Development and structure of *Bacillus subtilis* biofilms manipulated by Iron(II) addition during cultivation at different shear stress conditions

Luisa Gierl[1], Harald Horn[2], and Michael Wagner[3]

[1]Karlsruher Institut für Technologie Engler-Bunte-Institut
[2]Karlsruhe Institute of Technology, Germany
[3]Karlsruhe Institute of Technology

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Abstract

Bivalent ions such as Ca2+ and Mg2+ are known to affect the structural and mechanical properties of biofilms. In order to reveal the impact of Fe2+ ions within the cultivation medium on biofilm development, structure and stability, Bacillus subtilis biofilms were cultivated in mini-fluidic flow cells. Two different Fe2+ inflow concentrations (0.25 and 2.5 mg/L, respectively) and wall shear stress levels (0.05 and 0.27 Pa, respectively) were tested. Biofilm structure was determined daily in situ and non-invasively by means of optical coherence tomography. A set of ten structural parameters was used to quantify biofilm structure, its development and change. Moreover, for each experiment ten replicates were cultivated and analyzed allowing for valid conclusions. Fe2+ addition influenced biofilm development (e.g., biofilm accumulation) and structure markedly. Experiments revealed the accumulation of FeO(OH) within the biofilm matrix and a positive correlation of Fe2+ inflow concentration and biofilm accumulation. Even at elevated shear stress levels this correlation was valid. In more detail, independent of the wall shear stress applied during cultivation over ten days biofilms grew approximately four times thicker at 2.5 mg Fe2+/L compared to low Fe2+ inflow concentrations of 0.25 mg/L. This finding hints on a higher stability of Bacillus subtilis biofilms against detachment when growing at elevated Fe2+ concentrations.

1. Introduction

Biofilms are aggregates composed of microorganisms, extracellular polymeric substances (EPS) as well as extracellular DNA. Mainly, they appear at interfaces in watery environments (Flemming, Wingender, & Szewzyk, 2007). Within the last decade, more attention was paid to biofilms due to their unique properties and potential applications as productive systems (Edel, Horn, & Gescher, 2019; Rosche, Li, Hauer, Schmid, & Buehler, 2009). On one hand, these communities have several beneficial features, e.g. (i) in cleaning waste water (Van Loosdrecht & Heijnen, 1993); (ii) by producing valuable (platform) chemicals (Cuny et al., 2019), (iii) methane (as fuel) (Yeung et al., 2017) or (iv) bioplastics (Hackbarth et al., 2020), too. On the contrary, biofilms can have adverse effects, i.e. on human health by growing on implants or by blocking industrial settings such as water pipes (Azeredo et al., 2016).

In order to optimize biofilm-technological processes it is necessary to understand biofilm proliferation and behavior under certain conditions.

Direct analysis of biofilm behavior or rather structure can be performed using different imaging techniques such as confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) or atomic force microscopy (AFM) (Allen, Habimana, & Casey, 2018; Azeredo et al., 2016; Bridier, Meylheuc, & Briandet, 2013; Dutta Sinha, Das, Tarafdar, & Dutta, 2017). While CLSM can be used for investigating the

biofilm matrix composition (e.g., DNA and EPS) in a range of several micrometers, SEM together with energy-dispersive X-ray spectroscopy (EDX) examines biofilms up to 1 nm resolution and their elemental composition. AFM determines for instance adhesion forces between biofilm and the substratum as well as cohesive strength (Allen et al., 2018; Azeredo et al., 2016). Optical coherence tomography (OCT) is an application becoming increasingly relevant for the analysis of biofilms' mesoscopic structure as seen from the raising number of publications (Blauert, Horn, & Wagner, 2015; Dreszer et al., 2015; Haisch & Niessner, 2007; Wagner & Horn, 2017; Weiss, Obied, Kalkman, Lammertink, & van Leeuwen, 2016). Advantages among other imaging techniques are the high optical resolution together with a fast acquisition of 3D datasets of translucent tissues and materials *in situ* despite large representative volumes. Mesoscopic biofilms are valuable for e.g., modeling of permeate fluxes in membrane systems (Derlon, Peter-Varbanets, Scheidegger, Pronk, & Morgenroth, 2012) or substrate turnover in biofilm reactors (Li, Wagner, Lackner, & Horn, 2016; Wagner & Horn, 2017). Since a statistical survey of biofilm replicates is inevitable for e.g., optimizing parameters in a biofilm production reactor, OCT is the imaging modality of choice for the identification and quantification of structural biofilm parameters.

Nutrients and hydrodynamics are some of the main effectors in biofilm lifecycle. Several studies have been performed which focus on the influence of different ions and flow velocities on biofilm behavior (Guvensen, Demir, & Ozdemir, 2013; Park, Jeong, Lee, Kim, & Lee, 2011; Paul, Ochoa, Pechaud, Liu, & Liné, 2012; Sehar, Naz, Das, & Ahmed, 2016; Song & Leff, 2006; P. Stoodley, Dodds, Boyle, & Lappin-Scott, 1999; P Stoodley, Cargo, Rupp, Wilson, & Klapper, 2002). Bivalent cations such as Ca^{2+} and Mg^{2+} are known to promote growth and stability of biofilms (Guvensen et al., 2013; Sehar et al., 2016; P. Stoodley et al., 1999). Additionally, iron (Fe^{2+}) may be of concern regarding biofilm development. Iron is an essential trace element and component of iron-sulfur-complexes in various enzymes. Moreover, a couple of bacteria utilize Fe^{3+} as an electron acceptor within the respiratory chain (Riemer, Hoepken, Czerwinska, Robinson, & Dringen, 2004). Furthermore, iron is essential for almost all living organisms and forms a cofactor in many cellular proteins, which are involved in electron transport, detoxification of reactive oxygen species (ROS) or DNA synthesis (Neilands, 1974). For that reason, at least a minimum of iron availability should be necessary for the maturation process of biofilms. The role of iron has been studied by several research institutes, too, mainly applying pathogenic *Pseudomonas aeruginosa* or static biofilms (e.g., agar plate- and microtiterplate biofilms) with diverging results (Banin, Vasil, & Greenberg, 2005; Berlutti et al., 2005; Kang & Kirienko, 2018; Musk, Banko, & Hergenrother, 2005; Ranmadugala, Ebrahiminezhad, Manley-harris, & Ghasemi, 2017; L. Yang et al., 2007). Additionally, most studies merely focus on the performance of those biofilms instead of physical structure - although structure and function are closely linked to each other. For instance, Möhle *et al.* (2007) described a positive effect on the biofilms' stability grown in a rotating disc reactor when higher amounts of iron sulfate (10 mg/L) were available (Möhle et al., 2007). Further studies showed that a limitation of iron (Singh, Parsek, Greenberg, & Welsh, 2002; Weinberg, 2004) as well as an excess of iron (Lin, Shu, Huang, & Cheng, 2012; Musk et al., 2005; L. Yang et al., 2007) in the environment inhibited the formation and development of biofilms in contrast to suspended cells.

In this study, the effect of Fe^{2+} in the cultivation medium on the development and maturation of *Bacillus subtilis*flow cell biofilms is investigated in order to evaluate biofilm behavior in terms of structure in 3D and non-invasively. Hence, two different Fe^{2+} concentrations were used to study the effect on the biofilm's morphology and maturation. This work strengthens the fundamental knowledge about biofilm physical structure and their interaction with interfaces. Furthermore, it highlights iron (Fe^{2+}) as an (trace) element, which can be used to control biofilm development and maturation.

2. Materials and Methods

2.1 Biofilm cultivation

Biofilms were cultivated in custom-made flow cells composed of sticky-Slides (sticky-Slide I 0.4 Luer, ibidi GmbH, Martinsried, Germany) glued to PVC slides (substrata). Sticky-Slides are made from transparent plastic and serve as the cover of the flow cell forming a flow channel with the size of $50 \times 5 \times 0.45$ mm^3(length $\times$ width $\times$ height, thickness of the sticky-Slide = 1 mm). A number of $N =$

10 flow cells were operated for each condition in parallel at volumetric flow rates of Q = 1 mL/min andQ = 5 mL/min, respectively (equals mean flow velocities of u = 0.75 cm/s and u = 3.75 cm/s, respectively).

Flow cells were inoculated with *Bacillus subtilis* pre-cultures grown at 37°C overnight in Luria Broth (LB) medium. Cells were grown to exponential phase; 10 mL of this pre-culture together with a minimal salts glycerol medium in a mixing ratio of 1:500 were used as inoculation solution. The cultivation medium was adapted from (Wang, Wang, & Hao, 2015) and contained (concentration in mg/L): $MnCl_2$ * 4 H_2O (10), L-phenylalanine (5), glycerol (5), $MgCl_2$ * 6 H_2O (4), L-tryptophane (3.5) with diverging concentrations of $FeCl_2$ * 4 H_2O ([?] 0.25 and 2.5 mg/L Fe^{2+}) in tap water. Salt concentrations of the tap water of Karlsruhe are accessible from the homepage of the Stadtwerke Karlsruhe (https://www.stadtwerke-karlsruhe.de) and contained (mg/L): Ca (112), Na (11), Mg (9.7), Si (5.4), K (1.7), P (< 0.01), Fe (< 0.01) and Mn (< 0.005).

Flow cells were flushed with the inoculum for 15 min. Afterwards flow was stopped for 1 h giving bacteria the possibility to settle. Then biofilm cultivation started in flow-through mode. Biofilm development 25 mm downstream the inlet was monitored daily for ten consecutive days by means of OCT using the EvoBot platform (Faina et al., 2016; Gierl, Stoy, Faina, Horn, & Wagner, 2020). An overview of the conducted experiments is provided in Table 1.

2.2 Optical coherence tomography and image processing

Optical Coherence Tomography (OCT) is a 3D imaging technique that allows for the non-invasive, real-time imaging of the mesoscopic biofilm structure (Martin, Bolster, Derlon, Morgenroth, & Nerenberg, 2014; Wagner & Horn, 2017). A brief summary regarding the working principle of OCT has already been provided in (Blauert et al., 2015; Gierl et al., 2020).

A spectral domain tomograph (GANYMEDE I, Thorlabs GmbH, Dachau, Germany) with an optical resolution of 8 x 8 x 2.1 μm^3 (x × y × z, LSM03 objective lens) in water (n = 1.33) was used to monitor biofilm development. OCT images (A-scan averaging = 3) with a size of 7 × 6 × 0.5 mm^3were acquired on a daily basis. Image post-processing included the calculation of structural biofilm parameters. OCT datasets were cropped to a volume of 7 × 5 × 0.25 mm^3 (due to autocorrelation artifacts). A mean filter with a radius of 2 px was applied and binary datasets were generated using Fiji (Schindelin et al., 2012). Substratum coverage (SC), mean biofilm thickness(L_F), textural entropy (TE), kurtosis (R_{KU}), skewness (R_{SK}), fractal dimension (FD), angular second moment (ASM), inverse difference moment (IDM) as well as average horizontal ($AHRL$) and average vertical run lengths ($AVRL$) were calculated from binary datasets according to (Wagner & Horn, 2017), (Blauert et al., 2015) and by use of the MiToBo plugin (Fiji) for biofilms (Möller, Glaß, Misiak, & Posch, 2016). In-house macros were used to render topographic representations of OCT C-scans (e.g., height maps representing the bulk-biofilm interface) (Wagner & Horn, 2017).

An overview of all structural parameters and their calculation is given in (Beyenal, Donovan, Lewandowski, & Harkin, 2004; Wagner & Horn, 2017; X. Yang, Beyenal, Harkin, & Lewandowski, 2000). ParametersSC, R_{KU} and R_{SK} were analyzed using Fiji's plugin function "analyze". An overview regarding the interpretation of the structural biofilm parameters is presented below (see Table 2).

2.3 Analysis of variance (ANOVA) and Scheirer-Ray-Hare

Grubb's tests and normality tests (Shapiro-Wilk) were performed in Origin to identify and discard outliers (OriginPro, Version 2018G, OriginLab 275 Corporation, Northhampton, MA, USA). Two factorial variance analyses with measuring repetitions were performed to evaluate the influence of Fe^{2+} and flow velocity on biofilm development and structure. In case of non-normality, a Scheirer-Ray-Hare test was applied. Three hypotheses were picked with the following predications:

H_1: No differences in structure due to usage of different flow velocities u

H_2: No differences in structure due to usage of different iron concentrations c

H_3: No correlation between both parameters u and$c$

To determine the approval or rejection of the hypotheses, calculated$p$-values were compared to a significance level of $\alpha< 0.01$, whereby values $p < \alpha$describe the rejection of the individual hypothesis. Variance analyses were performed in Excel (Excel version 15.11, Microsoft Corporation, Washington, USA).

3. Results and Discussion

3.1 Structural differences in OCT-imaged biofilms

Four growth conditions were applied to biofilms that differed in volumetric flow rate Q and the Fe^{2+} concentration$c$ (see Table 1). The aim of the study was the identification of a dependency of biofilm development and structure in terms of inflowing Fe^{2+} as well as of different flow regimes. Figure 1 illustrates height maps showing the topography of the developed biofilms at day 10 for each flow cell under each applied condition. Images of all flow cells on each day are given in Supplementary Information Figure 1.

Figure 1 reveals biofilm growth in all flow cells. To be noticed, FC 9 of E4 had a different appearance among the other flow cells for these experimental conditions. The same is visible from E3 in FC 5 among FCs 8 and 10. Experiment E1 ($c = 2.5$ mg/L Fe^{2+}, $u = 0.75$ cm/s) depicts biofilms with a mean biofilm thickness$L_F =$ 75 µm in all replicates. Additionally, under condition E1, biofilm aggregates of L_F up to 200 µm established and the substratum was covered with heterogeneous aggregates, ranging from smaller and spherical colonies to longer (> 2 mm) and elliptic colonies. In experiment E2 ($c = 0.25$ mg/L Fe^{2+}, $u = 0.75$ cm/s) less biofilm developed and merely FC 1, FC 2 and FC 5 to 7 are covered with biofilm ($L_F = 50$ µm). In comparison to E1, biofilm growth within E2 started delayed and only small colonies of minor widths and lengths, as well as of minor heights accumulated. In E3 ($c = 2.5$ mg/L Fe^{2+}, $u = 3.75$ cm/s) in turn, biofilm growth seemed to be more dense at day 10 of the growth course. Likewise E2, bacterial colonization was first visible around days 3 and 4. Here, accretion of the flow chamber took place from the walls to the center of the FC, probably due to the higher flow velocity. In E4 (c= 0.25 mg/L Fe^{2+}, $u = 3.75$ cm/s), coverage of the substratum was again low as in E2 and only a few colonies randomly developed with high biofilm thicknesses of$L_F = 200$ µm.

Figure 1 already provides visible differences in growth patterns for different experimental conditions. However, for quantification of the influence of flow velocity (shear stress) and iron (Fe^{2+}) dosage on biofilm structure and development several structural parameters have been calculated (confer Materials & Methods). Those are presented in Figure 2 and discussed in the following.

In Figure 2, the development of 10 different structural biofilm parameters for all conditions E1-E4 are illustrated. As already visible in Figure 1, the effect of the high (E1 + E3, $c = 2.5$ mg/L Fe^{2+}) and the low iron(II) concentration (E2 + E4,$c = 2.5$ mg/L Fe^{2+}) is visible. While mean biofilm thickness L_F and substratum coverageSC of E1 and E3 show a steady increase until the end of the experiment, those parameters stay at a minimum level in E2 and E4. A similar trend is distinct for the parameters textural entropyTE, average horizontal AHRL and average vertical run length AVRL. Additionally, stated parameters of E3 (c= 2.5 mg/L Fe^{2+}, $u = 3.75$ cm/s) exceed those of E1 ($c = 2.5$ mg/L Fe^{2+}, $u = 0.75$ cm/s) at the end of the experiment (from day 8 to day 10). The same trend is shown for biofilms grown with $c = 0.25$ mg/L Fe^{2+}, whereas mentioned structural parameters in E4 ($u = 3.75$ cm/s) exceed those of E2 ($u = 0.75$ cm/s). While mean biofilm thicknessL_F and substratum coverageSC indicate an early accumulation of biofilms with high biofilm volume in E1 and E3 ($c = 2.5$ mg/L Fe^{2+}), higher values of textural entropy TE exhibit more heterogeneous biofilms. Thus, biofilms grown with 2.5 mg/L Fe^{2+} show more differentiated and partially distributed structures. Furthermore, average run lengths AHRLand AVRL of biofilms in E1 and E3 display longer and wider biofilm aggregates resulting in the largest aggregates regarding biofilm volume in E3 ($c =$ 2.5 mg/L Fe^{2+}, $u = 3.75$ cm/s) (compare Figure 1).

As seen from the structural parameters skewness R_{SK} and kurtosis R_{KU}, biofilms in E4 ($c = 0.25$ mg/L Fe^{2+}, $u = 3.75$ cm/s) displayed random distributed and high biofilm hills with a low substratum coverage SC at the beginning of the experiment. From day 5 to the end of the experiment (day 10), values of R_{SK} and R_{KU} under all conditions approximate and stay near zero. Thereby, a homogeneous distribution of biofilm

as well as an equalized ratio of colonies with either low or high biofilm thickness L_F, except for E2 ($c = 0.25$ mg/L Fe^{2+}, $u = 0.75$ cm/s) where only several high and small biofilm aggregates occurred, is confirmed (compare Figure 1). Again, structural biofilm parameters fractal dimension FD, angular second moment ASMand inverse difference moment IDM prove a lucid differentiation between biofilms grown with $c = 0.25$ mg/L Fe^{2+} and $c = 2.5$ mg/L Fe^{2+}. Higher values of FD up to 1.8 in E1 and E3 ($c = 2.5$ mg/L Fe^{2+}) explain more irregular surfaces of the aggregates (compare Table 2). Here, the parameter ASMdescribes a change in growth direction (additional growth in y-direction (width)) and IDM proves additional growth in width, since distances between cell clusters are minimizing to the end of the experiment, compared to E2 and E4 ($c = 0.25$ mg/L Fe^{2+}).

As can be seen, biofilm thickness $L_F$and/or surface coverage SC, which are typically used for characterization of biofilm structure, do not provide the complete information on biofilm development. Therefore, only the joint consideration of all evaluated structural parameters leads to a complete overview of biofilm development over the cultivation period of 10 days. Figure 2 shows, that at the beginning, biofilms cultivated at $u = 3.75$ cm/s (E3, E4) developed more slowly due to the higher shear stress. However, towards the end of the experiment (days 8 to 9) those biofilms became more stable in terms of L_F,SC, ASM and IDM in relation to biofilms in E1 and E2 ($u = 0.75$ cm/s). With regard to the different iron(II) concentrations, more unambiguous differences in mostly all structural parameters could be identified (correlation of E1 + E3 and E2 + E4). The results confirm the positive influence of Fe^{2+} on biofilm accumulation and differentiation. Likewise (Körstgens, Flemming, Wingender, & Borchard, 2001) showed, that bivalent cations (e.g., Ca^{2+}) enhance the stability of the biofilm matrix in *Pseudomonas aeruginosa*biofilms which could explain the increased adhesion and biofilm accumulation in E1 and E3 ($c = 2.5$ mg/L Fe^{2+}) of the presented study. Additionally, in the study by (Möhle et al., 2007), increased concentrations of iron sulfate in the nutrient medium ($c = 10$ mg/L) prevented sloughing of microbial biofilms (activated sludge) in a rotating disk reactor. Furthermore, the authors documented the dependence of the biofilm thickness from the substrate concentration*c* as well as from the shear stress on the biofilm surface. Beyond, in studies with iron complexing agents it was found that a minimal concentration of soluble iron is necessary for the formation of *P. aeruginosa* biofilms in flow cells (Renslow, Lewandowski, & Beyenal, 2011; Singh et al., 2002; L. Yang et al., 2007). Thereby, one theory is, that iron regulates the surface motility of the bacteria and again promotes the biofilm formation by stabilizing the EPS matrix, which mainly consists of negatively charged polymers (Berlutti et al., 2005; Lin et al., 2012; Singh, 2004; Weinberg, 2004).

These studies further show, that an excess of iron concentrations inhibits biofilm formation, too, since the release of DNA from dead*P. aeruginosa* cells is suppressed. This release is an important structural component of biofilms (Lin et al., 2012; L. Yang et al., 2007). However, an ideal iron concentration in culture medium cannot easily be determined. While (Berlutti et al., 2005) define "high" iron concentrations in a range of 0.55 – 5.5 mg/L as positive in terms of aggregation and manipulation of biofilm development and structure in different reactor systems and tests, (L. Yang et al., 2007) reported an inhibition of biofilm growth in microtiter plates and flow cells in this concentration range. In the present study, this inhibition by high iron concentrations cannot be proven. Presumably, an addition of iron (Fe^{2+}) does not stimulate every bacterial biofilm system or possibly optimum iron amounts can vary among different biofilm species. Nevertheless, (Weinberg, 2004) confirmed that zinc, manganese and iron have key functions in biochemical as well as in morphological conversion of pro- and eucaryotes, respectively. Since soil carries high amounts of iron, a positive influence on growth of the used soil bacterium *Bacillus subtilis* could be demonstrated in the present study (Kolodkin-Gal et al., 2013; Pelchovich, Omer-Bendori, & Gophna, 2013; Rizzi, Roy, Bellenger, & Beauregard, 2019).

Likewise, the influence of hydrodynamics on biofilms is well-known and documented in several studies (Manz, Volke, Goll, & Horn, 2003; Park et al., 2011; Paul et al., 2012; Purevdorj, Costerton, & Stoodley, 2002; P. Stoodley et al., 1999; P Stoodley et al., 2002; Teodósio, Simões, Melo, & Mergulhão, 2011; Weiss et al., 2016). These studies verify the formation of streamers at higher flow velocities meaning increased growth in length, which is best visible in E3 for FC 8 and E4 for FC 9 ($u = 3.75$ cm/s, see Figure 1 and Figure

2 AHRL). Statements about the viscoelastic properties and strength of the cell clusters characterize the positive influence of the hydrodynamics furthermore (Allen et al., 2018; Peterson et al., 2015; Rupp, Fux, & Stoodley, 2005; Safari, Tukovic, Walter, Casey, & Ivankovic, 2015; P Stoodley et al., 2002; Paul Stoodley, Lewandowski, Boyle, & Lappin-scott, 1999; Towler, Rupp, Cunningham, & Stoodley, 2003).

3.2 ANOVA-confirmed effects of iron(II) on biofilm structure

Two factor-based variance analyses were performed to verify the results of the structural biofilm parameters given in Figure 2. These evaluations determine to which extent a correlation of the conditions (c, u) took place. Furthermore, they provide insight on the differentiation of structural biofilm parameters between conditions with statistical certainty.

A p-value above the significance level $\alpha_{0.01}$ proves that the hypothesis is accepted and that no differences in structural biofilm parameters can be estimated by the chosen condition. This was shown by both the ANOVA and the Scheirer-Ray-Hare test for all structural parameters in hypothesis H_1 (Table 3).

With a minimum value of the skewness parameter with $R_{\mathrm{SK}}(p = 0.07 > \alpha)$ up to a maximum value of the substratum coverage SC ($p = 0.84 > \alpha$), there were no statistically significant differences between the low ($u = 0.75$ cm/s) and high flow velocity ($u = 3.75$ cm/s). On closer consideration of R_{SK} in Figure 2, it gets visible that this parameter showed the lowest p-value in H_1 because there was no overlapping of the standard deviations of E1 + E3 as well as of E2 + E4 at the end of the experiment. This documents that the volumetric flow rate and resulting shear stress τ_w have a slight influence on the biofilms regarding skewness R_{SK} and the fractal dimensionFD. Nevertheless, this could not be confirmed with statistical significance compared to the influence of Fe^{2+} on the biofilm aggregates (see Table 3, H_2). In comparison to Figure 2, Fe^{2+}has a positive effect on the mean biofilm thicknessL_F and substratum coverageSC, which basically means on biofilm accumulation.

In contrast, diagrams of skewness R_{SK} and kurtosisR_{KU} revealed the smallest differences between conditions E1-E4 at the end of the experiments. This is confirmed by the ANOVA forR_{SK} with the closest value to α ($p = 0.18{\cdot}10^{-2} < \alpha$) in H_2. The value of R_{KU}, on the other hand, is far from the significance level ($p = 0.56{\cdot}10^{-5} < \alpha$), although the experimental course ofR_{KU} resembled the development of R_{SK} in Figure 2. One reason might be the use of the Scheirer-Ray-Hare test, which is generally considered to be less accurate than the ANOVA (Dytham, 2011). The results of H_3 again show, as is evident from H_1, p-values above the significance level α. This verifies the assumption that the influence of the volume flow and the addition of Fe^{2+} do not correlate with one another. Solely, the value of the average horizontal run lengthAHRL is relatively approximate to the level of significance ($p = 0.04 < \alpha$). An explanation is given by the non-changing direction of water flow in the channel: preferably, the aggregates are growing in x-direction if sufficient addition of Fe^{2+} is present, as the flow is unidirectional.

The investigation of the fluid-structure interaction demonstrates the need to analyze a large number of biofilm structural parameters since the evaluation of one parameter may (possibly) not be sufficient to determine differences or rather dependencies and correlations (e.g.,R_{SK}, R_{KU}). Additionally, the choice of a statistical test like an ANOVA will prove the occurrence of underlying relations.

3.3 Influence of Fe amounts and compounds on biofilm development

Further gravimetrical determinations as well as ion chromatography (ICP-OES) verified that Fe^{2+} is somehow stored into the biofilm matrix and not rinsed out with the nutrient medium flowing through (data not shown). Here, most of the iron was incorporated in biofilms of experiments E1 and E3 ($c = 2.5$ mg/L Fe^{2+}). Several hypotheses were postulated that describe the uptake and influence of iron into the biofilm. For instance, (Kang & Kirienko, 2018) confirm an uptake of iron via siderophores (iron carriers) as well as the storage of iron in*Pseudomonas aeruginosa* biofilms. (Rizzi et al., 2019) report that*Bacillus subtilis* utilizes the formation of biofilms and the production of siderophores to take up iron (Fe) from the medium, likewise to ensure normal growth. Thereby the authors define iron (Fe) as the most important metal in biology (Rizzi et al., 2019). Additionally, (Oh, Andrews, & Jeon, 2018) found out that iron promotes biofilm formation through oxidative

stress and that it stimulates EPS production in *Campylobacter jejuni* . Hence, in their study an addition of iron significantly supported the formation of microcolonies in the early stage as well as the differentiation into mature biofilm structures, which is reflected here both by the OCT as well as the analysis of the structural biofilm parameters.

Additionally, measurements of the individual biofilms via attenuated total reflectance infrared spectroscopy (ATR-IR) were performed (data presented in SI Figure 2). It was verified that experiments E1 and E3 ($c = 2.5$ mg/L Fe^{2+}) as well as E2 and E4 ($c = 0.25$ mg/L Fe^{2+}) are correlating, respectively, which coincidences with the results of the growth experiment (Figure 2). According to literature, Fe is mainly incorporated into the biofilms via polymorphs of iron oxide-hydroxides (x-FeOOH) (Chan, Stasio, Welch, Fakra, & Banfield, 2004; Florea et al., 2011; Neu et al., 2010; Omoike, Chorover, Kwon, & Kubicki, 2004). Thereby, in experiments E1 and E3 an incorporation of α-FeOOH took place, whereas in E2 and E4 β-FeOOH was stored into the biofilms. This was found in (Wagner, 2011) and (Ivleva, Wagner, Horn, Niessner, & Haisch, 2010), too, whereby the authors documented an incorporation of γ-FeOOH into waste water biofilms. As maintained by (Wagner, 2011) and (Mohle et al., 2007), cross-linking of iron with the biofilm matrix ensured an increased stability of the biofilms. α-FeOOH is a highly reactive compound and (Omoike et al., 2004) proved that an interaction of *Bacillus subtilis*biofilms with α-FeOOH ensures an energetically stable connection for further EPS- and cell adhesion. This made it possible for the biofilms in E1 and E3 ($c = 2.5$ mg/L Fe^{2+}) to grow increasingly and to remain stable even at a high flow velocity ($u = 3.75$ cm/s) without sloughing events of microcolonies. With its loose structure, β-FeOOH exhibited the ability to store high amounts of water (Mei, Liao, Wang, & Xu, 2015). Thus, the low addition of iron(II) in E2 and E4 ($c = 0.25$ mg/L Fe^{2+}) as well as the storage of β-FeOOH into the matrix could be reasons for the reduced accumulation of biofilm mass. Potentially, the type of x-FeOOH incorporation is concentration-dependent and dependent of the bacterial organism or its (cell) surface because absorptions were clearly distant from each other, as pointed in (Mei u. a., 2015), too.

Conclusion

Bacillus subtilis biofilms were cultivated at Fe^{2+} inflow concentrations of 0.25 and 2.5 mg/L, respectively, as well as at wall shear stress levels of 0.05 and 0.27 Pa, respectively. Structure development was successfully monitored by means of optical coherence tomography and quantified by a large set of ten structural parameters. Through analysis of ten replicates the following conclusions are drawn:

1. Iron is - independently of its concentration - incorporated and accumulated within the biofilm matrix as modifications of FeO(OH).*Bacillus subtilis* biofilms cultivated in this study showed the accumulation of α- and β-FeO(OH).
2. FeO(OH) seems to provide cross-linking abilities and may thrive EPS production enhancing the adhesive as well as cohesive strength of investigated biofilms.
3. A positive correlation between the Fe^{2+} inflow concentration and biofilm development/accumulation partially compensates increased detachment of biofilm at elevated wall shear stress levels.

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There is no conflict of interest to disclose.

Nomenclature

α significance level

AFM Atomic Force Microscopy

ANOVA ANalysis Of VAriance

ATR-IR Attenuated Total Reflectance Infrared Spectroscopy

c concentration (mg/L)

CLSM Confocal Laser Scanning Microscopy

E1-E4 Experiments E1 to E4

EDX Energy-Dispersive X-ray microanalysis

EPS Extracellular Polymeric Substances

FC Flow Cell

Fe^{2+} Iron(II)

H_1-H_3 Hypotheses 1 to 3 for the ANOVA

ICP-OES Inductively Coupled Plasma Optical Emission Spectroscopy

LB Luria Broth

MIP Maximum Intensity Projection (height map)

OCT Optical Coherence Tomography

p exceedance probability (significance value)

PVC Poly-Vinyl-Chloride

ROS Reactive Oxygen Species

Q volumetric flow rate (mL/min)

SEM Scanning Electron Microscopy

τ_w wall shear stress in flow cells (Pa)

u flow velocity (cm/s)

x-FeOOH iron oxide-hydroxide polymorph

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Tables

Table 1: Overview of conducted experiments.

	$\mathbf{c_{Fe^{2+}}}$		$\mathbf{u}$	$\tau_\mathbf{w}$
Experiment	(mg/L)	**Q** (mL/min)	(cm/s)	(Pa)
E1	2.5	1.0	0.75	0.05
E2	0.25	1.0	0.75	0.05
E3	2.5	5.0	3.75	0.27
E4	0.25	5.0	3.75	0.27

Table 2: Overview of all structural parameters used as well as their abbreviation, unit and interpretation.

Parameter	Abbr.	Unit	Interpretation
Mean biofilm thickness	L_F	µm	Biofilm height in z-direction; calculated from the bulk-biofilm interface to t
Substratum coverage	SC	%	Coverage of the flow cell bottom with biofilm; 100 % minus this parameter
Textural entropy	TE	-	Is a measure of the randomness of the pixel intensity distribution and thus
Fractal dimension	FD	-	Describes the irregularity of the aggregates‘ surfaces; higher values equal h
Skewness	R_{SK}	-	Determines the occurrence of low (valleys; $\backslash nR_{\mathrm{SK}}<0$) and high biofilm colo
Kurtosis	R_{KU}	-	Defines the distribution of these occurred valleys and hills on the biofilms'
Average second moment	ASM	-	Direction orientated indicator of the cell clusters (higher values describe di
Inverse difference moment	IDM	-	Similar to ASM but distance orientated (lower values indicate that distanc
Average vertical run length	AVRL	µm	Mean colony width in y-direction; calculated from separated biofilm aggreg
Average horizontal run length	AHRL	µm	Mean colony length in x-direction; calculated from separated biofilm aggre

Table 3: Two-factorial variance analysis of calculated structural biofilm parameters shown as p-values. $p < \alpha$corresponds to a rejection of the hypothesis. H_1: no difference in structural biofilm parameters due to flow velocities. H_2: no differences caused by varied Fe^{2+} concentrations. H_3: no correlation between the two parameters c and u.

Two-factorial variance analysis (α = 0.01)

	ANOVA	ANOVA	ANOVA
Structural parameter	$\mathbf{H_1}$	$\mathbf{H_2}$	$\mathbf{H_3}$
L_F	0.45	$0.90{\cdot}10^{-4}$	0.24
SC	0.84	$0.10{\cdot}10^{-4}$	0.57
TE	0.35	$0.30{\cdot}10^{-5}$	0.24
R_{SK}	0.07	$0.18{\cdot}10^{-2}$	0.61
FD	0.11	$0.12{\cdot}10^{-4}$	0.43
ASM	0.63	$0.27{\cdot}10^{-5}$	0.32
IDM	0.79	$0.97{\cdot}10^{-5}$	0.55
	Scheirer-Ray-Hare	Scheirer-Ray-Hare	Scheirer-Ray-Hare
Structural parameter	$\mathbf{H_1}$	$\mathbf{H_2}$	$\mathbf{H_3}$
R_{KU}	0.16	$0.56{\cdot}10^{-5}$	0.21
AHRL	0.17	$0.12{\cdot}10^{-6}$	0.04
AVRL	0.78	$0.17{\cdot}10^{-4}$	0.21

Figures

Figure 1: Biofilm height maps (maximum intensity projections, MIPs) of day 10 comparing biofilms of each condition. The calibration bar resembles the biofilm height (L_F) in µm. The scale bar equals 1 mm.

Figure 2: Comparison of the structural biofilm parameters mean biofilm thickness L_F, substratum coverage SC, textural entropy TE, skewnessR_{SK}, kurtosis R_{KU}, fractal dimensionFD, angular second moment ASM, inverse difference moment IDM, average horizontal AHRL and average vertical run length AVRL over the course of the experiments (10 days). Inoculation took place on day 0. An overview and meaning of the parameters is provided in Table 2. $N = 10$ replicates; E1/E3, [?]$c = 2.5$ mg/L Fe^{2+}; E2/E4, [?] $c = 0.25$ mg/L Fe^{2+}; opened data points [?] $u = 0.75$ cm/s; closed data points [?] $u = 3.75$ cm/s.