

Earthworm mucus contributes to the formation of organo-mineral associations in soil

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ABSTRACT

Earthworms are considered as “ecosystem engineers” impacting soil properties as well as nutrient and element cycles. As they move through soil, earthworms secrete cutaneous mucus which is metabolized by soil microorganisms and a source of plant-available nutrients. Earthworm-processed soil contains carbon enriched, earthworm-specific soil aggregates (e.g. casts and middens) in comparison to earthworm unaffected soil. The reason could be that organic polymeric substances in earthworm mucus bind to soil minerals. The objective of this study was to investigate the small-scale interactions between earthworm mucus and secondary soil minerals, e.g. goethite and illite, leading to the formation of organo-mineral associations. We characterized the chemical composition of earthworm mucus by FTIR and ¹³C-NMR spectroscopy and compared spectra of mucus to microbial extracellular polymeric substances (EPS), an abundant and well-known type of organic matter that binds with soil minerals. Mucus from anecic (*Lumbricus terrestris* L.) and endogeic (*Aporrectodea caliginosa* Sav.) earthworm species was dominated by proteins and carbohydrates. Between 21 and 36% of the total organic carbon in the mucus containing treatments adsorbed to illite and goethite, and most of the binding with goethite was associated with phosphorus containing mucus compounds. The surface charge of newly-formed organo-mineral associations was determined by measuring the isoelectric point (IEP). The IEP of mucus-goethite associations was 6.8, which was lower than the bare goethite IEP of 8.4. The zeta potential of mucus-illite associations was greater than bare illite. We conclude that the specific adsorption of earthworm mucus constituents to soil minerals leads to the formation of mucus-mineral associations. These associations contribute to retention of organic substances from earthworm mucus in soil (micro-)aggregates and explain the altered physicochemical properties of earthworm-formed aggregates in comparison to the earthworm unaffected “bulk” soil material.

1. Introduction

The soil fauna significantly contributes to the formation of stable soil aggregates, impacting the turnover rates, porosity and coherence of soil as well as water infiltration (Lee and Foster, 1991). Among the ecological groups, anecic and endogeic earthworms affect the soil structure as “ecosystem engineers” (Jones et al., 1994) by forming casts, middens or burrow wall linings, and serve as important biogenic agents of aggregation (Six et al., 2004). Specifically, the drilosphere (Bouché, 1975), defined as the zone of 2 mm to 1 cm thickness around the burrow walls (Andriuzzi et al., 2013; Lipiec et al., 2015) is directly affected by earthworm activity due to the deposition of earthworm-formed soil aggregates (Six et al., 2004).

When earthworms burrow through soil, they deposit nutrient-rich mucus, and excrete casts, a mixture of digested soil material, organic matter (OM) and microorganisms, on the burrow walls (Lee, 1985; Brown et al., 2000). The released cutaneous mucus is a lubricant to facilitate earthworm movement through the soil and protects the earthworm from being encased by soil particles (Lee, 1985). The cutaneous and intestinal mucus consist of water, carbohydrates, proteins, lipids and polysaccharides (Pan et al., 2010; Zhang et al., 2016). In consequence, earthworm activity and mucus excretion shape soil and soil aggregate properties and processes (physically, chemically and biologically) e.g. nutrient element storage (P and C accumulation), habitat formation (beneficial conditions for microbial growth and root penetration), infiltration and transport (burrows as preferential flow

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pathways) (Lee and Foster, 1991; Brown et al., 2000; Six et al., 2004). The OM inside the drilosphere and earthworm-formed aggregates is attributed to cutaneous and intestinal mucus, together with earthworm processed plant debris (Shipitalo and Protz, 1989; Six et al., 2004; Brown et al., 2000) and builds the fundament for microbial hotspots in earthworm formed structures (Edwards and Fletcher, 1988; Aira et al., 2009). The incorporation of OM is reported to cause changes of the physicochemical properties of the earthworm-formed aggregates in comparison to the surrounding soil material (Brown et al., 2000; Six et al., 2004).

Earthworms may release 0.2–0.5% of total animal C per day via mucus excretion or cast formation (for *Octolasion lacteum*, see Scheu, 1991). Given this impressive numbers, we intend to expand the studies on biogenic OM by identifying the role of earthworm mucus with respect to microbial EPS, a well-studied fraction of OM in soil. The objective of our study is to elucidate the formation of mucus-mineral associations due to adsorption, as so far neglected but essential process during the formation of earthworm-formed soil structures. We aim to contribute to a better understanding of the role of earthworm derived mucus-mineral associations in the element cycling and as reactive component in soil. Several authors demonstrated the complex interactions of microbially produced OM, especially extracellular polymeric substances (EPS) of bacterial origin, with typical constituents of the soil mineral phase like iron oxides, clay minerals and quartz (Omoike and Chorover, 2004; Cao et al., 2011; Fang et al., 2012). We hypothesize, regarding their specific composition, a comparability between microbial EPS and earthworm mucus in terms of their interaction with the soil mineral phase and the formation of organo-mineral associations. Additionally, we presume a similar composition and mucus-mineral interactions of mucus obtained from different ecological functional groups due to maintaining their function as drag reducing lubricant found for mucus of different earthworm species (Zhang et al., 2016).

Specifically, we investigated the interactions between earthworm cutaneous mucus extracts of *Lumbricus terrestris* L. (anecic) and *Aporrectodea caliginosa* Sav. (endogeic) with goethite and illite, i.e., typical secondary minerals of temperate soils. We compared our results with results obtained for EPS from *Bacillus subtilis* of the late (Narvekar et al., 2017; Guhra et al., 2019) and early stationary phase (Liu et al., 2013). The mucus composition was characterised by spectrometric methods. We conducted completely mixed batch adsorption experiments of earthworm cutaneous mucus with the named secondary soil minerals. The composition of mucus before and after reaction with minerals was investigated by attenuated total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR). The potential adsorption of mucus compounds to the bare mineral phase of illite or goethite was measured as change of the total organic carbon (TOC) concentration (TOC analyzes) as well as the change of element concentration like phosphorus and potassium by inductively coupled plasma optical emission spectrometry (ICP-OES). The formation of organo-mineral associations was indirectly evidenced by the determination of an altered surface-charge of organo-mineral associations in comparison to the bare minerals by zeta potential measurements.

2. Material and methods

2.1. Earthworm mucus extraction

Earthworms were sampled on pasture and cropland sites located within the Hainich Critical Zone Exploratory (Küsel et al., 2016) in north-west Thuringia, Germany. Soil groups varied between Rendzic Leptosols, Cambisols and Luvisols originating from limestone and Loess cover (Kohlhepp et al., 2017). Endogeic earthworms were sampled by hand-sorting of the top 15 cm of the soil. Anecic earthworms were sampled after Chan and Munro (2001), applying a mustard solution which was poured into the remaining soil pit. Earthworm species were determined according to Sims and Gerard (1999). Cutaneous earthworm

mucus of endogeic (EN) *A. caliginosa* and anecic (AN) *L. terrestris* was extracted according to Eisenhauer et al. (2009). To prevent contamination of mucus with cast material, earthworm species were kept separately on wetted pulp (XXL2 Blue; Roth, Karlsruhe, Germany) to void their guts for approximately 60 h. Coprophagy was prevented by regular exchange of the pulp. Before the extraction, the earthworms were washed with pure water (Milli-Q, Integral 5, Elix Technology Inside, Merck Millipore, Darmstadt, Germany) and placed inside beakers. According to Eisenhauer et al. (2009), we utilized 30 individuals of *L. terrestris* for mucus extraction and used the equivalent biomass of *A. caliginosa* to maintain comparability between the two treatments. Hence, the extraction took place with the resulting ratio of 1.3 g (earthworm biomass):1 ml (pure water). Pure water was added to the beakers and earthworms were stirred with a glass rod for 15 min (every third minute for 1 min). This leads to tactile skin irritation, which provoked the mucus release. The extract was centrifuged twofold at 20,000 g at 20 °C for 30 min (Sorvall RC 6 Plus Zentrifuge, Thermo Scientific, Waltham, USA) to remove remaining mineral particles. The obtained mucus was frozen (temp.: -20 °C) overnight and subsequently freeze-dried (Alpha 1–4 LSC, CHRIST, Osterode am Harz, Germany).

2.2. Characterization of mucus

The freeze-dried stocks of anecic and endogeic mucus were analyzed by Fourier-transform infrared spectroscopy (FTIR) (Nicolet iS10 spectrometer, Thermo Fisher Scientific, Dreieich, Germany) with the attenuated-total-reflection (ATR) option (Smart iTX, Thermo Fisher Scientific, Dreieich, Germany), CHNS elemental analyser (Euro EA, Euroveector, Pavia, Italy) and solid-state ¹³C-nuclear magnetic resonance (NMR) (DSX 200, Bruker, Billerica, USA) spectroscopy. Measurements of the chemical shifts were done at a resonance frequency of 50.3 MHz with cross-polarization magic angle spinning technique. We measured at a spinning speed of 6.8 kHz and pulse delays of 0.4 s. A line broadening of 50 Hz was applied to the spectra. We compared the NMR spectra with data obtained at similar acquisition conditions as for EPS from *Bacillus subtilis* 168 (DSM 402), in the late (Narvekar et al., 2017; Guhra et al., 2019) and early stationary phase (Liu et al., 2013). We computed the integrals of chemical shift regions of aliphatic C (0–45 ppm), αC of amino acids (45–65 ppm), O-alkyl-C (65–90 ppm), anomeric C (90–110 ppm), aromatic as well as olefinic C (160–110 ppm) and amides as well as carboxyl C (160–200 ppm) according to Metzger et al. (2009).

2.3. Mineral characterization

Goethite was synthesized according to Schwertmann and Cornell (2000). In short, a solution of 1M Fe(III)nitrate-nonahydrate (EMSURE 99–101% for analysis, Merck, Germany) was prepared and rapidly mixed with 5M NaOH (from sodium hydroxide pellets ≥ 98%, CARL ROTH, Karlsruhe, Germany). Subsequently, ultrapure water was added in a ratio of 100:16. The precipitated ferrihydrite was aged to goethite by storage in a drying chamber (Binder, Tuttlingen, Germany) at 60 °C. Needle-shaped goethite was obtained from this procedure with an average length of $0.9 \pm 0.3 \mu\text{m}$ as measured by scanning electron microscopy (see Guhra et al. (2019) for details). To remove nitrate, the material was rinsed repeatedly with deionized water and freeze-dried (Alpha 1–4 LSC, CHRIST, Osterode am Harz, Germany) for storage.

Chunks of Illite (INTER-ILL, Mernöki Iroda, Általános és Kereskedelmi Kft, Hungary) were crushed and dry sieved to a maximum size of $36 \mu\text{m}$. Mid particle size of illite was $9.0 \pm 0.6 \mu\text{m}$ as measured by laser diffraction (Analysette 22 compact, Fritsch, Nagytarcsa, Hungary). Minerals were characterized by X-ray diffraction (XRD, D8 Advance, Bruker, Karlsruhe, Germany) and specific surface area by N₂-BET (Autosorb-1, Quantachrome, Odelzhausen, Germany) (see Guhra et al. (2019) for details).

2.4. Mucus-mineral adsorption experiment

The adsorption of mucus to the mineral phases of illite and goethite was investigated with a series of completely mixed batch reactor experiments using aqueous suspensions of mucus and illite or goethite. To produce the mucus-extract suspension, freeze-dried mucus was successively dispersed in 1 mM NaCl solution (99.7%, VWR, Leuven, Belgium) in a cooled ultrasonic bath (VWR ultrasonic cleaner; ratio: 1 mg mucus/7.5 ml NaCl_{aq}). Illite and goethite was suspended in 1 mM sodium chloride solutions with/without mucus. The solid (mg):liquid (ml)-ratio of the batch-experiments containing illite and goethite was adjusted to 2.5:1.

As reference, blank samples consisting of only endogeic mucus and anecic mucus as well as bare mineral phases mixed with sodium chloride solution were processed in the same way as the mineral-mucus containing variants. Batches of mucus-free and mucus-containing variants were shaken (115 rounds/minute) for 24 h. Afterwards, the solid and liquid phase were separated by centrifugation at 20,000 g at 20 °C for 30 min (Fang et al., 2012; Cao et al., 2011) to estimate the adsorbed amount of carbon and phosphate originating from endogeic and anecic mucus. For this, the supernatant was analyzed with a TOC- and DOC-Analyzer (Multi N/C 2100s, Analytic Jena GmbH, Jena, Germany) and inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian, Varian 725 ES, Darmstadt, Germany). An aliquot of the supernatant was freeze-dried and analyzed by ATR-FTIR. As reference for any amendments in the band intensities within the mucus spectra of mineral treated and untreated samples, the band assigned to the carboxylic groups at 1402 cm⁻¹ (Abdulla et al., 2010) was used for normalization. The P:C ratio was calculated by the P concentrations (in mmol/l) divided by the TOC-values (in mmol/l). The standard deviations given as a measure for uncertainty result from the measurements of three replicates. The significance of treatment effects was tested by Student's two-tailed *t*-test with *p* < 0.05.

2.5. Surface charge and isoelectric point

The isoelectric point (IEP) of the pure and mucus-associated minerals as well as endogeic and anecic mucus references was calculated from zeta potential measurements (Nano ZS, Malvern Instruments, Malvern, UK) as function of the pH. We used freeze-dried and resuspended pellets of minerals and organo-mineral associations after the conduction of the batch experiments. These were washed for several times with pure water to remove loosely bound mucus to obtain potentially strong mucus-mineral associations. Suspensions of 100 mg/l pure minerals (goethite or illite) and the mucus-mineral associations were prepared in 10 mM sodium chloride solution. Subsequently, the pH was adjusted by the addition of HCl (from hydrochloric acid 37%, VWR AnalAR NORMA-PUR, Paris, France) or NaOH (from sodium hydroxide pellets ≥ 98%, CARL ROTH, Karlsruhe, Germany) to the mucus references and the mineral or organo-mineral associations containing suspensions. To equilibrate the surface charges of bare minerals and the associations (Kosmulski, 2016), the suspensions were shaken (115 rounds/minute) for 24 h. After equilibration, pH (WTW pH 197 with PLUS WTW, pH-Elektrode Sen Tix 41, Weilheim, Germany) and zeta potential (Nano ZS, Malvern Instruments, Malvern, UK) were measured. The intersection of the stepwise linear interpolation of zeta potentials as a function of pH was used to calculate the IEP.

3. Results

3.1. Mucus characterization and comparison to microbial EPS

The freeze-dried stock endogeic and anecic earthworm mucus provided narrow C:N ratios of 5.1 ± 0.2 (endogeic mucus) and 3.67 ± 0.03 (anecic mucus) and were composed of a mixture of proteins, carbohydrates and polysaccharides as attributed to the chemical composition by

ATR-FTIR analysis. In both mucus samples, typical bands of the stretching vibration of O–H bonds assigned to surface water (Socrates, 2001) were observed with high extinctions between approximately 3500 to 3100 cm⁻¹ (Fig. 1). Bands at 2920 cm⁻¹ and 2860 cm⁻¹ are typical for C–H asymmetric and symmetric stretching vibrations of methylene groups (Socrates, 2001). The most prominent bands in both samples were at 1631 cm⁻¹ (Amid I) and 1544 cm⁻¹ (Amid II), which represent typical bands of proteins as the stretching vibration of C=O (Amid I) and the N–H deformation and C–N stretching in –CO–NH (Amid II) (Omoike and Chorover, 2004; Jiang et al., 2018). These bands were more distinct in anecic mucus. The band at 1449 cm⁻¹ is attributed to symmetrical deformations of CH₂ and C–OH (Omoike and Chorover, 2004) and more apparent in anecic mucus as in endogeic mucus. The band at 1402 cm⁻¹ represents COO-groups (symmetric stretching) in both samples (Abdulla et al., 2010). The band at 1347 cm⁻¹ is assigned to O–H in-plane bending vibration of carbohydrates (Abdulla et al., 2010). Both bands were more pronounced in endogeic mucus. The band at 1300 cm⁻¹ is attributed to the Amid III band (Socrates, 2001). The wavenumber of 1242 cm⁻¹ was allocated to the asymmetric stretching vibration P=O of the phosphodiester backbone of nucleic acid or phosphorylated proteins (Omoike and Chorover, 2004). The band at 1078 cm⁻¹ is attributed to phosphate symmetric stretching bands C–O–P as well as C–O–C ring vibration of polysaccharides and the band at 1028 cm⁻¹ to an asymmetric stretching of C–O (Omoike and Chorover, 2004; Abdulla et al., 2010).

The results of the ATR-FTIR spectroscopy were supplemented by NMR measurements. According to Metzger et al. (2009), the two chemical shift regions between 110 and 65 ppm (anomeric C and O-alkyl C) are mainly assigned to carbohydrates. Similar proportions of anomeric C (110–90 ppm) values were found in anecic and endogeic mucus, whereas the proportion of O-alkyl C (90–65 ppm) was slightly higher in endogeic mucus (Table 1). In comparison to the carbohydrate related regions, higher proportions mainly referred to proteins, fatty acids and peptidoglycan (amides, carboxyl C, aliphatic C, aromatic C, olefinic C and αC of amino acids) were detected in mucus samples. Anecic and endogeic mucus had similar proportions of αC of amino acids, which can be predominantly related to the proteins, whereas the proportions of aromatic C and olefinic C were the highest in anecic mucus. Amides, carboxyl C and aliphatic C were similar for both mucus samples.

Additionally, we compared the NMR data of mucus samples with EPS samples from previous studies of the early (Liu et al., 2013) and late stationary phase (Fig. S1; Narvekar et al., 2017; Guhra et al., 2019). The microbial EPS of the early stationary phase exhibited relations of anomeric C and O-alkyl C in the same magnitude as anecic mucus, whereas the EPS of the late stationary phase showed approximately twofold higher values of the chemical shift regions of carbohydrates. Proportions of αC of amino acids were slightly higher in microbial EPS of both phases compared to the mucus samples (Table 1). One of the most distinct differences between mucus and EPS could be verified for aromatic C and olefinic C, where approximately only half of the proportion of the mucus was found in microbial EPS. The early stationary phase EPS showed highest values for the chemical shift regions of amides and carboxyl C, whereas the EPS of the late stage had the lowest amounts of carboxyl C and aliphatic C.

3.2. Completely mixed batch reactor experiments

3.2.1. Experimental conditions

The initial total and dissolved anecic and endogeic mucus-C concentrations in the mucus references were higher for anecic mucus than for endogeic mucus (Table 2). In comparison to TOC and DOC concentrations, P, Ca, S, Mg, Si, Mn, Fe and Al concentrations exhibited lower amounts (Table 2). The concentration of K was highest between the inorganic constituents in both samples.

The highest pH were detected in the anecic mucus containing

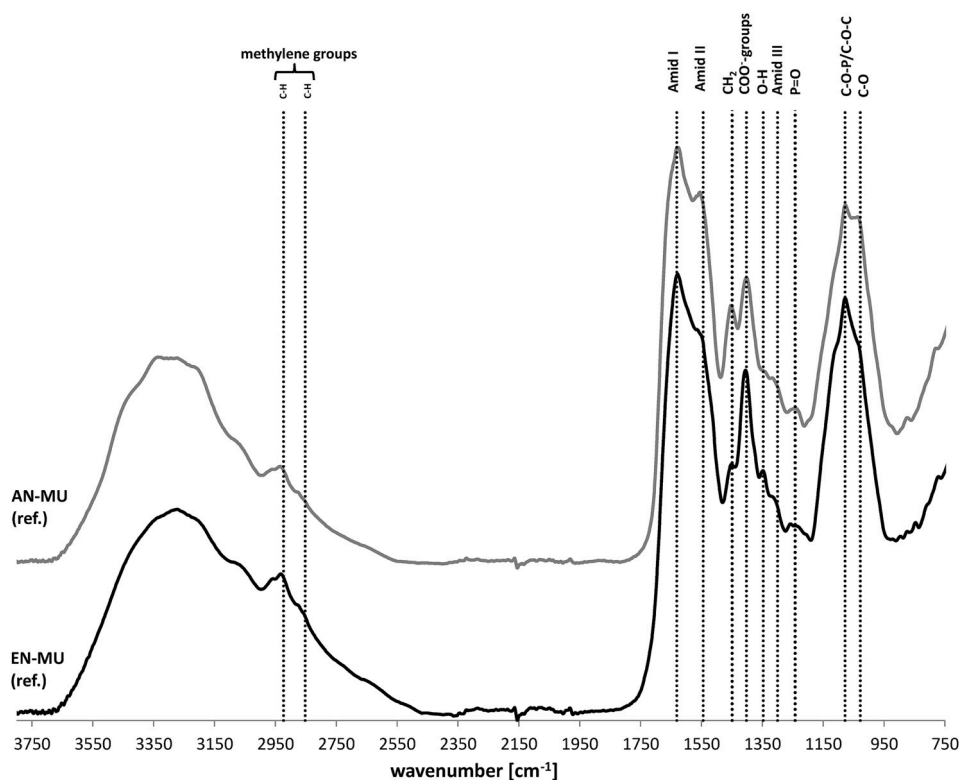


Fig. 1. Attenuated total reflection – Fourier-transform infrared spectroscopy (ATR-FTIR) spectra of the freeze-dried anecic *L. terrestris* (AN-MU) and endogeic *A. caliginosa* (EN-MU) mucus reference (ref.).

Table 1

Integral regions of solid-state¹³C-nuclear magnetic resonance spectroscopy (NMR) of the freeze-dried mucus of anecic *L. terrestris* and endogeic *A. caliginosa* after extraction. For comparison, additional spectra of microbial EPS were added from previous studies: *Bacillus subtilis* 168 (DSM 402) (EPS), late (Narvekar et al., 2017; Guhra et al., 2019) and early (Liu et al., 2013) stationary phase.

Sample	percent [%]					
	chemical shift [ppm]					
	200–160	160–110	110–90	90–65	65–45	45–0
amides, carboxyl C	aromatic, olefinic C	anomeric C	O-alkyl C	αC of amino acids	aliphatic C	
anecic mucus	16.6	18.4	5.6	11.7	18.4	29.3
endogeic mucus	17.6	12.1	5.6	17.0	19.4	28.2
EPS (early growth)	21.7	7.5	3.4	10.9	23.8	32.8
EPS (late growth)	7.4	4.4	12.0	43.7	21.2	11.3

Table 2

Total organic carbon (TOC) and dissolved organic carbon (DOC) as well as inductively coupled plasma optical emission spectrometry (ICP-OES) measured element concentrations of K, P, Ca, S, Mg, Si, Mn, Fe and Al in the dispersed anecic *L. terrestris* and endogeic *A. caliginosa* mucus references. Uncertainty is given as standard deviation (n = 3).

Sample	[mg/l]										
	TOC	DOC	K	P	Ca	S	Mg	Si	Mn	Fe	Al
anecic mucus	23.12 ± 0.05	16.5 ± 1.0	5.65 ± 0.04	1.065 ± 0.005	0.973 ± 0.007	0.83 ± 0.01	0.342 ± 0.003	0.06 ± 0.01	0.0231 ± 0.0001	0.0211 ± 0.0006	LOD
endogeic mucus	15.37 ± 0.07	14.8 ± 0.3	4.77 ± 0.06	0.653 ± 0.004	0.54 ± 0.04	0.551 ± 0.009	0.233 ± 0.009	0.125 ± 0.001	0.0080 ± 0.0001	0.053 ± 0.008	0.011 ± 0.001

treatments followed by endogeic mucus containing treatments and the bare minerals without mucus (Table 3). Between the different treatments (anecic mucus, endogeic mucus and mucus-free), goethite-containing batches showed the highest pH in comparison to illite and the mucus references.

3.2.2. Mucus adsorption

In goethite-containing treatments, 35 ± 1% of anecic mucus TOC and 35.8 ± 0.4% of endogeic mucus TOC were adsorbed (Fig. 2). For illite, an adsorption of 32 ± 2% and 21 ± 4% was detected. Phosphorus (P) vanished almost entirely (P concentration below the limit of detection of 0.03 mg/l) in anecic and endogeic mucus containing batches. We explain this by a rather complete adsorption of P to goethite. For illite, 5

Table 3

Physicochemical parameters, pH and electrical conductivity of mucus-free and -containing batches treated with goethite and illite as well as mineral-free references (ref.) of mucus of anecic *L. terrestris* and endogeic *A. caliginosa* after conducting the batch reactor experiments. Uncertainty is given as standard deviation (n = 3).

Type	Sample	pH	Electric conductivity [$\mu\text{S}/\text{cm}$]
anecic mucus	ref.	6.76 ± 0.05	183.0 ± 0.1
	goethite	7.10 ± 0.04	183.3 ± 0.2
	illite	6.77 ± 0.02	183.7 ± 0.1
endogeic mucus	ref.	5.69 ± 0.02	192.4 ± 0.4
	goethite	6.71 ± 0.08	192.5 ± 0.4
	illite	5.84 ± 0.09	194.9 ± 0.6
bare minerals	goethite	6.3 ± 0.1	125.9 ± 0.2
	illite	5.90 ± 0.06	127.2 ± 0.3

$\pm 1\%$ and $7.5 \pm 0.6\%$ adsorption of P was observed. In comparison to P, only $24 \pm 3\%$ and $47 \pm 2\%$ of sulfur (S) were adsorbed to goethite. The adsorption to illite was below 5% for anecic and endogeic mucus.

The P:C ratio of the anecic mucus reference was slightly higher than that of endogeic mucus. If mucus was treated with illite, the P:C ratio was higher in comparison to the mucus reference solution. However, the calculation of the P:C ratios of goethite treated samples was not possible due to P concentrations below the limit of detection (LOD). Consequently, low P:C ratios can be assumed and were estimated by the utilization of the LOD of 0.03 mg/l P as minimum value (Fig. 2). The S:C ratios of the mucus reference and illite were similarly to the respective P:C ratios (Fig. 2). For the goethite treated system, higher values were observed for anecic mucus and reduced values for endogeic mucus in comparison to the mineral-free mucus reference.

3.2.3. ATR-FTIR spectra of freeze-dried supernatants

For the freeze-dried references and supernatants after the batch experiments, three main constituents were attributed to the bands between 1750 and 900 cm^{-1} : proteins (1700–1500 cm^{-1}), carbohydrates (1460–1200 cm^{-1}) and polysaccharides as well as nucleic acids (1300–900 cm^{-1}) (Fig. 3; Socrates, 2001; Jiao et al., 2010). In comparison to goethite treated samples, for illite only small changes were observed in the protein area of anecic and endogeic mucus, which does not allow for a clear distinction of Amid I and Amid II bands. In the wavenumbers related to polysaccharides and carbohydrates, only slight changes could be established. For anecic and endogeic mucus containing batches treated with goethite, clear intensity decreases were noticeable in the protein and polysaccharide region in comparison to the carboxyl groups within the carbohydrate area. The bands assigned to proteins decreased in their intensity near (anecic mucus) or below (endogeic mucus) the level of the carboxylic groups. This observation was more pronounced for polysaccharides which are notably below the reference level for both treatments. In comparison to the anecic mucus, endogeic mucus treatments containing illite or goethite showed clear intensity decreases in the Amid I band of the protein area in relation to the Amid II band.

Dominant protein related FTIR bands were also found for EPS of the early stationary phase. The bands allocated to the asymmetric stretching vibration P=O (1242 cm^{-1}), P-O symmetric stretching (1031 cm^{-1}) and asymmetric stretching of C-O (1028 cm^{-1}) (Abdulla et al., 2010; Cao et al., 2011) were slightly more pronounced in early stationary phase EPS than mucus. The EPS sample from the late stationary phase revealed dominantly polysaccharide assigned bands in relation to those attributed to proteins and carbohydrates (Fig. S2).

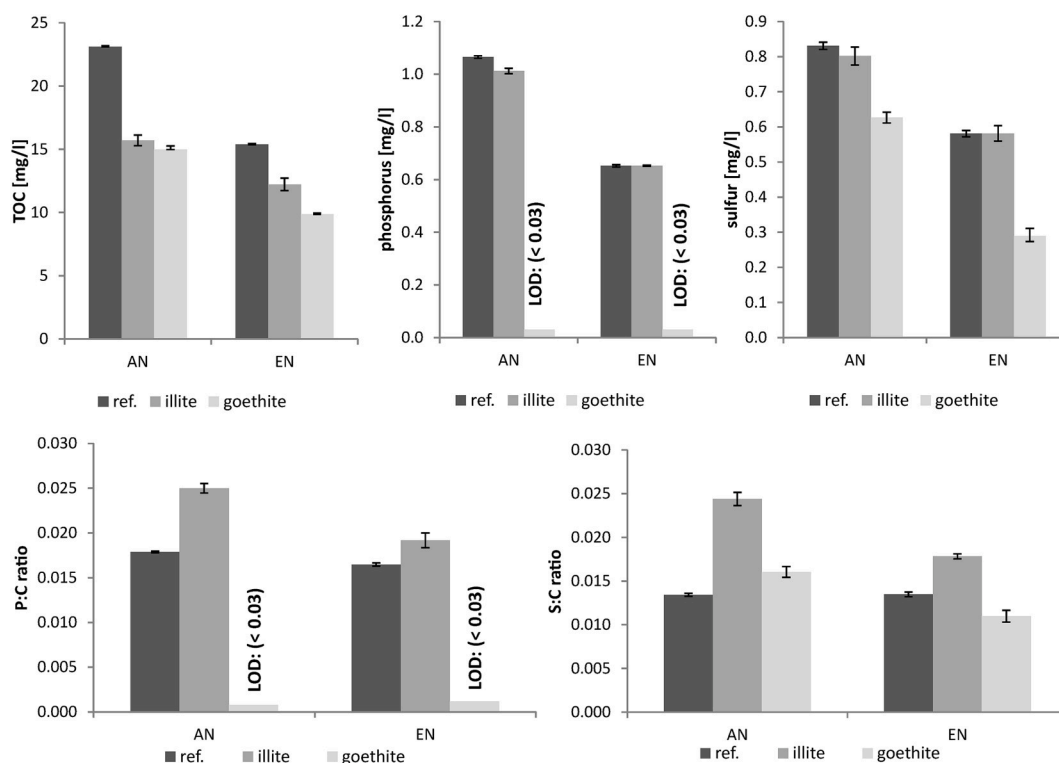


Fig. 2. Total organic carbon (TOC) and inductively coupled plasma optical emission spectrometry (ICP-OES)-measured P and S from the mineral phase separated supernatants after batch adsorption experiments of treatments containing anecic *L. terrestris* (AN-MU) and endogeic *A. caliginosa* (EN-MU) mucus. The reference (ref.) is assigned to the mineral-free solution which was processed in the same way then the other treatments. The P:C ratio and S:C ratio was calculated for mineral-free and -containing treatments of AN and EN-MU. For the calculation of the P:C ratio of the goethite-containing treatment the limit of detection (LOD) of the device was used for the estimation for P concentration.

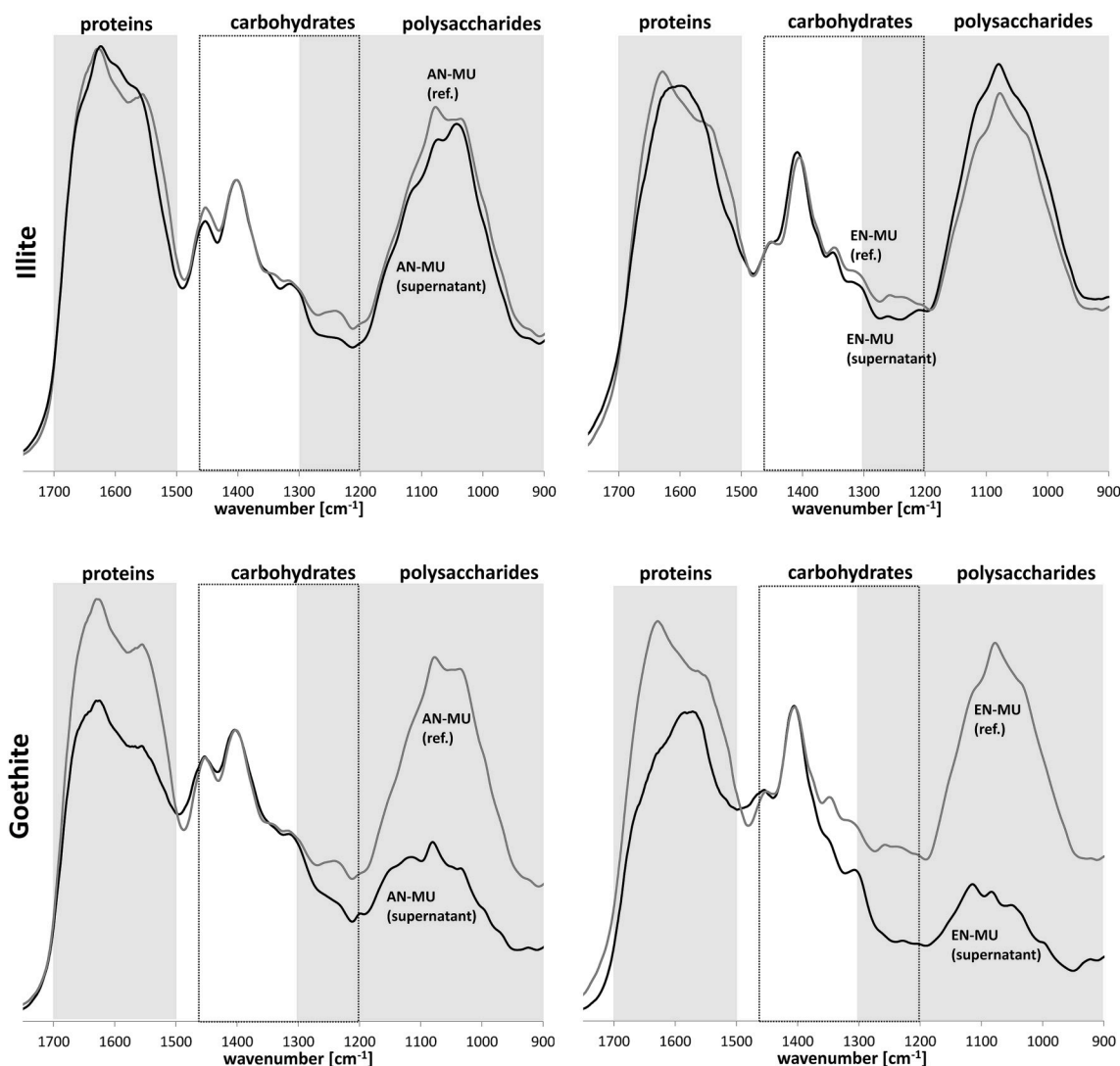


Fig. 3. Attenuated total reflection – Fourier-transform infrared spectroscopy (ATR-FTIR) spectra of the anecic *L. terrestris* (AN-MU: left) and endogeic *A. caliginosa* (EN-MU: right) mucus treated (black) with goethite (Gt: top) and illite (Il: bottom) as well as reference (ref.) without a mineral treatment (grey). For comparison of spectra, intensities were normalized to the carboxylic group (1402 cm^{-1}). Main composites were classified in proteins ($1700\text{--}1500\text{ cm}^{-1}$), carbohydrates ($1460\text{--}1200\text{ cm}^{-1}$) and polysaccharides as well as nucleic acids ($1300\text{--}900\text{ cm}^{-1}$) according to Jiao et al. (2010) and Socrates (2001).

3.3. Surface charge of organo-mineral associations

The surface charge of pure illite was net negative in comparison to the positively charged goethite at experimental pH (range between grey vertical dashed lines, Fig. 4). Zeta-potentials near pH of 2 were not measurable due to the limitations of the device. The mucus reference samples exhibited an IEP at 2.4 for anecic mucus and values below 2 for endogeic mucus. In comparison, bare goethite showed an ongoing decrease from $51 \pm 2\text{ mV}$ (pH: 3.5) to $-32 \pm 2\text{ mV}$ (pH: 10.5) with an IEP at 8.4, the IEP of endogeic mucus-goethite associations and anecic mucus-goethite associations decreased in a parallel manner with approximately 10 mV lower zeta potentials for the same pH and reached the IEP at 6.8. The lowest zeta potential was observed for illite. The zeta potentials of anecic mucus-illite associations and endogeic mucus-illite associations were above the values of bare illite and below mineral untreated anecic mucus and endogeic mucus. All samples containing illite showed an IEP below 2.

4. Discussion

4.1. Mucus composition

We show by NMR spectroscopy, that cutaneous mucus samples extracted from *L. terrestris* and *A. caliginosa* were composed of chemical moieties assigned to proteins, peptidoglycans, fatty acids and carbohydrates. The dominance of proteinaceous molecules in the mucus (Cortez and Bouché, 1987) of both ecological groups was ascertained, too, by ATR-FTIR spectra which exhibited distinct amid bands typical for proteins (Socrates, 2001). The application of spectroscopic methods showed a coexistence of different structural groups (e.g. amides, aliphatic C and carboxyl groups) in cutaneous mucus of *L. terrestris* and *A. caliginosa* which corroborates the suggestions of a rather complex composition reported by Richards (1978) for mucus of lumbricids based on wet chemical methods.

Despite the high similarity between anecic and endogeic mucus, we observed higher carbohydrate contents in endogeic mucus as highlighted by the peaks of methyl groups of polysaccharides between 62 and 55 ppm (Mao et al., 2007), while more aromatic and olefinic C as potential hydrophobic parts of proteins and nucleic acids were found in

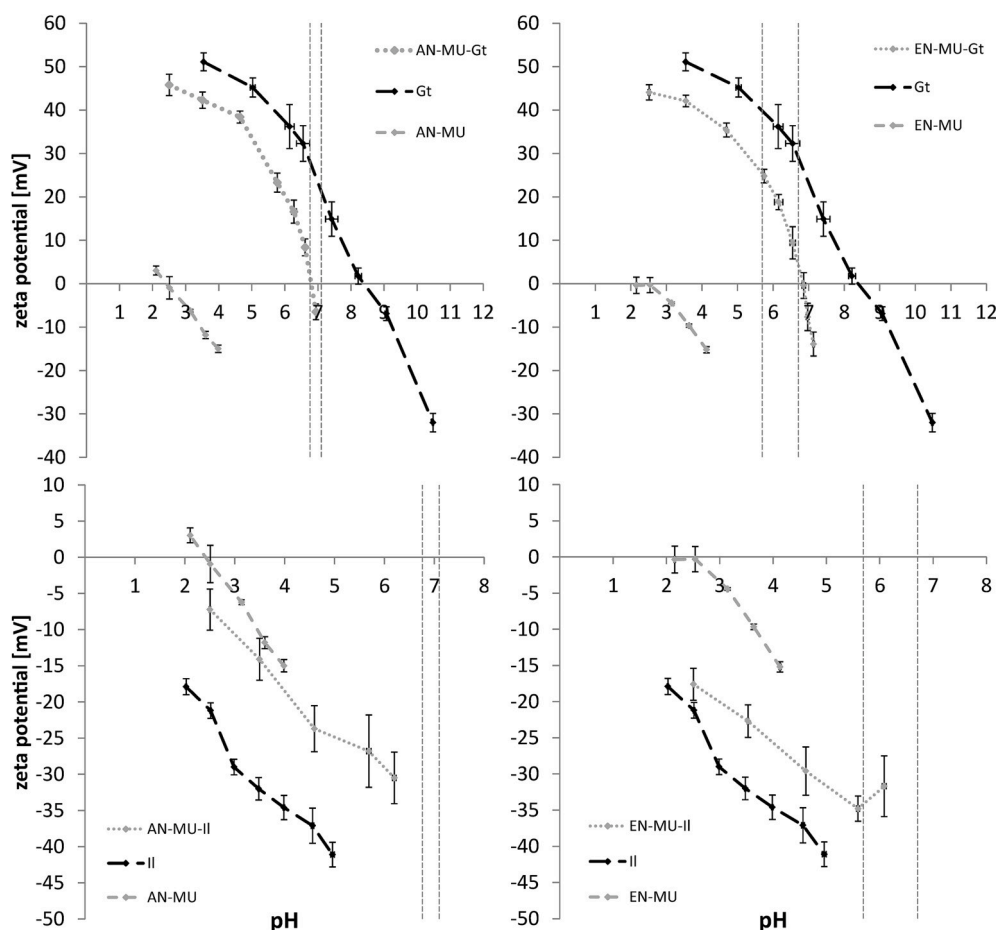


Fig. 4. Zeta potential measurements as function of pH for bare minerals (black dashed line), mucus (grey dashed line) and mucus-mineral association (grey pointed line) for anecic *L. terrestris* (AN-MU) mucus (left) and endogeic *A. caliginosa* (EN-MU) mucus (right) as well as for goethite (Gt) (top) and illite (II) (bottom). Areas between vertical grey dashed lines indicate pH during the experiments.

anecic mucus. Hydrophobic mucus components are reported as beneficial for stabilization of permanent vertical burrows which are characterized by higher water repellency in comparison to the bulk soil (Ellerbrock et al., 2009; Leue et al., 2015). Compositional differences in cutaneous mucus could originate from the adaption to different digging and feeding behavior of ecological groups. This was proposed already by Zhang et al. (2016) who connected amino acid composition to different rheological properties of cutaneous mucus.

We found that the ATR-FTIR and NMR spectra of cutaneous earthworm mucus were similar to those of microbial EPS of the common soil microorganism *Bacillus subtilis* obtained from the early stationary phase (Liu et al., 2013) in comparison to those of the late stationary phase (Narvekar et al., 2017; Guhra et al., 2019). Compared to earthworm mucus, however, both EPS types showed fewer structural groups assigned to proteins and nucleic acids between 160 and 110 ppm as revealed by NMR. Additionally, as shown by ATR-FTIR, the amide bands of EPS were shifted in comparison to those of mucus, which suggests different composition and structures of proteins (Socrates, 2001). The high number of structural groups assigned to amino acids (65–45 ppm) which are similar in both EPS types can be attributed besides proteins to glycosaminoglycans, typically produced by bacteria (Metzger et al., 2009). Based on our findings we substantiate the hypothesis of compositional similarities between mucus and microbial EPS of the early stationary phase. This implies a similar fate in soil, similar functionalities for soil microorganisms and interactions with the soil mineral phase. Consequently, the findings of microbial EPS and their environmental relevance might also apply for mucus. Yet, we need to take into account that both the amount and the composition of microbial EPS strongly

depends on various factors, e.g., microbial strain and growth phase, wetting/drying cycles, temperature, elemental composition of the environment and pH (More et al., 2014; Costa et al., 2018). In contrast, a much more uniform and stable composition is to be expected for the cutaneous earthworm mucus to preserve the function as lubricant (Lee, 1985). We thus suggest that earthworm mucus provides a compositionally less variable and - due to earthworm's active soil processing - a more readily available and widespread source of biotic OM in soil. This, in turn, may point to a more important role of earthworm mucus than microbial EPS in the formation of organo-mineral associations and even larger aggregated soil structures.

The investigation of freeze-dried anecic and endogeic mucus exhibited high contents of total N and C which lead in turn to low C:N ratios around 3.7 (anecic mucus) and 5.1 (endogeic mucus). These low C:N ratios of cutaneous mucus are in line with the results of Scheu (1991) and are reported as beneficial for microbial activity, increasing respiration and mineralization rates in the burrow system (Tiunov and Scheu, 1999; Brown et al., 2000; Don et al., 2008). Besides organic constituents, earthworm mucus contains a variety of electrolytes (Pan et al., 2010). Within the inorganic mucus constituents, K showed the highest concentrations. As outlined by Lemtiri et al. (2014), K can be released from clay minerals due to a pH decrease in the earthworm gut passage and thus serves as nutrients benefiting plant growth.

4.2. Mucus adsorption to secondary soil minerals

In relation to the FTIR bands attributed to proteins and polysaccharides, the band assigned to the carboxylic groups at 1402 cm^{-1}

(Abdulla et al., 2010) remained rather unchanged in the supernatants after adsorption. This coincides with the findings for EPS of Omoike and Chorover (2004): They did not find any evidence for the contribution of carboxyl groups to the adsorption of EPS to goethite. Furthermore, the decreased P:C-ratio of the goethite-treatment indicates a preferential adsorption of phosphorus-containing mucus constituents. We explain this by the specific adsorption via the formation of Fe–O–P bonds. These are likely formed due to the complexation between phosphoryl groups and Fe metal centers on the goethite surface as known for microbial EPS (Omoike and Chorover, 2006). Additionally, hydroxyl groups are released from the goethite surface during the formation of such complexes, which results in an increase of the pH in the solution (Omoike and Chorover, 2006). This is consistent with the increased pH in treatments containing goethite and mucus in comparison to the goethite-free or mucus-free treatments. Consequently, this affinity of phosphorus-containing mucus constituents to goethite points to the immobilization of P on the soil mineral phase (containing secondary iron oxides) in earthworm-formed aggregates and thus supports the suggestions of Scheu (1987) that P-immobilization in earthworm casts is mainly caused by abiotic processes.

In contrast, ATR-FTIR spectra of illite-containing samples showed only minor compositional change in response to the adsorption. Yet, we observed a clear increase of the P:C- and S:C-ratios for the illite treated anecic and endogeic mucus-samples compared to goethite treated samples and the untreated mucus reference. Hence, we conclude a reduced contribution of P- and S-containing mucus constituents in the adsorption to illite. A fractionation of phosphorus was also reported for microbial EPS, where a reduced adsorption of P to clay minerals in comparison to goethite occurred (Cao et al., 2011; Lin et al., 2016). The most conspicuous change of ATR-FTIR spectra of illite-containing mucus treatments in comparison to the mucus references was the absence of the separation of the bands corresponding to amid I and amid II of proteins (Socrates, 2001). Clay minerals provide variably charged silanol and aluminol groups on the edges and constantly negatively charged siloxane groups on the basal planes as adsorption sites (Hong et al., 2013; Yu et al., 2013). The basal planes contribute massively to the net charge of illite. Consequently, we observed negative charges for illite at ambient pH of the batch experiments where mucus was also negatively charged. Thus, we assign the adsorption between proteins and the basal planes of clay minerals to the impact of hydrophobic interactions. This may counterbalance the electrostatic repulsion between the equal charges (Yu et al., 2013). Additionally, a preferred adsorption of amino groups, as part of proteins, to variably charged clay mineral aluminol groups under the formation of hydrogen bonding and electrostatic interaction (Cao et al., 2011; Lin et al., 2016) could cause these changes in amid band distribution. Additionally, the high S:C ratio suggests that S-containing mucus constituents like the amino acids cysteine and methionine, which are common in the mucus of different earthworm species (Zhang et al., 2009, 2016), are unfavored in the adsorption processes to illite, in turn leading to the compositional fractionation of dispersed mucus.

Considering that earthworm-formed and physically stabilised organo-mineral associations inside casts and burrows may outlast their creating earthworms (Brown et al., 2000), the observed adsorption of molecules from mucus contributes to nutrient element storage and fertilisation due to earthworm activity (Lee and Foster, 1991). The selectivity of adsorption of cutaneous mucus of both earthworm species to goethite and in lesser extent to illite ascertains our proposition on similar mucus-mineral interactions of mucus obtained from different ecological functional groups. These mineral specific interactions are particularly relevant considering the mineral composition of the interacting soil material and potential release and translocation of unassociated mucus constituents inside earthworm burrows (Edwards et al., 1993; Simard et al., 2000).

4.3. The role of earthworm mucus for the formation of organo-minerals associations

Brown et al. (2000) outlined that earthworm-produced structures provide physicochemical and biologically changed properties in comparison to the initial soil materials. Schrader (1994) reported a buffering effect of cutaneous mucus on soil substrates to circumneutral pH, which we found for the anecic mucus extract (pH: ~6.8) in comparison to the endogeic mucus extract (pH: ~5.7). At this pH, the net charge of anecic and endogeic mucus (IEP: anecic mucus 2.4; endogeic mucus >2) was negative in comparison to goethite (IEP 8.4) which suggests additional adsorption via electrostatic attraction. The acidic IEP of mucus implies the contribution of carboxylic groups which are known to protonate at low pH in microbial EPS (Wang et al., 2012). Omoike and Chorover (2006) also showed an increased adsorption of microbial derived molecular mixtures if opposite charges are present. The amount of adsorbed OM to illite (IEP < 2) was like that of goethite, despite net negative charges of mucus and illite during the experimental conditions which force electrostatic repulsion. However, Illite-mucus associations are formed during electrostatically unfavourable conditions which points to mechanisms other than electrostatic interactions and specific adsorption which we expected for goethite. For instance, Kwon et al. (2006) demonstrated that hydrogen bonds provided by lipids are important for the adsorption to silanol groups of silica surfaces. Those lipids were also found in mucus as peak near to 30 ppm attributed to lipids as part of the NMR integral region for aliphatic C (Metzger et al., 2009). In addition, hydrophobic interactions with the constant negatively charged basal planes caused by long-chained aliphatic compounds such as lipids and high molecular weight OM like proteins (Yu et al., 2013; Kleber et al., 2015) could be an explanation for the, in comparison to endogeic mucus, higher adsorption of anecic mucus to illite due to its higher content of proteinaceous mucus constituents. This agrees with the findings on the preferred accumulation of proteins on clay mineral surfaces reported by Lin et al. (2016) for microbial EPS. The interaction between mucus and minerals is additionally affected by earthworm mucus provided electrolytes (Pan et al., 2010) such as K, polyvalent cations (Ca and Mg) as well as water-rich molecules which are known to bridge between organic and inorganic soil constituents and permit the formation of organo-mineral associations, i.e. via cation bridging (Shipitalo and Protz, 1989; Totsche et al., 2018). In summary, the physicochemical properties of mucus shape the reaction environment during the formation of earthworm-generated soil structures like casts and the innermost burrow walls of up to 1 cm soil volume (extended drilosphere) (Schrader, 1994; Andriuzzi et al., 2013; Lipiec et al., 2015).

Organo-mineral associations formed during our experiments revealed altered IEPs in comparison to the pure minerals, even after conducting a threefold washing step. This implies the formation of water stable organo-mineral associations. Hence, the formation of a surface coating and screening of the charge of the bare minerals due to mucus adsorption is suggested. Similarly, an alteration of the surface charge of goethite by the adsorption of microbial EPS of the early stationary phase (Fang et al., 2012) or humic substances (Assemi et al., 2004) led to a charge reversal under constant experimental conditions. However, EPS of the late stationary phase was shown to adsorb in a clearly lesser extend to illite and alter the surface charge of illite inconsiderably (Guhra et al., 2019). In this study, we provide evidence for the screening of the surface charge of illite due to the formation of mucus-mineral associations. This points additionally to the importance of a higher content of proteinaceous mucus constituents for the adsorption to clay minerals.

5. Conclusion

We found that earthworm cutaneous mucus was similar in its chemical composition, adsorption behavior and ability to form organo-mineral associations as microbial EPS secreted in the early stationary

phase. Yet, earthworm mucus had similar chemical composition in two ecological groups (anecic and endogeic), whereas microbial EPS composition varies according to the microbial growth stage and environmental conditions. This implies that the functions of earthworm mucus in soil could be consistent and predictable. Adsorption of earthworm cutaneous mucus depended on the type of soil mineral, which could determine how much and which fraction of mucus becomes associated with nascent organo-mineral associations. The unassociated mucus potentially remains in the soil liquid phase where it could be used for microbial metabolism or is transported in the soil pore system. Under field conditions, earthworm mucus will be deposited throughout the soil profile. For instance, the vertical digging of anecic earthworms forms biopores that are coated with mucus and operate as preferential flow-paths, while the horizontally burrowing endogeic species are known to form stable, OM-enriched (micro-)aggregates during their bioturbation process. The preferential adsorption of the organic carbon and organic phosphorus compounds in the mucus on soil minerals could enrich the newly-formed organo-mineral associations with biogenic nutrient elements. If this occurs in field environments, it suggests that earthworm mucus contributes to nutrient redistribution throughout the soil profile and implies a biogeochemical mechanism to retain the phosphorus secreted in earthworm mucus. Furthermore, the screened surface charge of mucus-mineral associations could explain the often discussed, but unsubstantiated claim that earthworm-processed soil has unique (micro-)aggregation and altered physicochemical properties when compared to bulk soil that was not in contact with earthworms. In conclusion, our work on the small-scale interactions among earthworm mucus and soil minerals provides an intriguing explanation for the beneficial effects of earthworms on pedogenesis and soil quality that were observed repeatedly in lab and field scale studies.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2020.107785>.

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