research, 19, 111--119, 1991.

Tiessen, H. and Stewart, J.: Particle-size fractions and their use in studies of soil organic matter: II. Cultivation effects on organic matter composition in size fractions, Soil Science Society of America Journal, 47, 509--514, 1983.

Tiessen, H. and Stewart, J.: Light and electron microscopy of stained microaggregates: the role of organic matter and microbes in soil aggregation, Biogeochemistry, 5, 312--322, 1988.

Tisdall, J.: Fungal hyphae and structural stability of soil, Soil Research, 29, 729--743, 1991.

Tisdall, J.: Possible role of soil microorganisms in aggregation in soils, Plant and soil, 159, 115--121, 1994.

Tisdall, J.: Formation of soil aggregates and accumulation of soil organic matter, Structure and organic matter storage in agricultural soils, 57--96, 1996.

Tisdall, J. and Oades, J.: Organic matter and water-stable aggregates in soils, Journal of soil science, 33, 141--163, 1982.

Torsvik, V. and Øvreås, L.: Microbial diversity and function in soil: from genes to ecosystems, Curr. Opin. Microbiol., 5, 240--245, 2002.

Traoré, O., Groleau-Renaud, V., Plantureux, S., Tubeileh, A. and Boeuf-Tremblay, V.: Effect of root mucilage and modelled root exudates on soil structure, European Journal of Soil Science, 51, 575--581, 2000.

Uroz, S., Calvaruso, C., Turpault, M.-P. and Frey-Klett, P.: Mineral weathering by bacteria: ecology, actors and mechanisms, Trends in microbiology, 17, 378--387, 2009.

Van Der Heijden, M. G., Bardgett, R. D. and Van Straalen, N. M.: The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems, Ecology letters, 11, 296--310, 2008.

Van Loosdrecht, M., Heijnen, J., Eberl, H., Kreft, J. and Picioreanu, C.: Mathematical modelling of biofilm structures, Antonie van Leeuwenhoek, 81, 245--256, 2002.

Vargas, R. and Hattori, T.: The distribution of protozoa within soil aggregates., The Journal of General and Applied Microbiology, 37, 515--518, 1991.

Venette, R. and Ferris, H.: Thermal constraints to population growth of bacterial-feeding nematodes, Soil Biology and Biochemistry, 29, 63--74, 1997.

Von Mering, C., Hugenholtz, P., Raes, J., Tringe, S., Doerks, T., Jensen, L., Ward, N. and Bork, P.: Quantitative phylogenetic assessment of microbial communities in diverse environments, Science, 315, 1126--1130, 2007.

Votselko, S., Pirog, T., Malashenko, Y. R. and Grinberg, T.: A method for determining the mass-molecular composition of microbial exopolysaccharides, Journal of microbiological methods, 18, 349--356, 1993.

Wagai, R., Mayer, L. M. and Kitayama, K.: Nature of the “occluded” low-density fraction in soil organic matter studies: a critical review, Soil Science and Plant Nutrition, 55, 13--25, 2009.

Walker, S. L., Fourgialakis, M., Cerezo, B. and Livens, S.: Removal of Microbial Biofilms from Dispense Equipment: The Effect of Enzymatic Pre-digestion and Detergent Treatment, Journal of the Institute of Brewing, 113, 61--66, 2007.

Wallace, H.: Movement of eelworms, Annals of applied Biology, 46, 86--94, 1958.

Wallace, H.: The dynamics of nematode movement, Annual Review of Phytopathology, 6, 91--114, 1968.

Ward, N. L., Challacombe, J. F., Janssen, P. H., Henrissat, B., Coutinho, P. M., Wu, M., Xie, G., Haft, D. H., Sait, M., Badger, J. and others: Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils, Applied and environmental microbiology, 75, 2046--2056, 2009.

Wargo, M. J. and Hogan, D. A.: Fungal—bacterial interactions: a mixed bag of mingling microbes, Current opinion in microbiology, 9, 359--364, 2006.

Wasikiewicz, J. M., Yoshii, F., Nagasawa, N., Wach, R. A. and Mitomo, H.: Degradation of chitosan and sodium alginate by gamma radiation, sonochemical and ultraviolet methods, Radiation Physics and Chemistry, 73, 287--295, 2005.

Watts, C. W., Whalley, W. R., Brookes, P. C., Devonshire, B. J. and Whitmore, A. P.: Biological and physical processes that mediate micro-aggregation of clays, Soil Science, 170, 573--583, 2005.

Weitere, M., Bergfeld, T., Rice, S. A., Matz, C. and Kjelleberg, S.: Grazing resistance of Pseudomonas aeruginosa biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode, Environmental Microbiology, 7, 1593--1601, 2005.

Weng, L., Temminghoff, E. J., Lofts, S., Tipping, E. and Van Riemsdijk, W. H.: Complexation with dissolved organic matter and solubility control of heavy metals in a sandy soil, Environmental Science & Technology, 36, 4804--4810, 2002.

Wilkinson, J.: The extracellular polysaccharides of bacteria, Bacteriological Reviews, 22, 46, 1958.

Wright, S. and Anderson, R.: Aggregate stability and glomalin in alternative crop rotations for the central Great Plains, Biology and Fertility of Soils, 31, 249--253, 2000.

Wright, S., Franke-Snyder, M., Morton, J. and Upadhyaya, A.: Time-course study and partial characterization of a protein on hyphae of arbuscular mycorrhizal fungi during active colonization of roots, Plant and Soil, 181, 193--203, 1996.

Wright, S., Green, V. and Cavigelli, M.: Glomalin in aggregate size classes from three different farming systems, Soil and Tillage Research, 94, 546--549, 2007.

Wright, S., Starr, J. and Paltineanu, I.: Changes in aggregate stability and concentration of glomalin during tillage management transition, Soil Science Society of America Journal, 63, 1825--1829, 1999.

Wright, S. and Upadhyaya, A.: A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi, Plant Soil, 198, 97--107, 1998.

Wu, Q.-S., Cao, M.-Q., Zou, Y.-N. and He, X.-h.: Direct and indirect effects of glomalin, mycorrhizal hyphae, and roots on aggregate stability in rhizosphere of trifoliate orange, Scientific reports, 4, 2014.

Yeates, G., Bongers, T., De Goede, R., Freckman, D. and Georgieva, S.: Feeding habits in soil nematode families and genera—an outline for soil ecologists, Journal of nematology, 25, 315, 1993.

Yeates, G. W.: Nematodes in ecological webs, eLS, 2010.

Yoder, R. E.: A direct method of aggregate analysis of soils and a study of the physical nature of erosion losses, Agronomy Journal, 28, 337--351, 1936.

Zech, W., Schad, P.and Hintermaier-Erhard, G.: Böden der Welt: ein Bildatlas, Springer-Verlag, 2014.

Zelles, L.: Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review, Biology and fertility of soils, 29, 111--129, 1999.

Zhang, H.: Organic matter incorporation affects mechanical properties of soil aggregates, Soil and Tillage Research, 31, 263--275, 1994.

Zhang, X., Bishop, P. L. and Kupferle, M. J.: Measurement of polysaccharides and proteins in biofilm extracellular polymers, Water science and technology, 37, 345--348, 1998.

Zhang, Z.-Q.: Animal biodiversity: An update of classification and diversity in 2013. In: Zhang, Z.-Q.(Ed.) Animal Biodiversity: An Outline of Higher-level Classification and Survey of Taxonomic Richness (Addenda 2013), Zootaxa, 3703, 5--11, 2013.

Zhu, W.: p< 0.05,< 0.01,< 0.001,< 0.0001,< 0.00001,< 0.000001, or< 0.0000001…, Journal of Sport and Health Science, 5, 77--79, 2016.

Zhu, Y.-G. and Miller, R. M.: Carbon cycling by arbuscular mycorrhizal fungi in soil--plant systems, Trends in plant science, 8, 407--409, 2003.

Zuckerman, B. M. and Jansson, H.: Nematode chemotaxis and possible mechanisms of host/prey recognition, Annual review of phytopathology, 22, 95--113, 1984.