An Imaging Mass Spectrometry Study on the Formation of Conditioning Films and Biofilms in the Subsurface (Äspö Hard Rock Laboratory, SE Sweden)

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Received October 2013; Accepted March 2014

Conditioning films and biofilms forming on surfaces of solid materials exposed to aqueous media play a key role in the interaction between the geo- and biospheres. In this study, time-of-flight secondary ion mass spectrometry and scanning electron microscopy were used to investigate the time scale, mode of formation, and chemistry of conditioning films and biofilms that formed on Si substrates exposed to aquifer water in the subsurface Åspö Hard Rock Laboratory, SE-Sweden. The detection of fragment ions of amino acids, carbohydrates, and carboxylic acids revealed that different types of organic compounds had adhered to the Si surface already after 10 min of exposure to the aquifer fluids, whereas the attachment of microbial cells was first observed after 1000 min. The organic compounds first formed isolated μm-sized accumulations and subsequently started to distribute on the wafer surface more homogeneously. Simultaneously further microorganisms attached to the surface and formed biofilm-like cell accumulations after 3 months of exposure to aquifer water.

Keywords: biofilm, groundwater, subsurface microbiology

Introduction

Biofilms (Costerton et al. 1995), consisting of microbial cells enclosed in extracellular polymeric substances (EPS), are of major interest to various scientific fields as they may form on nearly every solid surface. Biofilms may cause serious health problems (Costerton et al. 1999; Keevil 2003) when growing on indwelling medical devices like catheters and prostheses (Donlan 2001) and lead to increased corrosion damage of colonized metallic components (Beech et al. 2006; Geesey et al. 1996). By growing on hulls they can considerably increase the power needed to maintain ships at a given speed (Cooksey 1996). By growing on hulls they can considerably increase the power needed to maintain ships at a given speed (Cooksey 1996). By growing on hulls they can considerably increase the power needed to maintain ships at a given speed (Cooksey 1996).

As constructors of microbialites, such as stromatolites, biofilms can be traced back in the geological record to the Archean era (Schopf et al. 2007; Tice et al. 2011) thus suggesting that microorganisms have been organizing themselves in this way during most of Earth’s history.

The formation of biofilms can be divided into four steps, namely (i) transport, (ii) adhesion, and (iii) attachment of cells to a solid surface, followed by (iv) colonization of the surface (van Loosdrecht et al. 1990). Apart from the characteristics of the substratum, aqueous medium, and cell surfaces, the rate and extent of cell attachment is affected by so-called conditioning films (Donlan and Costerton 2002). These thin organic films begin to form from dissolved organic matter quickly after the exposure of a solid surface to an aqueous medium and are considered as an important prerequisite before the establishment of complex biofilms can take place (Cooksey and Wigglesworth-Cooksey 1995; Loeb and Neuhof 1975).
Using techniques like fluorescent spectroscopy (Loeb and Neihof 1975), infrared spectroscopy (Baier 1980), and X-ray photoelectron spectroscopy (Taylor et al. 1997) compound groups like proteins, lipids, nucleic acids, polysaccharides, amino acids (Taylor et al. 1997), glycoproteins (Baier 1980), and humic material have been identified in conditioning films. However, these methods are limited in their ability to provide detailed information on the molecular composition of conditioning films. Time-of-flight secondary ion mass spectrometry (ToF-SIMS, Benninghoven 1994), when used in static mode, is a quasi-nondestructive technique that allows analyzing the chemical composition of the uppermost atomic or molecular monolayer of a solid surface with high mass accuracy and at a $\mu$m-resolution (Belu et al. 2003; Benninghoven 1994). In ToF-SIMS, a pulsed primary ion beam is rastered over the sample surface and leads to the emission of secondary ions. These ions are then analyzed according to their flight time, which is a function of their $m/z$ ratio.

The capabilities of ToF-SIMS have prompted researchers to study the chemical composition of conditioning films and biofilms (Beech et al. 1999; de Brouwer et al. 2006; Poleunis et al. 2002; Poleunis et al. 2003; Pradier et al. 2000). Most of these earlier studies, using Cs$^+$ and Ga$^+$ ion sources, focused on the short-term (24-h) formation of conditioning films on stainless steel panels immersed in sea water. The observation of various small, amino acid-derived fragments indicated that proteins were the first compounds to adsorb on these surfaces. In recent years, the invention of cluster ion sources (e.g., Au$_n^{2+}$; Bi$_n^{2+}$) in liquid metal ion guns (LMIGs) has led to considerable improvements in the analysis of biological molecules by ToF-SIMS, as these sources provide dramatically higher yields for relatively large organic fragment and molecular secondary ions (Winograd and Garrison 2010). The cluster primary ion sources have also given rise to an increasing number of studies employing ToF-SIMS for the analysis of organic substances in geobiological systems (for a review, see Thiel and Sjövall 2011).

The purpose of the present study was to observe on an extended time scale (minutes to months) the chemical changes involved in the evolution from an initially formed conditioning film towards a biofilm in the subsurface. The formation of conditioning films and biofilms in the subsurface is of particular interest for geobiological research as the growth conditions and surrounding environment are thought to resemble the environments under which early life on Earth developed (Trevors 2002).

Our experiments were conducted in Aspö Hard Rock Laboratory (Aspö HRL), a research tunnel operated by the Swedish Nuclear Fuel and Waste Management Company (SKB) as a testing site for the long-term deposition of nuclear waste. The 3.6-km-long tunnel extends to a depth of 450 m below sea level under the island of Aspö located about 400 km south of Stockholm in SE-Sweden. The Aspö HRL offers a unique window into the subsurface. Groundwaters sampled from boreholes in the tunnel have been reported to contain, among others, homoacetogens, methanogens (Kotelnikova and Pedersen 1998), iron oxidizing bacteria (Anderson and Pedersen 2003) and sulphate reducing bacteria (Pedersen et al. 1996). Our study, combining ToF-SIMS and SEM, was aimed at gaining an insight into the growth pace of conditioning films and biofilms on pristine surfaces exposed to native fluids from the continental deep biosphere.

**Materials and Methods**

**Flow Reactor Setup**

Dark, airtight flow reactors (20 cm inner diameter × 9.5 cm high) with an effective volume of 77 mL were constructed from highly inert PTFE (Teflon) to avoid contaminations from organic plasticisers (Figure 1). Sample holders inside the flow reactor were manufactured from stainless steel bolts and PTFE washers, and are located within the reactor cap, so that samples were immersed from the top into the fluid. The inflow was installed below the outflow to keep the reactor free of air. Prior to use, all flow reactors were rinsed with acetone, cleaned with ethanol (70%) and autoclaved for 20 min at 121°C.

Silicon wafers (~30 mm × ~10 mm × 0.525 mm, Si-Mat, Silicon Materials, Landsberg/Lech, Germany) were chosen as substrate surfaces for conditioning and biofilm growth due to their conducting nature that allowed for uncomplicated analysis in the ToF-SIMS instrument without the need to use charge compensation. The wafers were rinsed with deionized water purified by a Milli-Q Plus 185 system and cleaned on both sides under an ozone producing mercury grid lamp

![Image](image_url)

**Fig. 1.** Flow reactors: a) open flow reactor with lid (view from inside), black arrows mark sample holders whereas white arrows indicate the in- and outflow openings. b) wafer samples in sample holders of flow reactor lid.
(emission wavelength: 185-579 nm, irradiance: \( \sim 7.5 \text{ mW/cm}^2 \), B.H.K. Inc., Ontario, CA, USA). After cleaning and sterilization, the flow reactors were equipped with up to 10 clean silicon wafers and filled with purified, autoclaved water. The flow reactors were connected with flexible PTFE tubes to a tapped fluid outflow at a depth of 69 m below sea level (Site 507A, REDOX-Site) in the tunnel of Aspö, allowing the wafers to be exposed to the aquifer water for 10 min, 1000 min, 10 d, and 90 d, respectively.

After 10 min and 1000 min, samples were taken out of the flow-reactors on site, gently immersed in purified, autoclaved water (3°C), and covered with a second, clean silicon wafer. The sandwiched wafers were plunge frozen in liquid nitrogen, and stored and transported at \(-80^\circ\text{C}\) on dry ice. Samples with longer exposition times were kept inside the water-filled flow reactors for transport (5 h) to the ToF-SIMS laboratory. Immediately before ToF-SIMS analysis, the samples were taken out of the reactors, gently immersed in purified, autoclaved water (3°C), and dried at room temperature in a laminar flow box. Parallel samples for scanning electron microscopy (SEM) were fixed in glutaraldehyde (2%) and stored at 4°C. Prior to SEM-analysis, samples were rinsed with purified water, dehydrated in a rising ethanol concentration (15% to 99%), dried in hexamethyldisilazane, glued on SEM sample holders, and sputtered with Au until a film thickness of \( \sim 10 \text{ nm} \) was reached. Control samples were

| Compound class | \( m/z \) | Tentative assignment | 10 min | 1000 min | 10 d | 90 d | Control |
|---------------|----------|----------------------|--------|----------|------|------|---------|
| Amino acids   | 30       | \( \text{CH}_4\text{N}^+ \) | x      | x        | x    | x    | x       |
|               | 44       | \( \text{C}_2\text{H}_8\text{N}^+ \) | x      | x        | x    | x    | x       |
|               | 58       | \( \text{C}_3\text{H}_10\text{N}^+ \) | x      | x        | x    | x    | —       |
|               | 61       | \( \text{C}_4\text{H}_12\text{N}^+ \) | x      | —        | x    | x    | —       |
|               | 83       | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | —        | —    | x    | —       |
|               | 84       | \( \text{C}_4\text{H}_10\text{N}^+ \) | x      | x        | x    | x    | x       |
|               | 86       | \( \text{C}_4\text{H}_10\text{N}^+ \) | x      | —        | —    | x    | x       |
|               | 87       | \( \text{C}_5\text{H}_12\text{N}^+ \) | x      | —        | —    | x    | x       |
|               | 88       | \( \text{C}_4\text{H}_8\text{O}^+ \) | x      | —        | —    | x    | x       |
|               | 102      | \( \text{C}_6\text{H}_14\text{O}^+ \) | x      | —        | —    | x    | x       |
|               | 107      | \( \text{C}_7\text{H}_16\text{O}^+ \) | x      | x        | x    | x    | x       |
|               | 110      | \( \text{C}_8\text{H}_18\text{O}^+ \) | x      | x        | x    | x    | x       |
|               | 120      | \( \text{C}_8\text{H}_20\text{O}^+ \) | x      | x        | x    | x    | x       |
|               | 130      | \( \text{C}_9\text{H}_22\text{O}^+ \) | x      | —        | x    | x    | x       |
|               | 136      | \( \text{C}_{10}\text{H}_{22}\text{O}^+ \) | x      | x        | —    | x    | x       |
|               | 145      | \( \text{C}_{10}\text{H}_{22}\text{N}^+ \) | x      | —        | —    | —    | —       |
| Carbohydrates | 31       | \( \text{CH}_2\text{O}^+ \) | x      | x        | x    | x    | x       |
|               | 43       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | x        | x    | x    | x       |
|               | 45       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | —        | —    | x    | x       |
|               | 55       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | x        | —    | x    | x       |
|               | 57       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | x        | x    | x    | x       |
|               | 59       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | —        | x    | x    | x       |
|               | 69       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | —        | —    | x    | x       |
|               | 81       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | —        | —    | x    | x       |
|               | 85       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | x        | —    | —    | —       |
|               | 87       | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | —        | —    | x    | —       |
|               | 105      | \( \text{C}_2\text{H}_4\text{O}^+ \) | x      | x        | —    | x    | —       |
| Carboxylic acids | 141     | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | x        | —    | —    | —       |
|               | 143      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | x        | x    | x    | —       |
|               | 157      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | x        | x    | x    | —       |
|               | 171      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | x        | —    | —    | —       |
|               | 181      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | —        | —    | —    | —       |
|               | 199      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | x        | —    | x    | —       |
|               | 213      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | —        | —    | —    | —       |
|               | 227      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | x        | —    | —    | —       |
|               | 253      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | —        | —    | —    | —       |
|               | 255      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | x        | —    | x    | —       |
|               | 281      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | —        | —    | —    | —       |
|               | 283      | \( \text{C}_4\text{H}_10\text{O}^+ \) | x      | x        | x    | x    | X       |

(x = detected; — = not detected).
immersed for 1000 min in purified, autoclaved water and prepared in the same way as described above for ToF-SIMS and SEM analyses.

**ToF-SIMS**

Wafer samples were analyzed with a ToF-SIMS IV instrument (ION-ToF, Münster, Germany), using 25 keV Bi3 primary ions at an accumulated dose density of $2.5 \times 10^{10}$ ions/cm². Areas of 500 μm × 500 μm were analyzed in bunched mode with a resolution of $128 \times 128$ pixels (lateral resolution ~4 μm) and a mass resolution of ca. $m/Δm$ 5000. Each analysis was conducted in positive and negative ion mode and the spectra were individually calibrated using CH$_2$C, C$_2$H$_2$C, C$_3$H$_2$C, C$_4$H$_3$C, and C$_4$H$_4$ peaks, respectively.

Ion images were acquired for fragment ions representing three different compound classes, namely amino acids, carbohydrates, and carboxylic acids (see Table 1). Summed ion images for each compound class were obtained by adding the signal intensities of the corresponding individual ion images.

**SEM**

SEM analyses were conducted using a LEO 1530 Gemini field emission SEM (Zeiss, Göttingen, Germany) at 3.8 kV.

**Results**

**ToF-SIMS**

**Positive ion mode**

The partial positive ion mode spectra ($m/z$ 20-80) of 500 μm × 500 μm areas on the wafers sampled after 10 min, 1000 min, 10 d, and 90 d, and the control wafer, respectively, are shown in Figure 2. All wafers displayed similar major peaks in the low-mass range. Ions of the silicon substrate were detected at $m/z$ 28 and 45 corresponding to Si$^+$ and SiOH$^+$, respectively.

Except for the samples exposed for 10 min in the flow reactors, all spectra showed Na$^+$ at $m/z$ 23 as the most intense peak. Other major peaks of inorganic species were observed at $m/z$ 39, 40, and 57 corresponding to K$^+$, Ca$^+$, CaOH$^+$, SiOH$^+$, and SiC$_3$H$_9$+$^+$.
and CaOH$^+$. Major peaks consistent with the hydrocarbon fragments C$_2$H$_3$$^+$, C$_3$H$_5$$^+$, C$_4$H$_7$$^+$, C$_4$H$_9$$^+$ and C$_6$H$_{10}$$^+$ were observed at \(m/z\) 27, 29, 41, 43, 55 and 57, respectively.

Minor peaks corresponding to fragment ions of amino acids (Wald et al. 2010) and carbohydrates (Sjövall et al. 2004) were detected by ToF-SIMS, but due to their low intensities, these peaks are not visible in the partial positive ion mode spectra shown in Figure 2 (see Supplementary Figures S1, S3, S5, S7, and S9). Furthermore, all spectra showed a peak at \(m/z\) 73 consistent with the SiC$_3$H$_9$$^+$ fragment ion, which is assigned to polydimethylsiloxane (PDMS) contamination, and a peak at \(m/z\) 28 consistent with the Si$^+$ ion from the silicon wafer substrate.

Negative ion mode
As in the positive ion mode, no major differences were observed for the partial negative ion mode spectra (\(m/z\) 10-80) of the wafers sampled after different exposure times to the aquifer water (Figure 3). All spectra showed major substrate peaks at \(m/z\) 60 and 77, corresponding to SiO$_2^-$ and SiOH$_3^-$. Further major inorganic ions detected at \(m/z\) 16, 17, 35, and 79 are consistent with O$,^-$, OH$^-$, Cl$,^-$, and PO$_3$$^-$, respectively. Intense organic fragment ions detected at \(m/z\) 25, 26, and 42 are consistent with C$_2$H$^-$, CN$^-$, and CNO$^-$, respectively. Minor organic ions detected in the negative ion mode include carboxylic acids in the C$_9$ to C$_{18}$ range (Passarelli and Winograd 2011; see also Supplementary Figures S2, S4, S6, S8, and S10). These ions are listed in Table 1.

Compound imaging
Ion images showing the spatial distributions of Si$^+$, SiC$_3$H$_9$$^+$, and three individual organic compound classes, i.e., amino acids, carbohydrates, and carboxylic acids, on the fluid-exposed surfaces are displayed in Figure 4. Images showing the distribution of the latter were obtained by adding the signal intensity from several fragment ions representing the corresponding compound class (the specific fragment ions used to produce the images are listed in Table 1 and corresponding spectral peaks are shown in Supplementary Figures S1-S10. On the wafer immersed for 10 min in the aquifer water, the ion images showed a spotlike distribution common for all three compound classes (i.e., amino acids, carbohydrates, and carboxylic acids), with only a few 20–40 $\mu$m diameter
spots displaying elevated signal intensities (Figure 4a-c). These spots are not colocalized with Si$^+$ or PDMS signals (Figure 4d, e).

On the wafers exposed for 1000 min to aquifer water, the ion images of amino acids, carbohydrates, and carboxylic acids showed a more uniform distribution (Figure 4f-h) compared to the 10 min wafer sample. A chemical contrast was observed in the ion images showing the distribution of PDMS and Si$^+$ (Figure 4i, j).

No chemical contrast was observed in the ion images of fragments of amino acids, carbohydrates, carboxylic acids, and Si$^+$ from the wafer immersed for 10 d (Figure 4k-n) whereas PDMS was unevenly distributed on the wafer surface (Figure 4o).

In contrast, the ion images obtained after 90 days of immersion in aquifer water showed a patchy distribution of amino acids and carbohydrates on the wafer surface (Figure 4p, q), with patches of about 10–20 μm being densely spread over the entire analysis area. The carboxylic acid fragments showed only very weak signals on this wafer. Low intensities of Si$^+$ signal were colocalized with areas showing high intensities of amino acid and carbohydrate signal (Figure 4p, q, s). The PDMS signal was patchy distributed but not colocalized with the signals of amino acid and carbohydrate fragments (Figure 4p, q, t).

The control wafers immersed for 1000 min in ultrapure, autoclaved water showed a non-uniform distribution of the Si$^+$ and PDMS signal, but no chemical contrast was
Fig. 5. SEM images of wafer surfaces. (a) wafer exposed for 10 min to aquifer water, white frame marks area enlarged in (b) showing a sodium chloride crystal, (c) wafer exposed for 1000 min to aquifer water, white frame marks area enlarged in (d) showing two solitary cells attached to the surface, (e) wafer exposed for 10 d to aquifer water, white frame marks area enlarged in (f) showing two solitary cells attached to the surface, (g) wafer exposed for 90 d to aquifer water, white frame marks area enlarged in (h) showing biofilm-like accumulation of cells enclosed in extracellular polymeric substances, (i and j) control wafer samples showing no attachment of microbial cells.
observed in the ion images of the three compound classes (Figure 4u-y).

In general the highest Si\textsuperscript{+} signal based on total counts (tc) was recorded for the wafer exposed for 10 d to aquifer water (9.78; Figure 4n), whereas the lowest tc was measured for the 90 d wafer sample (1.66; Figure 4s). The tc of the control wafer and the wafers immersed for 10 min and 1000 min in aquifer water, respectively, differed only slightly (3.16 and 3.22; Figure 4d, i, x).

The total SiC\textsubscript{3}H\textsubscript{9}\textsuperscript{+} signal was lowest for the wafer immersed for 1000 min (1.17; Figure 4j) and increased with longer time of immersion to 4.77 after 90 d of exposure (Figure 4t). The highest tc value (8.69) for SiC\textsubscript{3}H\textsubscript{9}\textsuperscript{+} was recorded for the control wafer (Figure 4v).

**SEM**

Representative SEM images obtained from the wafer surfaces displayed organic particles, minerals and microbial cells attached to the wafer surface (Figure 5).

On the 10-min wafer, exclusively sodium chloride crystals were found adhering to the surface (Figure 5 a, b). The first solitary cells attached to the wafer surface were observed after 1000 min of exposure to aquifer water (Figure 5c, d).

With increasing time of exposure, more solitary cells were found to be attached to the wafer surface (Figure 5e, f) and, successively, larger accumulations (10\textendash 100 \(\mu\)m in diameter) of cells enclosed in a matrix were formed on the wafer (Figure 5g, h). No cells were found to be attached to the surface of the control sample (Figure 5i, j).

**Discussion**

The fragment ions of amino acids, carbohydrates, and carboxylic acids showed a non-uniform distribution with spots of higher intensity (20\textendash 40 \(\mu\)m in diameter) on the wafer surface already after 10 min of immersion of the wafer in aquifer water. As no microbial cells were found to be attached to the wafer surface and known artificial contaminants such as PDMS were not colocalized with these spots, the most plausible explanation for the observation of these organic structures is the initial formation of a conditioning film on the wafer surface. Earlier studies on surfaces exposed to seawater have identified carbohydrates and proteins as the initial and most abundant constituents of the primary conditioning film (Compère et al. 2001; Garg et al. 2009; Jain and Bhosle 2009). Likewise, our finding of an immediate onset of the conditioning film formation after exposure of the wafer surface to the aquifer water is in agreement with earlier observations on surfaces immersed in sea water (Bakker et al. 2003) and conditioning films produced from artificial growth media (Chen et al. 2010).

With increasing exposure time (1000 min), carbohydrate and amino acid fragment ions showed a more continuous distribution on the wafer surface. The slight contrasts observed in the ion images can be plausibly explained by patches of PDMS contaminations (Figure 4j). The continuous distribution of carbohydrate and amino acid fragment ions is consistent with a report stating that the primary film forming on stainless steel surfaces immersed for 24 h in sea water is composed of proteins and shows a uniform distribution (Poleunis et al. 2002).

However, it has to be considered that potential inhomogeneities may be present at length scales shorter than the lateral resolution (\(\sim 4 \mu\)m) of our ToF-SIMS analysis. In fact, from atomic force microscopy analyses of stainless steel surfaces immersed in seawater it has been concluded that proteins are initially adsorbed in the form of 20\textendash100 nm diameter “islands” (Pradier et al. 2000), which are too small to be resolved in our ToF-SIMS ion images. Similarly, the first attachment of solitary microbial cells to the wafer surface observed in the SEM images after 1000 min of immersion may not have become evident in the ion images due to the limited lateral resolution of our ToF-SIMS analyses. Considering the low number of cells and their solitary distribution observed in SEM images, these cells are unlikely to be the source of the uniformly distributed organic signal in the ion images.

Based on the consistent Si\textsuperscript{+} signal recorded for the wafers after 1000 min and 10 d of exposure time, the surface coverage of the wafer remained largely constant. No major changes in the distribution of the ions of the three organic compound classes could be observed between the ion images obtained from the wafer samples exposed to aquifer water for 1000 min and 10 d. From the SEM images it can be concluded that in this time interval solitary cells increasingly attached to the surface. Again, these single cells may have been too small to be resolved in the ToF-SIMS ion images.

However, based on the higher Si\textsuperscript{+} signal from the wafer exposed for 10 d compared to the wafer exposed for 1000 min, the coverage of the substrate surface decreased in this time interval.

Between 10 d and 90 d of exposure, the cells, as observed by SEM, started to form larger accumulations of cells and to secrete EPS, and thus organize themselves in biofilm-like structures. These observations are consistent with the recording of the lowest Si\textsuperscript{+} signal by ToF-SIMS and thus the highest observed surface coverage compared to wafers exposed for shorter time periods. The low resolution of the ToF-SIMS images and the unspecific organic ions observed did not allow to identify microbial cells or surrounding EPS as specific sources of the organic signals imaged by ToF-SIMS. However, the main constituents of EPS typically include polysaccharides and proteins (Sheng et al. 2010). Hence, the island-like distribution of amino acid and carbohydrate fragment ions observed on the 90-d wafer can plausibly be explained by proteins and polysaccharides that make up the EPS matrix secreted around the \(\mu\)m-sized biofilm structures.

**Conclusions**

ToF-SIMS and SEM provided insights into the chemistry and time scales of the initial conditioning film and biofilm formation during exposure of a pristine surface to a subsurface aquifer. The organic matter that initially adhered in
small patches to the surface, prior to any attachment of microbial cells, was found to be rich in amino acids, carbohydrates and carboxylic acids. Following this initial stage, the conditioning film began to distribute on the substrate surface more homogeneously, and microbial cells started to attach after a few hours. The formation of actual biofilm-like structures, comprising accumulations of microbial cells enclosed in EPS, took place in the time interval between 10 d and 90 d of immersion in aquifer water. In conclusion, our results highlight that any native surface formed, e.g., by tectonic processes in the shallow, continental subsurface, will under exposure to aquifer water likely be covered by organic matter within minutes and by complex biofilms within a time scale of weeks to months.

Acknowledgments

We thank D. Hause-Reitner (University of Göttingen) for assistance with scanning electron microscopy, and E. Johanson, M. Kronberg, K. Nilsson, and M. Lundqvist from SKB for technical and logistic support at the Aspö Hard Rock Laboratory. We are also grateful to L. Laake (University of Göttingen) and his team for the construction of the flow reactors and the UV-ozone cleaning apparatus.

Funding

This study was financially supported by the German Research Foundation (DFG; Grant FOR 571). This is publication #74 of the DFG Research Unit “Geobiology of Organo- and Biofilms.”

Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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