Monitoring of bacterial community structure and growth: An alternative tool for biofilm microanalysis

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ABSTRACT

Microorganisms, such as bacteria, tend to aggregate and grow on surfaces, secreting extracellular polymeric substances (EPS), forming biofilms. Biofilm formation is a life strategy, because through it microorganisms can create their own microhabitats. Whether for remediation of pollutants or application in the biomedical field, several methodological approaches are necessary for a more accurate analysis of the role and potential use of bacterial biofilms. The use of computerized microtomography to monitor biofilm growth appears to be an advantageous tool due to its non-destructive character and its ability to render 2D and 3D visualization of the samples. In this study, we used several techniques such as analysis of microbiological parameters and biopolymer concentrations to corroborate porosity quantified by 2D and 3D imaging. Quantification of the porosity of samples by microtomography was verified by increased enzymatic activity and, consequently, higher EPS biopolymer synthesis to form biofilm, indicating growth of the biofilm over 96 h. Our interdisciplinary approach provides a better understanding of biofilm growth, enabling integrated use of these techniques as an important tool in bioremediation studies of environments impacted by pollutants.

1. Introduction

In medicine, natural science and engineering, strategies to monitor biofilm growth and behavior in response to stress have become a major challenge [1–5], and several methodological approaches are necessary to provide an accurate analysis of the role and potential use of bacterial biofilms.

In recent years, imaging techniques have become a great tool for ecological studies involving bacterial biofilms, since they allow visualization of bacterial biofilms as well as bacterial cells [6–8]. In addition, depending on the technique employed, other factors can be observed, such as the association of pollutants with biofilms and other substances, and the monitoring of biofilm development over time. For these reasons, imaging techniques that allow monitoring of bacterial biofilms in the environment have become extremely important tools for bioremediation studies [9]. The advent of X-ray techniques are a good alternative to visualize the bacterial biofilm, mainly since they provide 2D and 3D visualization of the biomass, indicating growth of the biofilm over 96 h. Our interdisciplinary approach provides a better understanding of biofilm growth, enabling integrated use of these techniques as an important tool in bioremediation studies of environments impacted by pollutants.
microorganisms, since it ensures the proximity of different metabolically-dependent physiological groups [15]. This matrix hosts the activity of extracellular enzymes in an area close to the cells, allowing efficient use of the products of enzymatic reactions for bacterial metabolism [16]. EPS therefore play a key role in biofilm formation, mass transfer through biofilms, adsorption by biofilms of different metals and organic/inorganic compounds and, most importantly, provides biofilms with structural support (shear resistance) [7,17–21].

The imaging techniques, such as computerized tomography, allow access to these biofilm properties and corroborate the results obtained by analytical quantification of standard microbiological parameters, providing a new way to monitor the biofilm growth in the environment. Thus, when associated with other types of analysis (e.g., enzymatic, biopolymer and cell biovolume), imaging techniques and especially non-invasive approaches may become important tools for the study of biofilm behavior in the environment and its potential role in bioremediation. The main objective of this work was to monitor the growth of a bacterial biofilm in porous media by integrating the X-ray imaging techniques with several techniques already recognized for their individual utility in microbial analysis.

2. Material and methods

2.1. Sampling

The bacterial consortia were isolated from surface sediments collected in the intertidal region of Jurujuba Beach, near the entrance of Guanabara Bay, Rio de Janeiro, Brazil (22°93'88.97"S; 43°11'28.03"W). This location was selected because the region presents a history of environmental degradation over recent years and therefore we could acquire consortia resistant to the stressors present in that area (Fig. 1), such as the metals Pb, Ni, Cu, Cr, Zn and Mn [22]. The sediment was stored in a sterile plastic bottle, and packed in a cool box with ice on the way to the laboratory, where the culture medium for the bioassay was prepared (the medium specifications are in the next section).

2.2. Bacterial consortia maintenance and isolation

The culture medium used for isolation and maintenance of the consortia included Bacto peptone (5 g L⁻¹, wt/vol) and urea (2 g L⁻¹, wt/vol) as carbon and nitrogen sources, respectively, as well as 75% seawater (75% seawater + 25% deionized water). Seawater was collected at Itacoatiara beach (22°58'28.3"S 43°02'20.2"W), area without a history of pollution, located in Rio de Janeiro State, Brazil, and it was pre-filtered (Millipore® Cellulose, 0.45 μm) to remove particulate material [23]. The culture medium was sterilized by autoclaving for 20 min at 120 °C [24].

An aliquot of the collected sediment was inoculated into an Erlenmeyer flask containing 250 ml of the culture medium and incubated in a bacteriological growth oven at 37 °C for 15 days before the start of the bioassay. This pre-inoculum procedure was necessary to attain a minimum biomass of bacteria, which was quantified by epifluorescence microscopy and analyzed for enzymatic activity at the time of being introduced into the bioassay (time zero).

2.3. Bioassay

Bioassays were adapted to allow visualization of biofilm extracted from the environment. Experiments were performed in microcosms consisting of glass cores (50 mm × 10 mm), which were filled with glass microspheres (Sö Esferas®) with diameter from 1.0 to 2.5 mm. A 150 μm pore size mesh was later fixed to both ends of the cores, which allowed fluid passage and retention of the microspheres throughout the bioassay. The microspheres were used as a substrate for biofilm growth, representing a porous medium such as compartmentalized coastal sediments. Each system was pre-washed with 70% ethanol (vol/vol) and the microspheres were autoclaved for 20 min at 120 °C. For the bioassay, the pre-inoculum was added to 3 L of sterile culture medium in a Kitazato flask. We inoculated 1.63 × 10⁹ cells. cm⁻³ at the beginning of the experiment. Analyses were conducted at 0, 24, 48, 72 and 96 h. At each time, three tubes were removed. At the base and at the top of each tube there were connections that allowed the opening or closing for the flow to pass, thus, the removal of one tube did not affect the continuity of the experiment in the other tubes until the end of 96 h. 1 g of the tubes content (microspheres + biofilm) was aliquoted for each triplicate of each microbiological analysis performed. All samples were analyzed in triplicates, with one control for each sample. Quantification of bacterial biomass, enzymatic activities (dehydrogenase and esterase) and microtomographic acquisition were performed at each time-point. Quantification of biopolymers was performed at time-point 0 and 96 h. To maintain bacterial growth throughout the experiment, an S160 submersible pump (Sarlobetter®) was used to maintain a constant flow (0.35 ml s⁻¹) of the solution containing the culture medium through the cores as shown in Fig. 2 [1]. Three independent bioassays, under the same conditions and using the same protocols, were performed using the same initial culture medium for all.

![Fig. 1. Map of the Guanabara Bay with the collection point represented in red at Jurujuba Beach, Niterói, Rio de Janeiro-Brazil. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](image1)

![Fig. 2. Illustrative Scheme of the bioassay. “N” represents the Number of glass cores in each experiment.](image2)
2.4. Quantification of microbiological parameters

2.4.1. Quantification of esterase enzyme activity (EST)
Quantification of EST was performed in triplicate according to Ref. [25]. The method is based on the estimate of the fluorescein produced in the sample treated with fluorescein diacetate (FDA) solution and incubated at 24 °C for 75 min on a mechanical shaker. The results were obtained using an optical spectrophotometer (Spectronic 20D®), the optical density (O.D.) was observed at a wavelength of 490 nm. The results are expressed in μg fluorescein. g⁻¹.

2.4.2. Quantification of the activity of dehydrogenase enzymes (DHA)
Quantification of DHA was performed in triplicate with the aid of an optical spectrophotometer (Spectronic 20D®) at 475 nm according to Ref. [26]. The method is based on the color change of INT (2(pyrdophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium), which works as an artificial electron acceptor. The product of the reaction is INT-F (iodonitrotetrazolium formazan chloride). The results are expressed as mg INT-F.mg⁻¹.

2.4.3. Quantification of bacterial cells
Bacterial cells number was established according to Ref. [27,28]. The samples were preserved in formaldehyde 4% (vol/vol). 75 μL of the chromophore Acridine Orange (100 μg ml⁻¹, wt/vol) was applied to a 2 mL sample after serial dilution. The sample was then stained for 5 min before filtration with a black nucleopore membrane (Isopore Membrane, polycarbonate, Hydrophilic, 0.22 μm, 25 mm, brown, plain). Quantification was performed by epifluorescence microscopy (x1000; Axios 50, Zeiss®, Texas Triple Red-Fluorescein-DAPI isotype), and the number of cells was estimated by calculating the total number of cells counted (sum of all fields counted), in triplicate. The results are expressed in cells. cm⁻³.

2.5. Quantification of biopolymers
Determination of total biopolymers (carbohydrate, lipids and proteins) was performed in triplicate. All determinations were done by the spectrophotometric method. Proteins (PRT) were analyzed by adapting the Hartree extraction [29]; with the modifications provided by Ref. [30]; using phenol compensation. For quantification, an optical spectrophotometer (Spectronic 20D®) was used at a wavelength of 650 nm. Bovine albumin, fraction V (Sigma), was used as a standard. Lipids (LPD) were analyzed according to Ref. [31] and tripalmitine was used as a standard. Carbohydrates (CHO) were quantified according to Ref. [32] and optimized by Ref. [33]; using glucose as standard.

2.6. 3D monitoring of biofilm growth

2.6.1. Microtomographic acquisition
A concentrated solution of BaSO₄ (0.3 g ml⁻¹, wt/vol) was added only to glass cores subjected to data acquisition, which had been removed from microcosms. The solution percolated between samples for 4 h prior to glass cores subjected to data acquisition, which had been removed. The system used for acquisition was the Skyscan microCT® Bruker microCT), with the objective of quantifying the porosity of the samples across the 96 h of bioassays. All calculations were performed based on the region of interest (ROI) and thresholding (TH). All tests performed in CTAn® for sample segmentation are described in the software manuals [35,36]. Porosity was determined by differentiating the sum of the spheres and the biofilm from the voids in the sample, from which the CTAn software could calculate the porosity (%) of each sample in 3D. Total porosity was established by the ratio of total pore space to total sample volume.

2.6.3. Image post-processing
Image post-processing was performed using the CT-Analyzer software (version 1.13.5.1, CTAn® - Bruker microCT), with the objective of quantifying the porosity of the samples across the 96 h of bioassays. All calculations were performed based on the region of interest (ROI) and thresholding (TH). All tests performed in CTAn® for sample segmentation are described in the software manuals [35,36]. Porosity was determined by differentiating the sum of the spheres and the biofilm from the voids in the sample, from which the CTAn software could calculate the porosity (%) of each sample in 3D. Total porosity was established by the ratio of total pore space to total sample volume.

2.7. Statistical treatment of data
All statistical analysis was performed using the R Core Team® software data package, Version R 3.48 3.3.2, Austria [38]. The Kolmogorov-Smirnov test was used to analyze the normality of the data and the Bartlett test for the homoscedasticity of variances [39]. As the data did not present a normal distribution and also did not present a homogeneous variance, the Kruskal-Wallis non-parametric test, ANOVA equivalent, was used for the analysis of microbiological parameters and porosity. The non-parametric test was applied in order to verify possible significant differences between the times in the bioassay and differences among the independent bioassays.

To observe the significant differences between the times within the bioassay, the Kruskal-Wallis test was applied. This test combines information from 1-way non-parametric analysis results with additional calculations to perform the non-parametric multiple comparison procedure [40]. Data were considered significant when p ≤ 0.05.

When comparing two paired samples, which replaces the Student’s t-test, when the data do not meet the requirements of the latter [39]. The data were considered significant when they reached p ≤ 0.05.

In the case of biopolymers, to assess whether there was a significant variation in the concentration between time 0 h and time 96 h, the Wilcoxon t-test was used. This test is a non-parametric method for comparing two paired samples, which replaces the Student’s t-test, when the data do not meet the requirements of the latter [39]. The data were considered significant when they reached p ≤ 0.05.

The results are illustrated by means in bar graphs, in which the average results and the standard deviation between the triplicates are presented (mean ± SD), and comparative graphs in lines, in which the means are presented for each time of the bioassay.

3. Results

3.1. Analysis of microbiological parameters and biopolymers
The results are represented by means in bar graphs with the standard deviation between the triplicates presented (mean ± SD). For the bioassay (Fig. 3A–D), we provided an inoculum of 1.63 × 10⁶ cells. cm⁻³ at time 0 h, representing the minimum biomass necessary for quantification of bacterial cells. During the bioassay, dehydrogenase activity exhibited the same trend as bacterial biomass, increasing exponentially in the first 24 h. The activity of the dehydrogenase enzymes

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increased from 0.004 ± 0.0 mg INT-F.mg⁻¹ to 0.20 ± 0.07 mg INT-F.mg⁻¹ in the first 24 h, and then, between 24 h and 48 h, decreased to 0.009 ± 0.0 mg INT-F.mg⁻¹. Esterase activity increased from 0.0001 ± 0.0001 μg FDA g⁻¹ at the beginning of the bioassay, to 0.0013 ± 0.0002 μg FDA g⁻¹ in 24 h, followed by a decrease to 0.0003 ± 0.0001 μg FDA g⁻¹ in 48 h. Bacterial biomass increased to 4.96 × 10⁶ ± 1.52 × 10⁶ cells cm⁻³ in the first 24 h of the bioassay, and then decreased to 2 × 10⁵±2.94 × 10⁶ cells cm⁻³ in the subsequent 24 h. After 48 h, dehydrogenase and esterase activities as well as biomass all increased again until the end of the bioassay (96 h), with biomass (7.37 × 10⁹±4.32 × 10⁹ cells cm⁻³) and esterase activity (0.0016 ± 0.0001 μg FDA g⁻¹) both reaching their maximum values at the 96 h time-point.

The Kruskal-Wallis test indicated that there was a significant difference between time-points for all microbiological parameters analyzed in the bioassay. The Kruskal-Wallis test showed that there was a significant difference between time-points for dehydrogenase activity (p = 0.0099), with the largest difference between the 0 h and 24 h time-points according to the post hoc KruskalMC test. A significant difference was also found for esterase activity (p = 0.0119), this time the largest difference was between the 0 h and 96 h time-points (KruskalMC test). Bacterial biomass also differed significantly between time-points (p = 0.0148) and, as for esterase activity, a KruskalMC test revealed this to be due to a large difference between the 0 h and 96 h time-points.

Biopolymers increased their respective concentrations at the end of the bioassay. Proteins increased from 0.007 ± 0.008 μg g⁻¹ at the beginning of the bioassay (0 h) to 0.113 ± 0.007 μg g⁻¹ at the end (96 h). Carbohydrates, from 0.014 ± 0.002 μg g⁻¹ to 0.057 ± 0.016 μg g⁻¹. And the lipids increased from 0.027 ± 0.003 μg g⁻¹ to 0.044 ± 0.021 μg g⁻¹. The Wilcoxon t-test showed no significant difference between biopolymer concentrations at time 0 h and 96 h. The Kruskal-Wallis test did not show any significant difference in the microbiological parameters evaluated between the 3 independent bioassays.

3.2. Computed microtomography analysis of porosity

Porosity was determined by differentiating the sum of the spheres and the biofilm (white) from the voids (black) in the sample (Fig. 4A and B), from which the CTA software could calculate the porosity (%) of each sample in 3D (Fig. 4C). Total porosity was established by the ratio of total pore space to total sample volume (Fig. 4D).

We found that total porosity had slightly decreased in the samples after 96 h of the bioassay. Porosity was 53.93% at time 0 h and declined to 48.21% by 96 h (a decrease of 5.72%), with the lowest porosity recorded at the 48 h time-point (44.99%). A Kruskal-Wallis test did not evidence a significant difference in porosity between the time-points (p > 0.05). However, the fact that porosity had decreased by the end of the bioassay suggests an increase in biofilm production over time. Although colonization of the biofilm was visually perceptible at the end of the bioassay, quantitative validation is necessary, such as can be provided by bacterial enzymatic analysis over time.

3.3. Linking microbial parameters with computerized microtomography

We found a correlation between our data on biomass and dehydrogenase activity (Fig. 3A and C) in that both increased over the 96 h of the bioassay. Biopolymer concentration also followed the same trend as esterase activity, increasing over the course of the bioassay (Fig. 3B and D).

Although the graphs show an inversely proportional correlation between enzymatic activity and porosity (Fig. 5), Spearman’s correlation test showed no conclusive evidence on the significance of the association between variables, with the exception of biomass and esterase (Table 1).
Increased enzymatic activity indicates an increase in EPS production and consequent biofilm formation, which could cause porosity to decrease. Thus, our quantification of enzymatic activity corroborated the results on porosity obtained through computerized microtomography.

4. Discussion

4.1. Microbiological parameters and biopolymers

The activities of the dehydrogenase and esterase enzymes presented the same pattern, as did bacterial biomass. Stressed communities, such as those present in Guanabara Bay, present altered energy demand, nutrients and their restoration. Energy demand among microbial communities can be measured through the activity of esterases, since these enzymes play a key role in the hydrolysis of organic matter and, consequently, in the energetic and nutrient cycles of the ecosystem [41]. Microbial cell viability and energy generation (ATP) can be assessed by the activity of dehydrogenase enzymes [25]. [41] reported that bacterial communities present in environments with high concentrations of toxic metals and organic matter exhibit increased esterase activity.

After 48 h of bioassay, the bacterial community began a new reproductive cycle, increasing its biomass (as shown by the enzymatic activity). Bacterial communities cycle through a sequence of phases [42], and subpopulations of biofilm cells coexist at different growth stages and, when exposed to subinhibitory concentrations of pollutants (such as metals), these cells die at different rates [43]. As a consequence, the surviving cells become less sensitive to toxic stress and the subsequent generation would likely present a genetic expression that is distinct from that of the previous generation [44]. According to Ref. [45]; this natural process of phenotypic diversification develops the resistance or tolerance of a biofilm to multiple metals, which can therefore be a useful
environmental indicator.

Although the values for enzymatic activity are lower than those found for other bacterial consortia isolated from Guanabara Bay sediments [41, 46,47], it was sufficient not only to sustain the bacterial community but also to increase the cellular biomass by the end of the bioassay [25]. demonstrated that the kinetics of enzymatic reactions track cell density over time, at least until the carbon source becomes a limiting factor, as observed in this bioassay.

The increased biopolymer concentrations we document here evidences synthesis of organic EPS components to sustain formation of the biofilm over the 96 h of bioassay. Although the increase was not statistically significant, we observed a higher amount of proteins relative to the other components at the end of 96 h, which has also been reported for other bacterial consortia [49–50]. The predominance of proteins in EPS may be due to the presence of a large amount of exoenzymes, as suggested by Refs. [51]. In addition, the higher protein content enhances hydrophobic interactions and bonding to polyvalent cations, facilitating cell aggregation and conferring greater stability to the biopolymer network [52], which are prime characteristics for the biofilm life strategy.

No significant difference was detected by the Kruskal-Wallis test between the microbiological results presented in the 3 independent bioassays, using the same initial inoculum for all, and also between times within a single bioassay. The standard deviation shown in the graphs demonstrates the expected variability of the replicates. In this way, the repeatability of the experiment is confirmed, demonstrating that there are no flaws even with less sampling effort.

4.2. Porosity analysis

Our quantification of biofilm porosity showed that there had been an increase in biofilm production by the end of the 96 h bioassay. Despite being efficient in other studies [53], here the porosity cannot be conclusively correlated with other analyzes, due to the lack of significant differences over time reported in this study. This lack of significant variation is probably linked to a limitation of the method and to the short duration of the bioassay, since injection of the chemical contrast agent causes detachment of weakly bonded biofilm fragments that predominate during the initial stage of growth [54]. In addition, in porous environments, space is much more limited, and biofilm growth tends to attenuate the fluid flow that supplies cells with nutrients, which makes their dispersion difficult [55].

In porous media (such as those of coastal sediments), biofilm growth can induce substantial changes in mass transport dynamics [56,57]. Variation over time of macroscopic parameters such as permeability, porosity and dispersion indicates biofilm development [1]. Thus, monitoring porosity over time using computed microtomography and corroborating the results by other techniques can be an effective ecological monitoring tool for sedimentary environments, providing a better understanding of biofilm behavior in such systems [58].

Both the spatial distribution of the biofilm and changes in porosity are important parameters to investigate the impact of biofilms on the hydrodynamics of porous media and mass transport, as well as the processes that occur during bioremediation. Our results demonstrate that computed microtomography can provide experimental data for ratiﬁcation of mathematical models of the porous media associated with biofilm growth [53,58,59].

Table 1

| Porosity | Esterase | Dehydrogenase | Biomass |
|----------|----------|--------------|--------|
|          | −0.30    | −0.60        | −0.30  |
| Esterase | 0.90     | 1.0*         | 0.90   |
| Dehydrogenase | −0.60  | 0.90         |        |
| Biomass  | −0.30    | 1.0*         | 0.90   |

*Significant p-values < 0.05.

4.3. Linking microbial parameters with computerized microtomography

Our comparative analysis revealed that the activity of dehydrogenase enzymes had increased by the end of the bioassay, evidencing growth of the biofilm through the energy demand (ATP). The increased biopolymer concentrations at the end of the 96 h bioassay indicated that there was a greater demand for the production of EPS to support the biofilm. This supposition is supported by the increased activity of esterase enzymes during the bioassay, indicating that there was a recruitment of substrate for the synthesis of EPS components.

Despite not showing significant differences, enzymatic activity was inversely proportional to the porosity of the samples, with the highest values of enzymatic activity occurring at the same time-point or before that of the lowest values of porosity. This finding can indicate that voids in the samples were colonized by biofilm throughout the bioassay [21]. suggested that energy investment under conditions of environmental stress is directed to the production of EPS. Thus, the evaluated microbiological parameters, together with our assessment of biopolymer concentrations, endorse the findings from microtomography, demonstrating how these techniques together can be used to monitor biofilm growth in porous media such as coastal sediments and soils.

Dehydrogenase and esterase activity are widely reported as indicators, respectively, of pollution and bacterial viability [7,21,41,46,47, 60–63]. According to Ref. [25]; assessment of dehydrogenase and esterase activity provides an effective means of monitoring microbial activity over time since these parameters are closely correlated with ATP content and cell density of pure and mixed microbial cultures.

Monitoring bacterial biofilms by means of integration of several correlated techniques allows better interpretation of results, leading to a better understanding of the role of biofilms in the process of bioremediation and bacterial behavioral responses to organic and inorganic pollutants. Imaging techniques such as computed microtomography enable 2D and 3D monitoring of biofilm samples, such as from contaminated soils and sediments, and quantification of their geometric and physicochemical properties.

5. Conclusions

Computed microtomography proved to be a viable technique for monitoring bacterial biofilm growth, with data generated by this technique being corroborated by other established methodologies. The microbiological parameters evaluated here, as well as biopolymer concentrations, served to corroborate microtomography data through their correlation with sample porosity. We also present dehydrogenase and esterase activity as good indicators, respectively, of environmental stress and bacterial viability.

Application of several integrated techniques, such as the evaluation of microbiological parameters and biopolymers and qualitative and quantitative analysis through microtomography imaging enables a better understanding of biofilm behavior and growth patterns of the same in different substrates, such as ducts and bioreactors. Thus, interdisciplinary environmental monitoring is an extremely important tool for the study and application of bioremediation techniques using bacterial biofilms in soils and coastal sediments.

CRediT authorship contribution statement

Guilherme O.A. da Silva: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft.
Simone Penna: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft.
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Miran A.C. Crapez: Conceptualization, Formal analysis, Funding.
acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing - original draft.

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