Bacteria-Mineral Colloid Interactions in Biofilms: An Ultrastructural and Microanalytical Approach

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Abstract

Simple reproducible experimental set-ups are described to study initial growth and interactions of bacteria with clay colloids and soil nanoparticles or with dissolved metal ions as primordial biofilms. These bacteria-nanoparticle constructs, exemplified by so-called clay hutches, are accessible to ultrastructural and microanalytical electron microscopical analysis. By this, the spatial arrangements and in part the physiological state of the involved autochthonous bacteria can be studied, leading to an estimate of the mineral-organic nutritional sphere the bacteria need for growth. It further leads to an entry to additional chemical, microbial and macromolecular traits of experimental follow-ups to analyse the mineral-organic chemistry, to isolate pollutant-adapted bacteria and to get information on the complex community structure of this kind of biofilms.

Keywords: Electron energy loss spectroscopy (EELS), 'In situ/in vitro' biofilms

1 Introduction

Clay minerals and other nanoparticles are known constituents of most soils and in the case of clay minerals encompass the smallest size fraction of clastic sediments, i.e. < 2 μm [1]. Because of their known high specific surface from 50 and more than 130 m^2/g [2, 3] and their high surface charge, they are broadly used for diverse sorption needs and applications in different industries. For instance, bentonite is widely used in purification of mineral oils, mineral fats and waxes or in water protection as sorbent of oil, floating on the water [4].

'Clay hutches' are an exemplifying term to describe a homogeneous arrangement of soil colloids and indigenous microorganisms attached to a hydrophobic surface of a suitable support (substratum). Originally, these associations of bacteria with soil colloids were grown from polychlorinated biphenyl (PCB)-contaminated soils as early biofilms [5, 6]. As such, they represent a closer, more detailed view to the general interaction and degradation of PCBs

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with soil constituents as this has been observed, for instance, in river sediments [7]. Ultrastructural analysis revealed the existence of particulate clusters of colloidal constituents associated with bacteria which were built up from a bacterial extracellular matrix, i.e. extracellular polymeric substances (EPS) and clay leaflets, occasionally accompanied by ironoxohydroxide colloids (Fig. 1). These studies showed that the experimental set-up is applicable to different analytical traits to analyse soil-microbe interaction with organic pollutants, i.e. polychlorinated biphenyls or polycyclic aromatic hydrocarbons (PAHs) [8].

In general, a sterile support with low surface energy (Permanox® slides, Melinex® stripes; see sketch of the construction in 'Notes') can be exposed to a natural environment, either submerged within the water column or placed on the sediment floor of a natural pond. Even when stuck into water-saturated soil, these supports should be good tools and opportunities to breed biofilms. Biofilms can be regarded as 'microbial landscapes' [9], grown autochthonously under natural conditions during a distinct period of time within a specific unique environmental milieu. Specific interactions such as metal cation adsorption by EPS can be monitored and studied as a trait of initial mineralogenesis.

The advantage of this simple experimental approach, i.e. a sterile, strict hydrophobic support either swimming on the water surface or submerged within the water of a pond, running water of a creek or buried into the soil, is based on its 'close-to-nature' character. It is the purpose of this methodological description of biofilm formation from the initial state to final confluent growth, thus to get high-resolution ultrastructural information of the intrinsic interactions between autochthonous bacteria with soil matrix colloids.

Hydrophobic surfaces, characterized by low surface energy, show short-term settlement and biofilm growth in contrast to high surface energies of hydrophilic glass slides. Nevertheless, floating, submerged or buried slides used as hydrophobic supports are to some extent selective for planktonic soil bacteria, which has to be considered.

The clarified bulk water of a before homogeneously and intensely mixed soil suspension in the experimental set-up contains the free porewater of soil, inhabited by planktonic, free-living bacteria and protozoa. It thus includes all autochthonous bacteria and further ingredients similarly needed for bacterial growth in soil. Thus, sterile floating supports are open for bacterial settlement and surface-associated growth. Since bacteria as well as clay phyllosilicates and other soil colloids are statistically distributed within the water body, multiples of swimming supports can be located on the water surface, suitable to get biofilms in parallel to characterize diverse state-of-the-art analytical traits [10]. As such, biofilms,

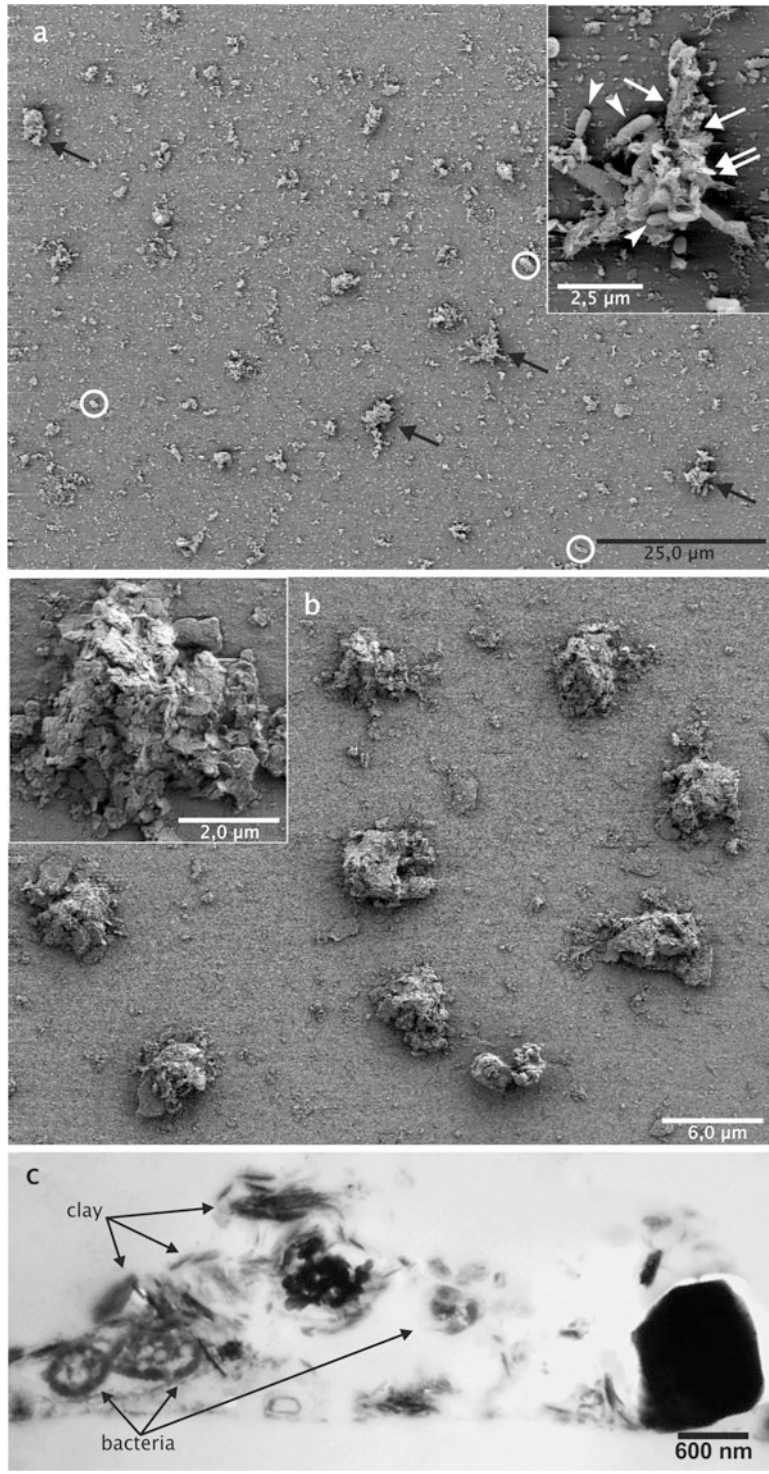

Fig. 1 Scanning micrographs of 'clay hutches'. (**a**) Initial state of 'clay hutch' formation; few bacteria are docked to the substratum (*circles*). 'Clay hutches' are indicated by *arrows*. A detailed view (*inset*) shows bacteria of different cell size (*white arrowheads*), which appear associated with a filigree EPS network (*white arrows*); only a few clay leaflets are visible (*double arrows*). (**b**) Compact 'clay hutches' after 14 days of exposition. No bacteria are visible in the periphery of the 'clay hutches'; here, a thin layer of particulate matter of different size covers the substratum background. *Inset* reveals the tight package of leaflets and compactness of a 'clay hutch'. (**c**) Corresponding ultrathin section, cut normally through a 'clay hutch', which shows the arrangement of bacteria and clay leaflets

highly similar and homogeneous in quality, can be analysed at the level of (a) their ultrastructure by light and electron microscopy; (b) elemental analysis by energy-dispersive X-ray (EDX) and/or electron energy loss spectroscopic analysis (EELS, ESI); (c) environmental nucleic acid analysis by SCCP, T-RFLP, DGGE and/or other macromolecular techniques [11–13]; and (d) finally isolation of bacteria, which are able to grow with hydrocarbons and other lipophilic substrates/pollutants.

In this chapter, I describe the making of 'in situ/in vitro' biofilm preparates for ultrastructure and microanalysis which describe microbial life in its close-to-natural context.

Though the experimental set-up for 'clay hutch' formation (and similarly the submerged hydrophobic supports in bulk water, e.g. of a pond, creek) is rather simple, it is not an easy task to proceed with the analysis of these microbe-soil colloid interactions on a micro- or even nanoscale. It would thus be of interest to study and understand the basics of communications, possibly by 'quorum sensing' of many bacteria or crosstalk at a drastic smaller level of only few, i.e. three to five, bacteria, within a settlement focus when attached to the hydrophobic substratum.

The question of how bacteria sense the specificity and quality of cargo from soil colloids in the water body as relevant for nutrition is of general interest in understanding bacterial life in context of the soil matrix. There is enough substantial reason for speculation but with the aid of optical tweezers or micromanipulators and suitable handling of individual 'clay hutches' with microcapillaries and/or bacterial consortia, this could be a further goal to study bacterial interactions, supplementing the primary view of light and electron microscopy.

2 Materials

2.1 General Material

250 ml Erlenmeyer flasks or glass Petri dishes of 5 cm height and of 10–12 cm diameter (www.neolab.de)

500 ml sterile glass beaker (www.neolab.de)

Sterile Teflon stirring rod (www.neolab.de)

Magnetic stirrer (www.neolab.de)

2 × 2 mm sieve for soil sieving (www.neolab.de)

Spoon or spatula (www.neolab.de)

Melinex® foil, sterile Permanox® slides (26 × 76 mm; www.thermofisher.com.au)

For submerged exposure of slides:

Suitable inert plastic ware (e.g. polypropylene; www.neolab.de)

Sterile 6.0–7.0 cm in diameter and 5.0 cm in height rubber plugs, used for clamping/fixing Permanox® slides for submerged substratum exposition (www.neolab.de)

Gelatine capsules, flat embedding moulds, forceps (for grid handling), Ni and/or Cu electron microscopic grids, 300 and 700 mesh size, thin bar (http://scienceservices.eu)

2.2 Reagents

Fixation buffer (1% (v/v) glutaraldehyde – 10 mM Hepes, pH 7.0)

Acidic, cationic ThO_2 colloid (0.04% (w/v) ThO_2 – 100 mM Na acetate, pH 3.0 (*see* **Note 1**))

Acidic washing buffer (100 mM Na acetate, pH 3.0)

Washing buffer 1 (20 mM Hepes, pH 7.0)

Washing buffer 2 (100 mM Na cacodylate, pH 7.2)

5% (w/v) aqueous osmium tetroxide

Dried acetone (over $CuSO_4$)

All chemicals are p.a. grade from Merck, Darmstadt, Germany (www.merck.de)

Spurr-epoxy resin (for embedment) (http://scienceservices.eu)

2.3 Microscopes (see Note 2)

Inverse light microscope

Scanning electron microscope (= SEM) (electron microscopic laboratory)

In-column energy filter transmission electron microscope (= EF−TEM), post-column filter with general TEM (electron microscopic laboratory) (*see* **Note 3**)

3 Methods

3.1 Soil Sample Preparation

Site material of interest should be rather fresh. It should be sufficiently dried and rough sieving at 2 mm mesh size is appropriate to get rid of plant materials and bigger sand granules. In order to get homogenized and mixed soil, samples should be additionally passed through a sieve of 0.5–1.0 mm mesh size. As such, an aliquot should be frozen and stored at −80°C for additional stock for total DNA extraction, needed for microbial community analysis. Doing so, the fraction of microorganisms, capable in soil-colloid hydrocarbon interactions and biofilm production, can be related to the total soilborne microbial community. Details of the soil type and soil horizon of the sampling site should be addressed. Treatment of the soil sample as is described below will lead to homogeneous starting conditions of statistical relevance, necessary for scaling up and/or multiplicity.

3.1.1 Preparation of the Soil Slurry

Four to six aliquots per type of soil (i.e. about 20 g per Erlenmeyer flask or glass Petri dish, suspended within 10–20 ml of sterile reverse osmosis purified water) are homogeneously mixed with the aid of a magnetic stirring bar at 50–150 rpm for 15–30 min at ambient temperature. One such aliquot of suspended soil should be sterilized by three to four heating-cooling cycles in an autoclave. This sterile soil sample is used to check for abiotic, physico-chemical interactions of the soil colloids with the substratum (when doing submerged experiment in resting or floating natural waters, this control cannot be done). The 'soil assays' are used as doubles or triplicates in Erlenmeyer flasks or adequate glass Petri dishes.

3.1.2 Handling of Permanox® Slides and Substratum-Grown Biofilms

Start the experiment by gentle floating sterile Permanox® slides on top of the water surface. This should only be done when the turbid bulk water has clarified after a resting period of 24 h at ambient temperature. In general, it is useful to only put in one slide per Erlenmeyer flask for a one-step experiment but – depending on the diameter of the glass Petri dishes – two or more may be layered on the water surface. This will be useful if samples have to be taken at different states of biofilm development. Slides have to be placed within an area of clean water surface, free from floating fine residual root or plant debris from the soil matrix which has passed soil sample sieving. If necessary, suck them off with a sterile vacuum pipette under a clean bench.

As such, biofilm growth of autochthonous, planktonic bacteria (and fungi) can start and a corresponding microbial community will be established within 7–14 days at ambient temperature in the laboratory without direct artificial or sunlight illumination. Development of 'clay hutch' biofilms can be roughly judged by inspection with the naked eye and is visible (under oblique illumination) as a faint turbid layer on the floating substratum surface. (Caution before picking up the biofilm-grown slide with a sterile forceps: if meanwhile neustonic biofilm has developed at the water-air interface in the vicinity of the floating slides or further plant-/root-derived debris or other aggregates, recognizable by eye, have accumulated, these have to be carefully sucked off first with a vacuum pipette in order not to contaminate the sample by partially flipping over and superimpose to the substratum-bound biofilms.) It is not recommended to intermittently take out the slides for light microscopical examination of unsterile handling and surface pressure impact or drying of the biofilm surface.

Sufficient biofilm substrata used for chemical, especially for microbial and community analysis, are gently picked up from the water surface with the aid of a suitable sterile forceps. Adhesive bulk water is shortly drained off from the short edge of the Permanox® slide with filter paper before they are frozen in liquid nitrogen for storage at −20°C until use for non-ultrastructural analysis

(those biofilms used for ultrastructural/elemental analysis never should be frozen and stored because this will lead to severe damage to ultrastructural details.)

3.2 Sample Handling and Preparation for Ultrastructural Analysis

3.2.1 Precheck of Biofilms

For ultrastructural analysis by either transmission (TEM) or scanning electron microscopy (SEM) (*see* Fig. 1), a floating Permanox® slide is picked up with a sterile forceps from the water surface, drained softly over the short edge in contact with filter paper and is roughly cleaned on its 'ungrown' backside with soft cleaning household paper to get rid of dust and other contaminants. As such, the slide is mounted on an inverse light microscope stage with its 'biofilm side' up and a few drops of clarified bulk water are added to prevent the biofilm to fall dry. Quality and dimensions of biofilm growth, i.e. development and density of 'clay hutches', can be observed with x20 to x40 (x63) objective lens at phase contrast-imaging conditions. Thus, the actual state can be observed and documented with the aid of a CCD camera. This light microscopic analysis will accurately show the unique growth and distribution of 'clay hutches' and/or the degree of biofilm heterogeneity or cluster formation by inhomogeneous growth. Further, a rough estimate will be given at x400 magnification on the amount and frequency of individual bacteria, not associated with soil aggregates.

3.2.2 Fixation, Dehydration, Embedment and Sectioning of Biofilms

Next, aliquots of the biofilm-grown substratum are cut as stripes, 1 × 2.6 cm in size, for electron microscopic analysis and are immediately transferred to a suitable Petri dish, partially filled with 20–25 ml fixation buffer, and let them float with biofilm side down. Glutaraldehyde fixation is performed at least for 20 min at ambient temperature and samples are stored and kept floating at 4°C until further processing up to 1 week.

For general embedding and ultrathin sectioning, subdivide the fixed biofilm in equal parts and process one half (as it is described in detail by [14]). For ultrastructural analysis, postfixation with 1% (w/v) OsO_4 – 100 mM cacodylate, pH 7.4 – is done for 30–60 min at ambient temperature after the sample has been washed for 10 min at ambient temperature in washing buffer 2. (Postfixation with osmium tetroxide is omitted if elemental analysis is done. Then, samples are washed twice in washing buffer 1 for 10 min at ambient temperature after glutaraldehyde fixation.) They are then dehydrated in an aqueous acetone series at ambient temperature. For this, submerge the Permanox® cutoffs with the biofilm side up in aqueous acetone in a glass dish (10%/30 min; 30%/10 min; 50%/10 min; 70%/10 min), stain with 1% (w/v) uranyl acetate in 70% acetone for 20 min at ambient temperature (this staining step is omitted when elemental analysis is done) and complete dehydration (100%/2 × 10 min). Infiltrate in an acetone-epoxy resin mixture (2 parts acetone + 1 part resin/30 min; 1 part acetone + 2 parts resin/60 min), followed by pure resin (2 × 30 min;

12 h/overnight). After transfer of samples to gelatine capsules or flat embedding moulds, prefilled with resin monomer, samples are degassed for some time with a rotary pump linked to a suitable glass exsiccator at residual pressure so air bubbles can come up smoothly from the resin, looking like foam, and care is taken by pressure handling not to make the resin overrun the gelatine capsule. The glass exsiccator is gently aerated and samples are polymerized at 70°C for 16 h in a laboratory oven/incubator.

General information of fixation of biological samples and resin embedment can be found in [14–17].

For conventional TEM, ultrathin sections (70–90 nm, also recognized as silver to golden shining sections) are cut with a diamond knife (*see* **Note 4**) with an Ultracut E® ultramicrotome (Leica, Austria). Sections, picked up with 300 mesh Cu-hexagonal Formvar-coated grids, are post-stained with aqueous uranyl acetate (4% (w/v); 5 min at ambient temperature) and lead citrate (0.3% (w/v): 5 min at ambient temperature) [18] and are analysed with an EF-TEM (CEM 902 or Libra 120; Zeiss, Oberkochen, Germany).

3.2.3 Sample Preparation for SEM

For SEM analysis process, use the second half of the biofilm sample, as is described in detail by [14].

In short, biofilm-grown Permanox® stripes, dehydrated in acetone, are transferred to a pressure chamber of a critical point drying unit (CPD030; Bal-Tec, Liechtenstein) filled with acetone at 10°C. After three to four washes with liquid CO_2 for each 10 min of equilibration time, raise temperature to 40°C and pressure to finally 80 bars. Within a period of 30 min, reduce pressure at constant temperature (40°C) to normal atmospheric. Mount the dried biofilms on aluminium stubs. In a sputter-coat unit (SCU040; Balzer Union, Liechtenstein), they then are coated with gold in an argon atmosphere (0.06 mbar; target distance, 10 cm; sputter current, 45 mA) for 54 s.

3.3 Submerged Exposition of Substratum in the Water Column

Similar to soil-derived biofilm growth on floating Permanox® slides on top of a water column which experimentally is defined by soil constituents, biofilm development and growth from bulk water, either from resting water of a pond or streaming water from a creek, are of general interest to study interactions of autochthonous microorganisms with solved or nanoparticulate minerals. Biofilm growth on submerged substrata with low surface energy can be studied over time, and thus, initial states of mineralogenesis, catalysed or initialized by bacterial impact, can be observed. These experiments to some extent simulate development of biofilms as these will grow on many submerged solid surfaces, such as inorganic stones or organic plant material, such as wood or leaves.

To do such experiments, submerged sterile Permanox® slides, fixed to 5.0 cm high rubber plugs (6.0–7.0 cm in diameter;

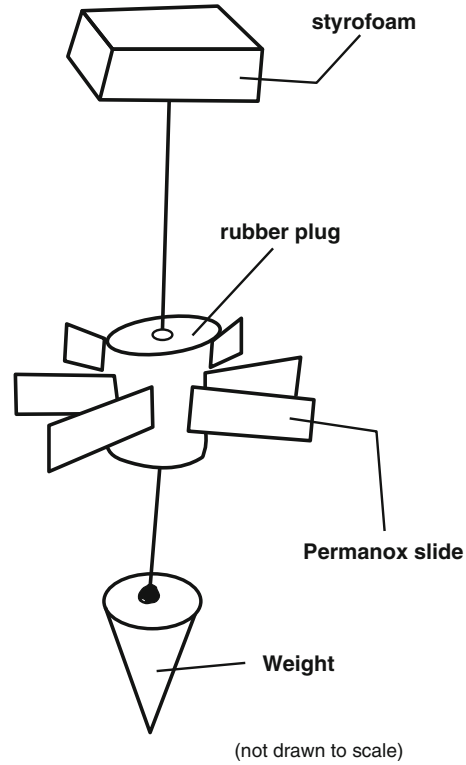

Fig. 2 Construction of rubber plug-based slide holdfast for submerged biofilm acquisition (Sketch). In order to position and fasten the slides, about 10 mm deep cuts are set into the rubber plug to a length of 30 mm by a short scalpellum

see Fig. 2), are exposed to different heights under the water surface in a resting pond or lake or laid down on the floor of a creek. Attention should be paid to keep these constructions in a stable position, not to get lost during the exposure period. Multiple slides should be inserted by their short edges to a rubber plug at sufficient distance, i.e. 10–15 mm, in order to prevent 'functional shading'. This way, a series of time points can be set and substrata with adhering biofilms can be sampled sequentially and fixed as is described for the 'clay hutch' set-up above.

3.4 Labelling of Acidic Groups in Biomatrices by Cationic ThO_2 Nanoparticles

3.4.1 Synthesis of Hydrous Cationic Thorium Dioxide Nanocolloids

According to [19], take 10 g of thorium nitrate hydrate (MM 480.06; Fluka, Switzerland) and dissolve in 50 ml water (distilled or reverse-osmophorese water) at ambient temperature to get a 20% (w/v) solution of pH 2.4 in a flask.

To 20 ml of this solution in a 250 ml round-bottomed flask, 0.4 ml aliquots of 25% (w/v) ammonium hydroxide are added dropwise under continuous stirring until pH 3.0 is reached and the solution turns slightly turbid.

Add more 0.4 ml NH_4OH so at pH 4.0, the solution turns intense turbid; further 0.4 ml NH_4OH are added to completely precipitate thorium hydroxide at pH 11.0.

In a flat funnel laid with filter paper (grade 3 hw; Sartorius, Göttingen, Germany), filter the milky suspension under slight vacuum.

Residual electrolytes are removed completely by washing with 100–150 ml distilled/reverse-osmophorese water until no ammonia is smelled.

Transfer thorium hydroxide paste with the aid of a spatula into a 100 ml Erlenmeyer flask and with 5–8 ml wash and add residual precipitate to the bulk.

Stir the slurry hydroxide and bring it to boil under reflux for 5 min (with the aid of a 40 cm Dimroth condenser).

Then, add 0.2 ml of 20% (w/v) thorium nitrate solution and continue to boil under reflux. Repeat this reflux boiling every time 0.2 ml thorium nitrate solution has been added.

Turbidity clarifies when two times 0.2 ml thorium nitrate has been added.

A final addition of 0.2 ml thorium nitrate did not increase turbidity and this is the last step in colloid peptization (1.2–1.6 ml of thorium nitrate on the whole will be sufficient), leaving the solution slightly opalescent at roughly 50% (w/v) colloidal ThO_2 solution at pH 2.0–2.5.

3.4.2 Acidic Group Labelling with ThO_2 Nanocolloids

For ultrastructural analysis of acidic extracellular polymeric substances (EPS), float a suitable cut-off segment of the Permanox® slide face down on acidic washing buffer in a small Petri dish and wash twice for 10 min at ambient temperature. Transfer to 0.04% (w/v) cationic colloidal thorium dioxide and let float and incubate for 30–60 min at ambient temperature or at 4°C overnight, to stain acidic EPS residues (for detail, *see* [19]). Next, float-wash the biofilm face down or submerge twice on 10 mM Na acetate, pH 3.0, as is described above. Start sample dehydration in an acetone-water series, according to [14].

3.5 Electron Energy Loss Spectroscopy

The presence of inorganic soil nanoaggregates as sorbents of organic substances and/or the formation of 'clay hutches' as active on growth on a low surface energy substratum gives the opportunity to study the interplay of soilborne indigenous bacteria with soil-derived colloids. These interactions, though on a static level in the fixed and embedded state, can be analysed by electron energy loss spectroscopy (EELS), applied in the (1) EELS mode to acquire spectra of an area/structure of interest or (2) in the electron spectroscopic imaging mode (ESI), which leads to elemental maps with high spatial resolution of key elements, such as Si, O, Al, Fe, etc. EELS is a useful means to get a nanometre-scaled view to individual bacteria-clay/nanoparticle associates (*see* Fig. 3).

30 to 40 nm ultrathin sections of unstained embedded biofilms are picked up with 700 mesh bare grids and are observed natively without post-staining. Either 'zero-loss elastic bright-field' images or 'inelastic images' at a corresponding electron energy loss of the element of interest are obtained by EF-TEM [20]. The specific settings for ESI recording in order to reveal high resolution of these spatial arrangements are described by [14, 19, 20]. Tracing cationic thorium dioxide colloids by ESI here outlines the distribution and local densities of negative charges of clay, EPS and the cell surfaces, directly observed at and linked to the macromolecular level.

Besides element mapping with ESI, electron energy loss spectra (EELS) can be acquired from dedicated areas of interest. This reveals insight into the chemistry and spatial coordination of the element of interest and shows EELS features next to the ionization edge (energy loss near edge structures; ELNES) as a fingerprint of the local chemistry, which can be used for comparison and differentiation of different 'clay hutches' (*see* **Note 3**).

3.5.1 Practice of EELS Acquisition with an 'In-Column' EF-TEM

As a rough guide to EF-TEM practice, the following steps should be addressed. In order to get suitable EEL spectra and elemental maps (electron spectroscopic imaging; ESI), it is a prerequisite to have the electron microscope perfectly adjusted according to the manual's instructions.

First, an unstained 30–40 nm ultrathin section, picked up by a 400 or 700 mesh 'thin bar' grid, is introduced into the EF-TEM. At low magnification (e.g. x3,000 to x5,000) and low beam intensities (beam current, 1–2 μA; illumination aperture, 80–200 mrad) to minimize beam damage, the sample is examined for 'biofilm features of interest' suitable. Generally, an electron dense motif (in the 'image mode') is centred and Gaussian-focused on the screen with the objective aperture, e.g. 60 μm in size, set precisely before (in the 'diffraction mode'). The image has been checked to be free/corrected from astigmatism. In the 'image mode', the 'spectrometer entrance aperture', which fits best to the motif's dimensions, is selected and centred to the 'index point' on the screen (this in general is the central small hole in small viewing screen of the EF-TEM). Then, the 'spectrum mode' is selected and the 'energy-selecting slit aperture' is removed by anticlockwise turns to the stop. The EEL spectrum now should be visible and is set to a suitable 'spectrum magnification', e.g. x100. It then has to be centred with its highest intensity edge, i.e. the 'zero-loss peak', to the 'index point' by the aid of x-y (spectrum shifting) knobs. Now, the CCD camera is started by the integrated application software for EEL spectrum registration. (See the 'EELS registration software manual', e.g. iTEM software

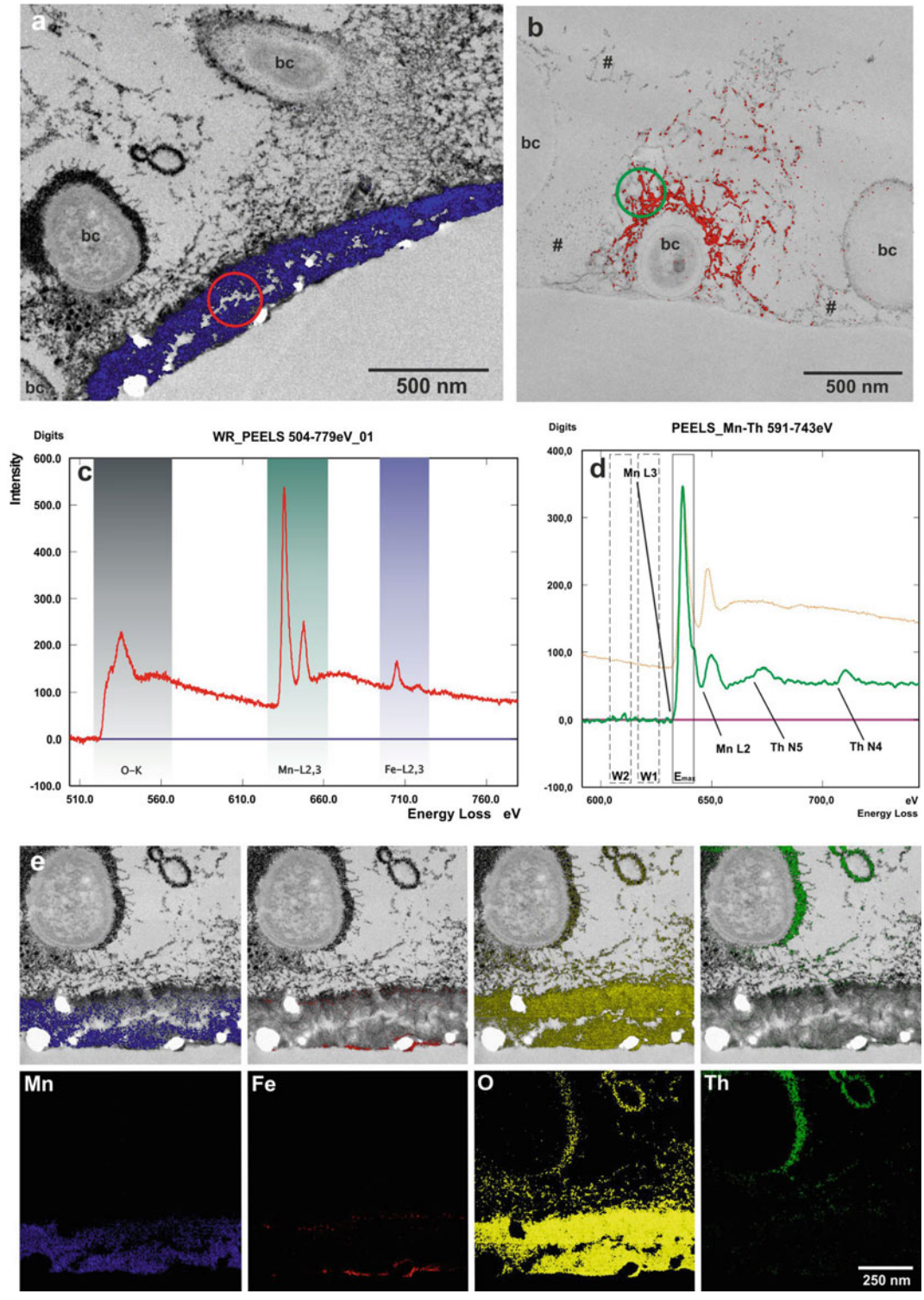

Fig. 3 Electron energy loss spectroscopic analysis of mineralogenesis in autochthonous biofilm. (**a**) Survey view of a biofilm, showing three bacterial cells (*bc*) in context with the heterogeneous EPS matrix, intensely

with the Zeiss Libra120Plus, used in our lab, for exact details and description to start this process. Further, suitable camera calibrations and settings have been done accordingly – see 'camera instruction manual' and 'EELS registration software'.) In the 'Wide Range PEELS' mode from about 2,500–0 eV spectrum, registration is done to get a rough overview of elements present within this range of electron energy loss (it is highly recommended to follow the instructions of the 'EELS registration software manual', since doing full-width spectrum registration takes some few minutes, i.e. 20–45 min [burning all the time with relatively high beam intensities on your sample motif]). If you look for and know the presence of 'indicator elements', characteristic for your sample, i.e. Fe, Mn, Al, etc., you can do short-time registration on the characteristic ionization energy range of your 'indicator element', the energy settings of which can be taken from the online 'EELS atlas'. After EELS registration, it is mandatory to switch back to the 'image mode' and check whether the motif has moved out of the spectrometer aperture partially or in total or not at all to be sure that your spectrum is valid. If not, you have to repeat the procedure with WR-PEELS registrations set at suitably smaller energy intervals, i.e. in the 'high energy loss' within 200–400 eV, 500–800 eV in the 'medium energy loss' and 600–1,200 eV from the 'low energy loss'.

A well-registered spectrum will (1) give you the exact elemental presence in your sample/motif and (2) show you the precise ionization energies and ELNES features (for instance, the suitable energy width of a maximum intensity peak, i.e. Mn-L3, Fe-L3, O-K edges; *see* Fig. 3d), which are mandatory for ESI parameters, set for high-resolution element mapping.

Element mapping or ESI can then be done in detail on your motif, used either at the same scale as during WR-PEELS

Fig. 3 (Continued) stained with ThO_2 nanocolloids. The *red encirled area* indicates the measuring area, used to WR-PEELS analysis and the corresponding spectrum shown in (**c**). The *deep blue* overlay at the contact interface of the biofilm with the Permanox substratum represents manganese distribution, as is additionally shown in part as the Mn elemental map in (**e**). (**b**) Further motif of Mn deposition within the EPS of an individual bacterial cell (*bc*), coloured in *red*. The *green circle* indicates the measuring and position of a PEEL spectrum, shown in (**d**). (**c**) Wide Range Parallel Electron Energy Loss Spectrum (WR-PEELS) of the encircled motif in (**a**). *Coloured rectangular areas* indicate the ionization edges and the corresponding ELNES features of oxygen, manganese and iron as shown in (**e**). (**d**) PEEL spectrum of the *green circled area* in (**b**). The *dashed line spectrum* shows the Mn-L2,3 reference. Spectra in (**c**) and (**d**) all have been background-subtracted according to the power law method. *Rectangular boxes* (E_{max}, W_1 and W_2) represent the energy slit width, set to 10 eV, and the positions along the energy axis (energy loss, eV), used to calculate the net elemental map of Mn-L3, according to the 'Three-Window Method' (after subtraction of background images (W_1, W_2) from the maximum intensity image (E_{max}) according to 'power law'). See Mn elemental map, colour-coded in *red* in (**b**) accordingly. (**e**) Gallery of unique biofilm motif, overlaid with the elemental maps (*first row*), as they are shown as colour-coded intensity signal maps (*second row*)

measurements or at lower magnification thus to encompass the motif's vicinity. Precise settings of the maximum intensities of the ionization edges of interest on the energy axis and the suitable energy width of the slit aperture should be set according to 'EELS acquisition software' manual. Before starting the ESI acquisition, the motif is set with appropriate magnification in the elastic bright-field mode; to get it precisely set to Gaussian focus, the low-loss range of 60–80 eV with suitable image intensities is chosen and ultrastructural features (partially in inverted contrast) are used for focusing, which are optimally Gaussian when recognized as sharp, detailed structures. With this focus fixed, the elastic bright field (= zero-loss image) is used for precisely positioning a 'detail of interest' close to the centre of the CCD detector (which is always readdressed for further element settings if further elements are constituents of the motif). Choosing the suitable ionization energy of the element's ionization edge to be mapped after starting the ESI application, follow/adjust parameter settings according to the 'EELS acquisition software' before the start of image registration. Acquisition of the ESI image stack (four images on the hole, one optimally set as elastic 'zero-loss' image at the end of registration) can be done within a few seconds (in the high-intensity 'low-loss' region) or can take up to 5–30 or more minutes at 'medium-electron energy loss' (finally, the strength of the sample/section will dictate how long and how many data sets can be acquired; but it is possible to collect data sets of different elements from one single motif up to more than 120 min; here, it is mandatory to check each ESI image stack on image drift; this is to decide whether the data set finally leads to a high-quality element map or whether ESI registration has to be repeated with different setting for that very element). The manual has to be followed in computational working out the 'background-corrected' element maps, either according to the 'Three-Window Method' with suitable mathematical models for background subtraction, i.e. 3 window power law, 3 window exponential law, etc., or with the 'Two-Window Method' as 'ratio imaging' (here, it is obligatory to learn more/get familiar with EELS theory, which is fundamentally given in [21]).

4 Notes

1. Cationic ThO_2 nanocolloids can be synthesized in a normal laboratory. A prerequisite however is the official permission from your local/institutional authorities to work with this radioactive compound. Synthesis of nanocolloids (from thorium nitrate × 5 H_2O; http://www.merck.de) is described in short under Sect. 3.4 and in detail by [19], and no special radiation protection is needed since Th is an alpha emitter and is non-toxic but has to be handled in a professional

manner, according to 'good chemical practice'. There can be a problem to get thorium nitrate hydrate as the starting compound because of its radioactivity.

2. Microscopical inspection, documentation and analysis at either low or high resolution should be done in an appropriate laboratory. Here, you can be trained in sample preparation if you are a novice.

   Further suitable equipment and expertise can be found in electron microscopic units, which should be equipped with sample preparation hardware such as a critical point drying apparatus and a sputter-coat unit for SEM analysis. An SEM equipped with FEG beam source is quite opportune to get images at high resolution (in our laboratory, we use a Zeiss Merlin for these purposes).

   Transmission electron microscopy (TEM) of ultrathin sections (70 nm thickness, bright silver interference colour) of embedded samples is a prerequisite to see and analyse interactions of bacteria with clay and other soil colloids. Sections should be post-stained with uranyl acetate for optimum contrast, thus making it possible to explore and understand the microbial motive. If this is understood and documented by medium (about x4,000 to x7,000) and high magnification (about x12,000 to x30,000), ultrathin sections 30–40 nm are cut with the same motive for elemental analysis by EELS. Either a post-column filter (Gatan system) or an in-column filter (Zeiss, JEOL) is suitable for electron energy loss spectroscopy. (EELS data presented in this article have been acquired with (in-column) energy filter transmission electron microscopes (EF-TEM) Zeiss CEM902 and Libra 120Plus (Zeiss, Oberkochen, Germany).)

   Further, you should be well trained and know how to adjust the optical instruments and how to work with them or you should get well trained for optical operations/observations or you should cooperate with the staff of a light or electron microscopical laboratory. Especially, electron spectroscopic analysis with an EF-TEM is not basic routine electron microscopy.

3. Theory of EELS is a sophisticated item and profound knowledge of electron scattering and ionization energies associated with energy levels of the outer valence electrons of the atom of interest should be understood in order to do right interpretation of spectrum features. It should be mentioned that X-ray absorption spectroscopy (XAS) and data obtained with this analytical methodology are highly similar and of good help as an alternative to EELS. A suitable entrance to the field of EELS is given by [21].

4. Though a diamond knife for cutting ultrathin sections is rather expensive, the use of self-made glass knives, as a much cheaper alternative, cannot be recommended. Here, the presence of soil colloids, such as clays and Fe and/or Mn containing colloidal aggregates, will immediately crash the sharpness of the knife and no fruitful sections will be obtained. These ingredients however should generally be withstood by the diamond, but extreme care should be taken not to include any sand granules (= quartz; Mohs's mineral hardness scale = 7; in comparison, diamond = 10) which immediately will destroy the sharpness and function of the diamond knife.

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