BAOJ Microbiology

Guilherme O A da Silva, et al, BAOJ Microbio 2017 3: 1
3: 026

Review Article

Imaging Techniques for Monitoring Bacterial Biofilms in Environmental Samples – an Important Tool for Bioremediation Studies

Guilherme O A da Silva[1*], Simone Pennafirme[1], Ricardo Tadeu Lopes[2], Inayá Lima[2] and Mirian A C Crapez[1]

[1]Universidade Federal Fluminense (UFF), Instituto de Biologia, Departamento de Biologia Marinha, e Programa de Pós-graduação em Biologia Marinha e Ambientes Costeiros, Niterói, RJ, Brazil.

[2]Nuclear Engineering Program/COPPE. Federal Univertsity of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

Abstract

Biofilm formation has been shown to be an efficient process for removing contaminants from the environment and has great potential as a bioremediation agent. This review aims to examine the advantages and limitations of imaging techniques currently used for biofilm visualization; namely, scanning electron microscopy (SEM) and transmission electron microscopy (TEM), epifluorescence (EPI), confocal laser scanning microscopy (CLSM), scanning transmission X-ray microscopy (STXM) and computed microtomography (μCT). The advent of X-ray techniques appears to be a good option, mainly for providing 2D and 3D images of biofilms, which allows faithful reconstruction of images that, represent the environment. μCT is a noninvasive technique that allows *in situ* monitoring of bacterial biofilms. STXM is an application of molecular environmental science, including studies of biofilms and bacteria–metal binding interactions. Combinations of several techniques, besides imaging, can be a more viable way to achieve reliable monitoring of bacterial biofilms in nature, facilitating considerable advances in bioremediation studies of environments impacted by heavy metals, oil spills and other pollutants.

Key Words: Bacteria; Biofilm; Bioremediation; Imaging Techniques; 3D Image

Introduction

Natural environments are intensely colonized by microorganisms. Bacteria are the most abundant and versatile of these colonizers and constitute a significant fraction of the entire living terrestrial biomass [1]. Most are organized into biofilms, i.e., complex associations of microbes embedded in an extracellular organic matrix consisting of extracellular polymeric substances (EPS) secreted by the cells [2]. By organizing into biofilms, organisms create their own microhabitats with pronounced gradients of biological and chemical parameters. Through these gradients, they can use substrates and energy effectively [3].

Perhaps the most important roles of biofilms are biosorption and biomineralization, which facilitates use of these organisms in bioremediation of areas impacted by heavy metals and other pollutants. Biofilms have been shown to be efficient in removing contaminants, while also being less damaging to the environment [4-9]. Furthermore, bacteria may be used as biosorbents because of their small size, ubiquity, ability to grow under controlled conditions, and their resilience to a wide range of environmental situations [10-13].

Importantly, EPS and bacterial cell walls can participate in metal adsorption reactions and potentially mediate mineral precipitation and dissolution reactions [8,14]. They can enrich some heavy metals, such as Pb, Cd, Cu and Zn, relative to the aqueous solute concentration of these elements [13,15]. Several studies have also addressed the hydrocarbonoclastic capacities of bacterial communities for recovery processes of environments impacted by oil [7,16-18]. Many of these studies report the oil degradation efficiency of such microorganisms and consequent increases in cell density and/or bacterial biomass [9-22]. EPS production with biosurfactant activity [23-26], and the use of dehydrogenase

***Corresponding author:** Guilherme O A da Silva, Universidade Federal Fluminense (UFF), Instituto de Biologia, Departamento de Biologia Marinha, e Programa de Pós-graduação em Biologia Marinha e Ambientes Costeiros, Niterói, RJ, Brazil, Tel: +55 21 99605 1726; E-mail: guilhermeoas@id.uff.br

Sub Date: June 16, 2017, **Acc Date:** June 28, 2017, **Pub Date:** June 29, 2017.

Citation: Guilherme O A da Silva, Simone Pennafirme, Ricardo Tadeu Lopes, Inayá Lima and Mirian A C Crapez (2017) Imaging Techniques for Monitoring Bacterial Biofilms in Environmental Samples – an Important Tool for Bioremediation Studies. BAOJ Microbio 3: 026.

enzymes as biomarkers of oil degradation [27-32].

EPS secretion is a general attribute of prokaryotic and eukaryotic microorganisms in natural environments [3]. The EPS matrix is comprised of different biochemical compounds secreted by microbes, including organic matter and cellular material/products arising from cell lysis [2,33]. As well as providing the protective physical infrastructure of biofilms, EPS also facilitates adhesion to surfaces [34,35] and promotes cell aggregation and biofilm accumulation [36-40]. Furthermore, EPS production by microbial cells can promote stabilization and protection of cells by increasing resistance to dehydration and biocides. The presence of cations and ions enables the EPS layer to retain organic and inorganic compounds, allowing the biofilm to tolerate higher contaminant and biocide concentrations [2,8,41].

Owing to their environmental importance, the role of biofilms has been studied in several ways, using differing techniques. In recent years, imaging techniques have become a great tool for these kinds of study because they allow visualization of the biofilm and the actual bacterial cells [42-44]. Furthermore, depending on the technique employed, other factors can be observed such as pollutants associated with biofilms and other substances and the monitoring of biofilm development through time. For these reasons, imaging techniques for monitoring bacterial biofilms in the environment have become extremely important tools for bioremediation studies of affected areas.

In this study, we have reviewed the imaging techniques used for biofilm visualization in environmental samples, describing their advantages and limitations, in order to support the choice of technique in accordance with the objectives of each study. Some techniques, such as microscopy, are already very widespread, while alternatives, such as X-ray sources, have also been shown to be important tools for the study of biofilms. Here, we describe applications of advanced microscopic techniques including confocal laser scanning microscopy (CLSM), epifluorescence (EPI), scanning and transmission electron microscopy (SEM and TEM, respectively), scanning transmission X-ray microscopy (STXM), and computed microtomography (μCT). This work aims to assist researchers in choosing the best imaging technique for a given study on bacterial biofilms, focusing on bioremediation processes. Next, we discuss six different proposals that could use several techniques for imaging bacterial biofilms or cells, depending on their individual goals.

Study Proposals

Biofilm Structure and Cell Morphology

Apart from traditional microscopic techniques, such as electron microscopy (SEM and TEM), new advanced techniques have been established to evaluate bacterial biofilms, including confocal laser scanning microscopy (CLSM) and X-ray computed microtomography (μCT). These techniques allow imaging of natural biofilm structure and morphology in variable ways, enabling both qualitative and quantitative analyses. Therefore, SEM, CLSM and μCT are being used in order to study complex microbial biofilm systems (Table 1) [42].

Electron microscopy uses an electron beam instead of photons, as in a conventional optical microscope. The use of an electron beam solves the resolution problem associated with the white light source [45]. SEM is widely used to image biofilms, but not for environmental samples. For example, SEM has been used to investigate capabilities of bioflocculant production and biosorption

Table 1: Objective and application of imaging techniques for bacterial biofilms in natural samples.

Technique	Objective/Application	References
CLSM	Biofilm structure; 3D biofilm morphology; Characteristics of biofilm growth; Quantification of biofilms; Study of biofilm edges	[51,52,58,42,64]
EPI	Differentiate bacterial cell morphotypes; Biomass quantification; Differentiate between healthy and dead cells	[76,78]
SEM	Quantify production of primary metabolites; Investigation of surface morphology; Imaging of biofilm growth	[59,46,87]
TEM	Investigate biosorption of heavy metals by bacterial strains; Investigation of EPS-produced by cyanobacteria for the bioremediation of heavy metals	[84,85]
STXM	Analyze structural and elemental composition	[70,72,42,44]
μCT	3D visualization in porous media	[54,55,56]

of zinc and lead by a novel bacterial species [46]. In that case, SEM was used to image the selected bacterial isolate, showing the surface morphology of the bacterial body with imminent production of extracellular bioflocculant, demonstrating rod shaped bacteria in clumps with prominent adhesion of extracellular products, most probably the bioflocculants [46,47]. This finding is important for bioremediation studies of areas impacted by heavy metals such as lead and zinc, and the use of bacterial extracellular bioflocculants as metal biosorbents [13,14,46,48].

Environmental SEM provides a fast, accurate image of biofilms, their spatial relationship to the substratum and elemental composition [49]. However, this technique requires prior preparation of the samples, with the risk of losing some EPS elements during this process [49]. Preparation of biological material for SEM requires extensive manipulation, including fixation, dehydration, and either air drying or critical-point drying because SEM operates in a high vacuum [49]. Non-conducting samples, including biofilms, must be coated with a conductive film of metal before the specimen can be viewed. Uncoated non-conductors build up local concentrations of electrons, referred to as "charging", that prevent the formation of usable images [49]. In addition, compared to other techniques based on X-rays, SEM does not provide a high resolution, creating difficulties for quantifying specific components on cell surfaces or biofilms and demonstrating the need for association of this technique with others to achieve study goals.

Due to the potential of laser microscopy, as well as more frequent availability of instruments and technological improvements, the number of CLSM manuscripts published in microbiology has increased exponentially [42]. CLSM is an optical imaging technique for increasing the optical resolution and contrast of a micrograph [50]. It enables reconstruction of 3D structures from obtained images by collating sets of images taken at different depths of field [50].

The applications of CLSM embrace structural examination of biofilms and bioaggregates, from 3D imaging of biofilm internal structures (including voids and channels) to imaging of EPS and its components [42]. Consequently, CLSM has kindled interest in the role of the EPS matrix in biofilms and could help deepen our understanding of the role of biofilms in bioremediation studies.

CLSM has been used to examine the morphology of bacterial cells in marine biofilms to analyze the role of these cells in the manganese cycle, as well as to evaluate the corrosion rate of metals immersed in sea water [51]. In that study, CLSM facilitated differentiation of filamentous and coccoidal cells, and suggested that filamentous organisms may be Mn metabolizers due to their location in the biofilm and based on other chemical analyses [51]. This finding could enable selection of an appropriate morphotype for reducing or avoiding corrosion of metallic structures immersed in sea water. For CLSM analysis, samples need prior preparation, based on fixation and staining with specific probe. Moreover, after microscopic acquisition, image post-processing is necessary [51].

Notwithstanding the immediate visual appeal of CLSM images, accurate reconstitution of biofilm morphology requires a lengthy and computationally-intensive succession of processing steps. Nonetheless, once performed, CLSM provides ample reward by enabling quantitative study of biofilm structure [52]. CLSM is the method of choice for monitoring structure formation of live biofilms in laboratory flow-cell reactors. As a result of its non-invasiveness and non-destructive character, CLSM enables in vivo reconstitution of microbial biofilm 3D structure in its naturally hydrated form [52,53]. However, despite its widespread use, the information captured by CLSM can only be very partially quantified, and even then quantification consists mostly of direct measures on the unprocessed images [52].

One of the greatest difficulties in monitoring environmental biofilms is the absence of 3D images, which would allow both better visualization and quantification of biofilms. However, this kind of analysis could be more accessible through X-ray computed microtomography (μCT). This technique uses an X-ray beam to provide 2D and 3D images at a micrometric scale, and permits quantification of the geometric properties of biofilms (e.g. area and volume) [54]. As a quali-quantitative and noninvasive technique, μCT can be a useful tool in bioremediation studies of bacterial biofilms from soil or aquatic sediments. Nevertheless, few studies have been published using μCT with this objective. Bioassays using microspheres as a substrate for biofilm percolation have been successfully performed, allowing quantification of the biofilm and analysis of growth patterns [54-56]. These studies are the basis for using μCT to monitor biofilm growth on natural sediments, as well as for bioremediation purposes of coastal areas. Addition of a chemical contrast agent is necessary for μCT imaging, but some such agents that do not significantly affect biofilm growth have already been used successfully [54-56]. The advantages and limitations of μCT are further discussed under Visualization of biofilms within porous media below.

Biofilm Quantification

Quantification of biofilms is of paramount importance in monitoring studies. Novel techniques such as CLSM and μCT enable this kind of analysis and have become essential tools in bioremediation studies. By providing 3D images, these techniques facilitate measurement of the geometric properties of biofilms and reduce associated errors. Approaches for quantification of biofilms through CLSM and μCT have evolved considerably, mainly due to the development of new image post-processing software. These

new advances allow evaluation of several geometric properties of biofilms, including volume, surface area coverage in each layer, biofilm thickness distribution, average biofilm thickness, volumes of microcolonies identified at the substratum, the fractal dimension of each microcolony identified at the substratum, roughness coefficient, distributions of diffusion distance and maximum diffusion distance, and surface to volume ratio from a 3D stack of biofilm images [57].

Quantitative parameters describing biofilm physical structure have been extracted from 3D CLSM images and used to compare biofilm structures, monitor biofilm development, and quantify environmental factors affecting biofilm structure [58]. Researchers have used biovolume, volume to surface ratio, and mean thicknesses to compare biofilm structures and have extracted quantitative parameters regarding morphological features from 2D and 3D images [58]. However, it will be necessary to develop more comprehensive parameters to describe heterogeneous biofilm morphology in three dimensions [58].

Both CLSM and μCT permit quantification of biofilm geometric properties. Besides these properties, μCT enables tracking of natural 3D biofilm growth over time with very high image resolution, representing a breakthrough in biofilm monitoring and assisting in investigations of bioremediation processes [56]. The technique is based on segmentation between empty and filled space (this latter representing the biofilm) to quantify the volume and area of the region of interest and also the porosity of the sample [54-56].

As for CLSM, μCT also needs image post-processing software for analyses and quantification of the biofilm, as well as the requirement for addition of a chemical contrast agent prior to image acquisition [54-56]. Although providing several advantages for the quantitative analysis of biofilms, as summarized in Table 2, both techniques require reasonable computational effort using analytical software to calculate the geometric properties of biofilms.

Biofilm Edge Structure and Elemental Composition

The ability of biofilm bacteria to sense and respond to molecular signals produced by neighboring biofilm cells is particularly important [59]. These characteristics make the edges of biofilms an important study area for a better understanding of cell-cell communication in biofilms. Also, biofilm edge studies allow investigation of the chemical signaling that occurs within these communities and intra-community EPS chemical compositions [60, 61]. Here, we present three examples using different techniques to assess this important topic, which have contributed to advances in bioremediation projects.

To understand microbial interactions between biofilms, it is necessary to perform rapid, real-time spatial quantification of small molecules in the microenvironment immediately surrounding biofilms [59]. Scanning electrochemical microscopy (SECM)—a variation of SEM— has been used to quantify small molecules surrounding a biofilm in 3D space, measuring concentrations of the redox-active signaling molecule pyocyanin (PYO) produced by biofilms of the bacterium *Pseudomonas aeruginosa* [59]. In other studies [62], authors addressed the challenge of metabolite spatial profiling by using SECM to quantify a primary metabolite produced by a bacterial biofilm in real-time [59].

SECM has the unmatched ability to determine the exact distance from an ultra-microelectrode sensing tip to a biological substrate through a feedback approach curve [63] and, thus, is able to measure the local concentration of redox-active small molecules over a biofilm. Using this technique, it is also possible to scan over a substrate in the x-y direction, providing a spatial concentration profile over the surface [59]. Using SECM it has been possible to demonstrate a paradigm for metabolite measurement and to show the possibility that electroclines and potentially other chemoclines produced by biofilms might play important biological roles, such as nutrient acquisition and toxic stress responses.[59]

It is also possible to use CLSM to study interfacial biofilms and to acquire time-resolved 3D data of biofilm structure [64]. CLSM can be used in a multi-channel mode, whereby the different channels map individual biofilm components with the aid of a software package for post-processing data and images [64]. Examination of the individual biofilm components using this multi-channel capability has allowed description of the spatio-temporal dynamic between diatoms and bacteria and between organic and inorganic matter during the phototrophic shift of a biofilm [64]. That study revealed new insights into the temporal development of a phototrophic biofilm, with multi-channel imaging facilitating parallel monitoring of the dynamics of individual biofilm components over time [64], showcasing CLSM as an important tool for bioremediation studies.

Scanning transmission X-ray microscopy (STXM) is an X-ray absorption technique that provides chemical and biochemical information on biological and environmental samples through the collection of sequences of images [42]. These sequences, over a range of energies, supply detailed quantitative mapping of chemical species because X-ray spectroscopy is based on the bonding structure of a particular chemical species [42, 65-67].

The fact that soft X-rays can penetrate water makes them ideal for studying hydrated samples, such as biofilms present in sediment and the water column [42]. With optimal calibration, this technique can provide a map of the chemical elemental composition of biofilm edges. Because the method uses the intrinsic X-ray absorption properties of the sample, there is no need to add reflective,

absorptive or fluorescent probes or markers that may generate artefacts and complicate analyses [42].

Many examples of STXM being applied to molecular environmental science already exist in the literature, including studies of biofilms and bacterial–mineral interactions [68]. The potential of STXM for examination of microbial communities and biofilms has been demonstrated in a series of recent publications with different aims, from mapping biomacromolecules [69] and metallic species (Fe^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}) [70] to the antimicrobial agent chlorhexidine in river biofilms [71].

STXM combined with X-ray fluorescence microscopy (SXFM) have been used to characterize the 3D structural and corresponding elemental distribution of bacterial biofilms of *Pseudomonas aeruginosa* [72]. The main advantage of this technique is that samples are fixed without contrast agents or microtomal sectioning, thereby maintaining an intact microbial community, which facilitates direct visualization of morphology together with elemental content [72]. This analysis is essential to gain a deeper understanding of biofilm evolution in order to develop potential strategies for biofilm monitoring [72], and can be used for studies of bioremediation. However, the combined techniques require an advanced X-ray source and a large computational effort to process the images.

Bacterial Cell Morphotypes, Cell Biomass Quantification, Identification and Quantification Of Healthy and Dead Cells

Quantification of bacterial cells and biomass are important to our understanding of the ecological role of bacteria in the environment [73]. Epifluorescent direct count techniques are frequently the methods of choice, yielding accurate estimates of total (including nonviable and viable) cell numbers in a wide variety of situations [73].

Epifluorescent direct counts are based on the property that, after excitation with low wavelength radiation, some substances absorb ultraviolet light and then emit radiation within the visible light spectrum [74]. For this reaction to take place, specific fluorochromes must be used. The two fluorochromes most often used in direct count methods are 3,6-bis[dimethylamino]acridinium chloride (acridine orange) and 4',6-diamidino-2-phenylindole (DAPI). With both stains, bacteria are identified on the basis not only of color but also of size and shape [73]. Direct count procedures involving acridine orange or DAPI have been used on a diverse array of samples, ranging from Antarctic soils [75, 76] to oyster tissue homogenates [77].

Bioremediation studies often use the epifluorescence technique to analyze variation in bacterial biomass in light of environmental pollution, such as by petroleum polycyclic aromatic hydrocarbons (PAH's) and heavy metals, as well as to determine the cells' metabolic condition (healthy or dead) [13,32,78]. Furthermore, a combination of EPI with others techniques can assist in determining the bacterial populations' resistance or tolerance to the presence of pollutants [13,79].

One of the greatest advantages of EPI is the practicality of sample preparation and, being a noninvasive technique, its efficacy for quantifying and evaluating cell condition and morphotype. In addition, bacterial physiology can be evaluated with the use of specific fluorochromes, with healthy cells emitting green light and dead cells emitting orange light [80]. However, a large amount of initial biomass is required due to the serial dilutions necessitated by the sample preparation process. Moreover, samples for bacterial enumeration should be preserved immediately following collection to avoid changes in numbers, sizes, and shapes of bacteria, which may occur rapidly (often in less than 1 day) with storage. The most commonly used preservative is formaldehyde [73].

Mapping of Metal Biosorption in Biofilms

Heavy metal biosorption by EPS is an innovative technology for removal of contaminants from the environment [13, 81,5,82]. As urbanization and industry has expanded, indiscriminate release and accumulation of heavy metals in the environment has increased, becoming a major concern that demands sustainable remediation technologies to rectify and reestablish natural conditions worldwide [15].

Biosorption by EPS includes a number of passive accumulation processes, such as ion exchange, complexation, microprecipitation, absorption and desorption, allowing its use as an effective alternative strategy for metal removal and recovery [83]. Therefore, techniques that allow visualization and mapping of these chemical compounds attached to the EPS are extremely important for assessment and monitoring of the techniques for heavy metal bioremediation in the environment.

TEM is frequently used to visualize the surface of a biosorbent and is a useful tool for evaluating the compositional characteristics before and after biosorption of heavy metals [84]. Batch experiments using metal solutions have been conducted to visualize changes in the cell walls of a biosorbent caused by the sorption of Pb2+ [84]. It was observed that many clumps of materials were concentrated at the boundary of the cell wall, and that the shape of the cell was distorted due to the Pb^{2+} adsorption on the strains, suggesting that most of the Pb ions were sorbed at the cell wall [84]. TEM studies have also been performed in order to investigate the sites of metal accumulation and structural changes occurring both in *Gloeothece* sp. (a wild-type cyanobacteria) and mutant cells [85]. As expected, TEM showed that large numbers of metal ions, either Pb^{2+} or Cu^{2+},

were adsorbed to the sheath of the wild-type cells [85].

Although successfully used in studies of biosorption of heavy metals by bacterial strains and in investigations of EPS produced by cyanobacteria for the bioremediation of heavy metals, TEM has some limitations including the need for an electron probe and the sample preparation and post-fixation processes with osmium tetroxide in order to preserve and contrast the membranes.

Correlation of metals with specific biofilm components is possible, but incorrect conclusions can be reached due to metal re-distribution, re-speciation, limited sensitivity or biofilm component modification [70]. Electron microscopies (TEM and SEM) coupled with energy dispersive spectroscopy (EDS) are capable of mapping metals in biofilms. Although SEM-EDS is well suited to the determination of elemental composition, morphology and crystallinity, as well as nanoscale localization, the requirement of dehydration and sectioning can create artifacts such as shrinkage and particle aggregation [70].

In aquatic ecosystems, studies of speciation and quantitative spatial distributions of metals are still scarce, mainly in the context of biofilm components, including biochemical information [70]. Such information would promote a better understanding of biogeochemical processes and the role that biofilms play in metal toxicity and bioavailability for remediation [70].

STXM techniques allow *in situ* sample examination without fractionation and extraction or sectioning, thereby avoiding artifacts associated with the separation, dehydration, and sectioning processes. Also, these methods can provide quantitative maps of chemical species at environmentally relevant concentrations [70]. STXM has been used to study a cultivated river biofilm that contained a variety of natural (iron and manganese) and artificially-introduced (nickel) metals, demonstrating that EPS have a high affinity for metal ions and enabling the use of biofilms as metal biosorbents [70].

Most heavy metals may be sequestered from the water column by the sediment compartment, becoming unavailable to aquatic biota [44,86]. Thus, the pollutant-removing process is an important topic for proposals for remediation in contaminated areas [44]. Using SXFM and SEM-EDS for the purpose of observing whether bacterial consortia formed biofilms and sequestered metals from seawater, researchers observed that *Nitratireductor* spp. and *Pseudomonas* sp. consortia isolated from marine sediments are suitable organisms for Zn^{2+} accumulation from contaminated environments [44]. Moreover, they also concluded that biofilms may act as an important mechanism of metal biosorption and that bacterial consortia isolated from contaminated marine soils may show considerable potential for use as bioreactors as well as in bioremediation programs [44].

Despite being a highly effective technique for mapping metallic elements in biofilms, with several advantages, SXFM and its technical variations have some limitations, such as the need for a Synchrotron light source and beam time, and the difficulty in finding an ideal substrate for the growth of biofilms that does not generate artifacts during sample acquisition. Further, maintenance of full hydration can be challenging, and damage or adverse effects caused by radiation and X-ray absorption saturation can bias results.

Visualization of Biofilms within Porous Media

In subsurface porous media, such as soil and marine or riverine sediments, microorganisms tend to attach to the media and grow as a biofilm [87]. Biofilm thickness, hydraulic conductivity, pore velocity distribution, and surface roughness can affect the transport of nutrients and substrates to the growing cells in porous media and, of course, the rate of bioremediation [87]. Many studies mention observations of biofilm in their system. However, the sampling process frequently disrupts the integrity of porous media for subsequent imaging [87].

A study comparing how endoscopic and SEM imaging perform for observations of biofilm growth after biodegradation of organic contaminants in porous quartz media was conducted in a simulated reactor [87]. Due to the limitations of low magnification and poor resolution of endoscopic images in that study [87], SEM image analysis was used to further identify the biofilm thickness. However, biofilm thickness measurements under SEM imaging were thinner than those calculated by endoscopic image analysis, probably because of dehydration and alteration of the biofilm material under SEM processing steps [87]. Furthermore, SEM does not permit temporal scale analysis and also does not provide high-resolution 3D images to reduce measurement error. Thus, new techniques that allow for direct visualization of biofilms *in situ* are required in order to characterize biofilm growth, surface architecture, and 3D spatio-temporal distribution within porous media [55].

Many of the aforementioned techniques are not applicable to generic porous media like soil and marine sediments due to their inherent opacity. These methods are also not well suited for imaging regions larger than across a few grains of a porous media [54]. Recent works have focused on imaging biofilms within porous media using monochromatic synchrotron-based X-ray μCT [54-56]. These works have demonstrated the ability of μCT to provide experimental data for validation of mathematical models of biofilm growth in porous media [54,56]. The main advantages of μCT include the variety of substrates it can be applied to and the fact that it is nondestructive, allowing for 3D *in situ* visualization and reconstruction of the solid, aqueous and biofilm phases percolated

in a porous matrix. μCT has been available for more than three decades and has proven a powerful tool for studying a wide array of processes in porous media systems [88-90].

Nevertheless, to date the method is based on physical straining or attachment of a chemical contrast agent to the biofilm surface. This is necessary because both the biofilm and aqueous phases have similar X-ray absorption properties [54]. Some contrast agents have been compared to establish the best agent for imaging biofilms [54-56]. Silver-coated microspheres have been used to differentiate the biomass from fluid-filled pore spaces [55]. Experimentation using this approach provided compelling evidence that this contrast agent produces realistic 3D representations of biofilms present in an experimental packed bead column system, and it accurately represented the solid biofilm-aqueous phase spatial arrangement [55]. Moreover, in order to validate their results, the researchers visualized the same sample using digital microscopy [55]. That study represented the first successful attempt at using high-resolution μCT to image three-dimensional biofilms *in situ* within intact porous media [55]. However, silver is a biocide and could be toxic to bacteria. For this reason, it must be added to sample specimens immediately prior to imaging, which may impair the quality of images [55].

Another experimental approach to using μCT for porous media imaging makes use of a medical suspension of barium sulfate to differentiate between the aqueous phase and the biofilm. Potassium iodide is also added to the suspension to aid delineation between the biofilm and the experimental porous medium [54]. This approach showed that the suspension mixture provides contrast between the biofilm, the aqueous phase and the solid-phase (beads), allowing quantification of changes in porous media (such as dispersion or permeability) induced by biofilm growth through use of specific upscaling techniques and numerical analysis [54]. Despite the toxicity of barium sulfate, its use to image the biofilm in 3D can be considered a success. In addition, this work allowed some specific questions to be answered about the use of μCT to image biofilms in porous media, such as the permeability of barium sulfate in EPS, the effects of contrast on biofilm geometry, and the optimal X-ray exposure time [54].

Although promising, both methodologies suffer from important limitations such as fast sedimentation and heterogeneous distribution of barium or uncontrolled deposition of silver spheres at the biofilm surface [56]. A new contrast agent, 1-chloronaphtalene, represents a novel methodological approach [56]. This methodology takes advantage of the contrast properties of 1-chloronaphtalene to prevent some limitations observed with the more classical contrast agents (barium sulfate and silver microspheres) [56]. The main advantage of 1-chloronaphtalene is that it is chemically different from bacterial biofilms and is immiscible with water. Compared with barium sulfate, the liquid phase in 1-chloronaphtalene-derived images appears more homogeneous and is easier to separate from the biofilm as the biofilm contours appear sharp in the images [56]. The results of that study showed the efficiency of 1-chloronaphthalene combined with μCT to demonstrate biofilm colonization in biofilters, while circumventing the drawbacks encountered with classical contrast agents, such as fast sedimentation and heterogeneous distribution of barium [56]. However, the study does not reveal whether there was some toxic effect of 1-chloronaphthalene on bacteria, nor did it validate the μCT approach against some other standard technique.

Despite the need for further studies on issues such as the influence of X-rays on biofilm growth and the toxic effects of the chemical contrast agents required for imaging, μCT has been shown to be a potential technique for monitoring biofilm growth in 3D, allowing both quantitative and qualitative analyses of samples [54-56,91]. Valuable insights have come from combining several techniques linked to microtomography, such as other imaging techniques, analytical experiments, and numerical simulations and visualization [91]. Such combinations allow for calculation of effective parameters of porous media, such as porosity, growth and percolation rate, as well as allowing measurement of the volume and coverage of biofilms in samples [54,91]. Due to its simplicity, accessibility and applicability to complex porous structures, μCT provides an interesting and versatile framework for studying biofilms within porous media [54]. Because it is a noninvasive technique that allows *in situ* monitoring of bacterial biofilms, its use is encouraged for studies employing bacterial biofilms as a bioremediation agent in impacted environments.

Discussion and Final Comments

Microbiological analyses and monitoring of biofilms are undergoing significant advances in imaging techniques. However, each technique presents its own particular advantages and disadvantages, which makes it very difficult to choose a technique. Depending on the purpose of each study, one technique may provide better information than others (Figure 1-3). For example, figure 1 shows the use of Scanning Eletron Microscopy (SEM) to access the biofilm attached with metals, in a superficial scale. Figure 2 shows the use of computerized microtomography (μCT) to access the 3D internal structure of the biofilm in a porous media, allowing the quantification of the geometric and physical properties of the sample. Figure 3 shows the use of a compilation of x-ray techniques and microscopy for the proposal to map the metal bound to the bacterial biofilm. Therefore, it is very important to take into account the advantages of each technique and, moreover, its limitations. Also, we would recommend conducting a preliminary study on the

Table 2: Advantages and limitations of imaging techniques for bacterial biofilms in natural samples.

Technique	Advantages	Limitations
CLSM	High resolution; 3D analysis of macromolecules, cells and communities; Quantification based on 3D images; Living, fully hydrated samples; Multichannel (up to 5) analysis; Noninvasive optical sectioning; Reflection and fluorescence modes; One-photon/two-photon excitation; Extensive range of associated analytical tools acquires time-resolved 3D data of the biofilm structure	Sample preparation; Pre-processing of CLSM data; Image post-processing; Depth of laser penetration due to absorption and scattering; Probe-dependent (except for autofluorescence and GFP); Poor resolution if there are extreme differences in fluorescence emission intensities in one channel
EPI	Non-invasive, Allows visualization of biofilms in vivo without losing the structural information; Practicality; Allows differentiation of healthy and dead cells	Sample preparation; Requires a minimum biomass (~10^8 cells.ml^{-1})
SEM	Real-time analysis; Great resolution	Sample preparation; Sample dehydration
TEM	Can evaluate the compositional characteristics before and after biosorption of heavy metals; Locates metal accumulation in the cell	Need electron probe X-ray micro-analyzer; Sample preparation; Post-fixation with osmium tetroxide in order to preserve and contrast the membranes
STXM	Non-invasive way to investigate elemental distribution; 3D structural and corresponding elemental distribution; Elemental analysis of inorganic and organic constituents; Samples are fixed without contrast agents or microtomal sectioning; Probe-independent; Fully hydrated samples	Synchrotron light source and beam time necessary; Difficulty in selecting the substrate; Maximum sample thickness is 300 nm; Maintaining full hydration can be challenging; Radiation damage; Absorption saturation
µCT	2D and 3D images; Non-invasive; Biofilm growth can be imaged in situ; Allows temporal scale analysis	Need contrast agents; Radiation damage

applicability of these techniques for each type of sample.

Based on the above-described examples, there is no standard or preeminent technique, but this group of techniques can help improve our understanding of the role of bacterial biofilms in nature and their potential as biological agents of bioremediation. We advocate the use of coupled techniques—imaging together with spectroscopy, biological and/or biochemical analyses—so that each additional technique acts as a validation of imaging data, thereby overcoming certain limitations and increasing the reliability of results. The importance of this combined analysis is given because each technique allows access to a specific information, and when used together, becomes a wider tool.

Table 3 shows some examples of interdisciplinary studies whereby several different techniques were employed for the study and monitoring of bacterial biofilms in nature. These types of multidisciplinary analyses have generated significant results regarding the role of biofilms in bioremediation processes, as well as presenting avenues of future research on bacterial biofilms in nature and the bioremediation process.

For example, combined use of STXM, CLSM and TEM provided a detailed correlative map of biofilm structure and composition, which has the potential for revealing the biochemical basis for biofilm organization and can assist in investigations and optimizations of biofilms for environmental remediation applications [69]. Similarly, application of STXM and SEM has suggested that EPS plays a key role in metal capture and precipitation, and has proven the resistance of bacterial consortia to metal contamination, which are crucial findings contributing to our understanding of the role of EPS in the biosorption of metals [44,92].

Another example of a combined technique approach to evaluating

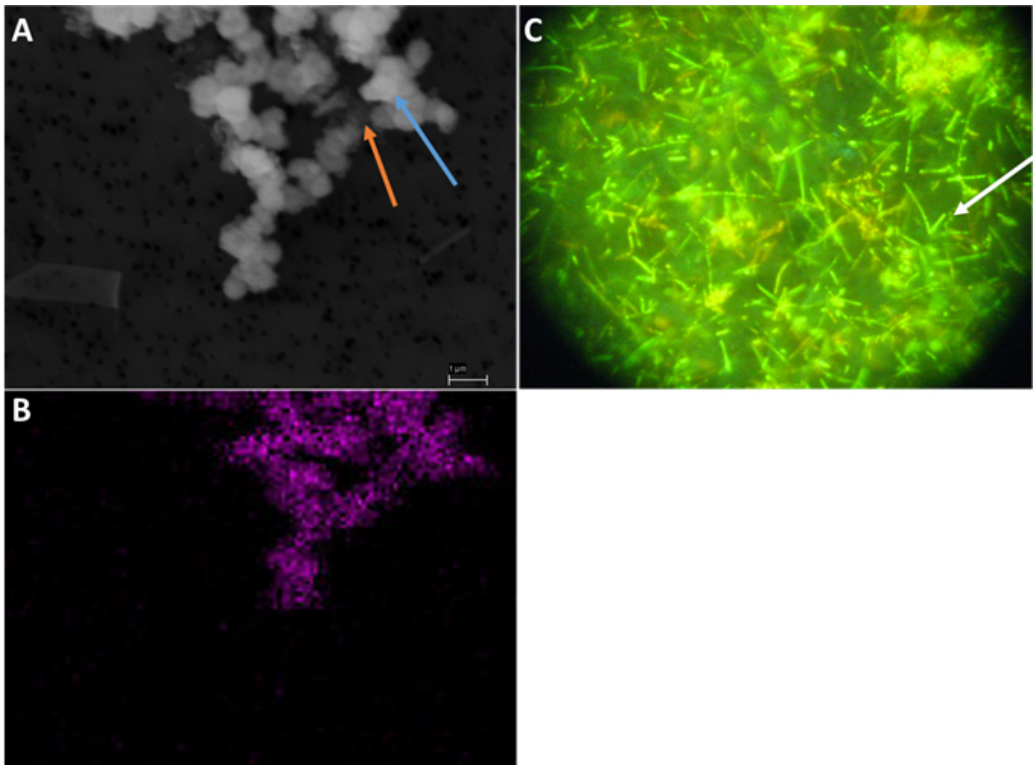

Figure 1: Examples of image analysis. A. Scanning Electron Microscope (SEM) analysis of copper resistant bacteria, isolated from marine sediment in southeastern Brazil (Orange arrow indicates biofilm while the blue arrow indicates bacterial cell); B. SEM-EDS of the same sample, with the copper adsorbed on the biofilm, marked by EDS; C. EPI Image of bacterial consortium, isolated from mangrove.

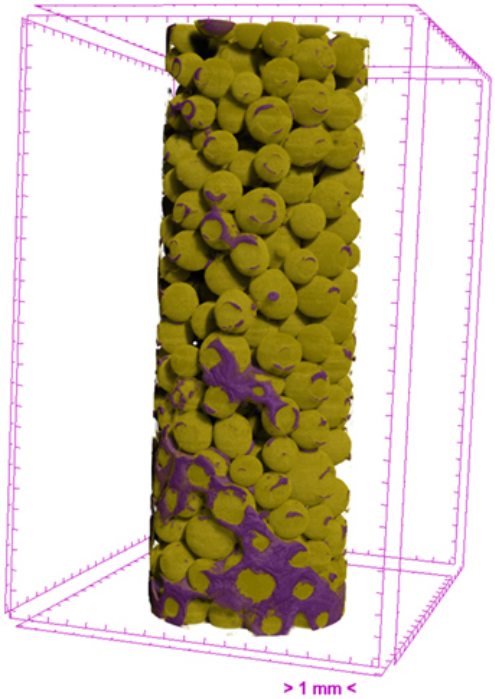

Figure 2: 3D µCT image of natural biofilm (Purple) isolated from marine sediment in a southeastern Brazilian polluted beach, in porous media (glass beads – yellow).

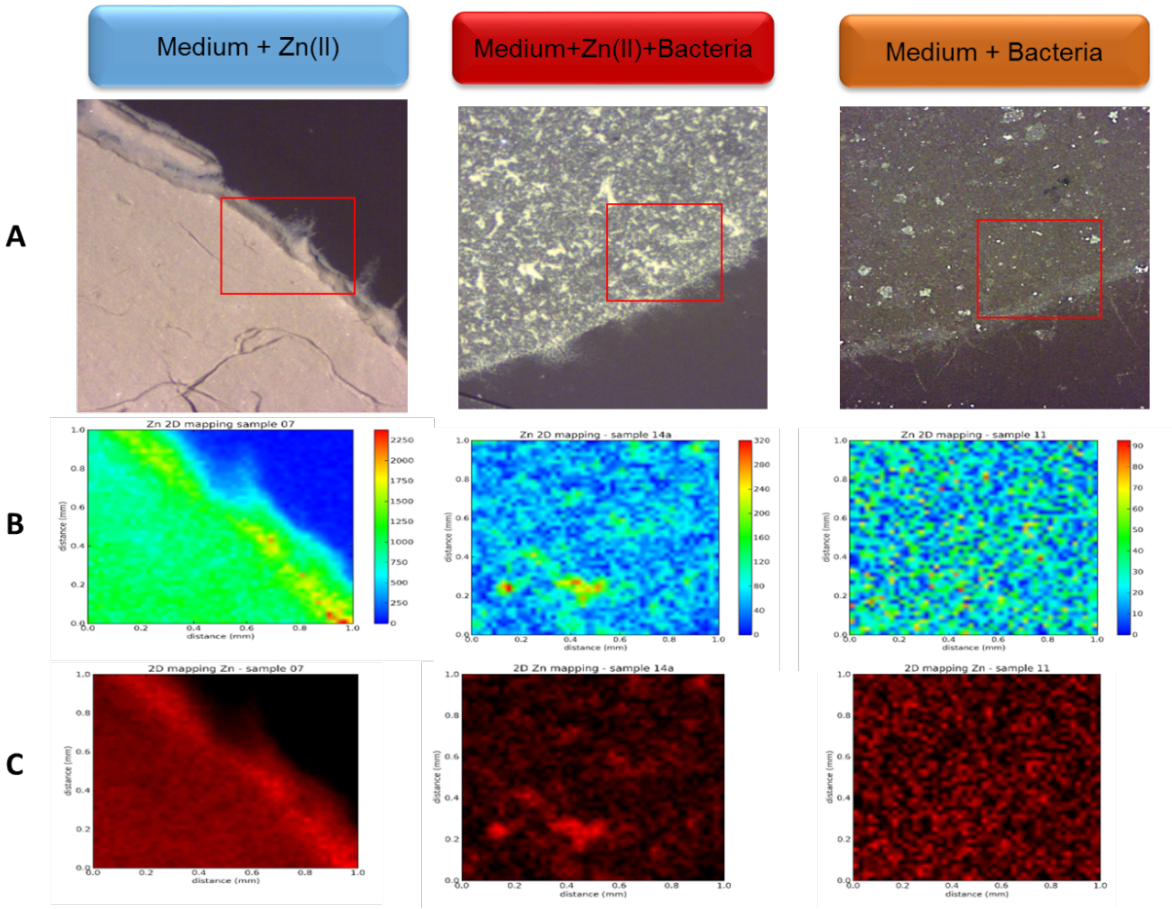

Figure 3: SXFM Analysis and optical microscopy images. A. Optical microscopy image of bacteria consortium in the presence of Zn in three different conditions (Left column with culture liquid medium and Zn(II) solution, middle column with liquid medium, Zn(II) solution and bacteria and right column with liquid medium and bacteria); B. 2D SXFM image of Zn mapping in the same sample (3A), showing the intensity levels in the three growing conditions; C. STXM 2D image of Zn presence map in the same sample, in the three growing conditions.

biosorption of metals by biofilms is use of SEM-EDX (Energy Dispersive X-ray) and the Fourier Transform Infrared (FTIR) spectroscopy technique [93]. FTIR spectroscopy provides more detailed information about specific metal–ligand interactions and, particularly, structural information on metal binding complexes associated with the various functional groups in EPS [94]. SEM in combination with EDX can provide information about the morphology and elemental composition of microbial biofilms, as well as extracted EPS [93, 95, 96]. These combined techniques can demonstrate the roles of microbial EPS in metal biosorption, providing vital data to implementation of metal biosorption technologies [93].

FTIR has also been used in combination with CLSM to distinguish the spatial chemical changes within multispecies biofilms grown from natural storm-waters in flow cells, providing information to better understand the heterogeneity of matrix components and correlating the functions of microbial species within complex natural biofilms [97]. Raman spectroscopy is another spectroscopic technique that can be used in combination with microscopy [98]. For example, combined use of CLSM and Raman spectroscopy overcomes the limitations of using CLSM alone and provides additional chemical information about biofilm components and their distribution, thereby enhancing our understanding of the composition and structure of the biofilm matrix [98].

Certain techniques can be combined to facilitate comparisons between different resolutions. Optical coherence tomography (OCT), CLSM and EPI have been used to investigate structural and spatial differences in biofilm growth at the top and bottom of a flow-cell model [99]. OCT uses near-infrared wavelengths to increase imaging penetration through highly scattering structures, enabling imaging at depths of several centimeters through transparent structures and of a few millimeters in highly scattering tissue [99]. OCT is a novel tool with great potential for use as a non-invasive, label-free, real-time, in-situ and/or in-vivo imaging

modality for biofilm characterization, particularly due to its high resolution compared to other techniques [99].

Electron microscopy techniques are widely used for environmental samples and have helped answer important questions about the behavior of biofilms in the environment. However, the advent of X-ray techniques appears as a good alternative, mainly for providing 2D and 3D images of biofilms and allowing reconstruction of images that are faithful to biofilms as found in the environment. The results of this methodology present a great advance for the application of biofilm use in bioprocesses, as bioclogging and bioremediation, since it opens possibilities for systematic studies of biofilm response, within porous media, to changes in physical, chemical and biological parameters. For example, modifications of local Reynolds and Péclet numbers, biofilm permeability and biomass growth rate, porosity, permeability and mass transport parameters, nutrient availability, temperature and pH stresses, and the impact of biofilm biodiversity on biofilm geometry within the 3-D porous media matrix can potentially be investigated [54,91,100,101,102].

In addition, non-invasive techniques such as μCT allow spatial monitoring of biofilms over time, which represents a major advance for bioremediation studies of sites impacted by metals or oil spills. Bacterial biofilms can interact with these pollutants, facilitating their removal from the water column, as well as from soil or sediments. Imaging techniques have corroborated results obtained by analytical quantification, evidencing the process of bioremediation in the environment. When associated with other types of analysis and methodologies, imaging techniques, and especially non-invasive approaches, can become important tools for the study of biofilm behavior in the environment and their potential role in bioremediation.

Acknowledgments

We thanks to CAPES for a scholarship to the first author. Also thanks to Dr. José A. P. Bitencourt for advice with the EPI images and Dr. Carlos Perez, from the LNLS/MCTI for advice with XRF and STFM analysis.

Table 3: Combinations of methods for combined analyses of biofilms.

Combination	Main Findings	Potential for Future Biofilm Research	References
STXM, CLSM & TEM	Created a detailed correlative map of biofilm structure and composition	Understanding the biochemical basis for biofilm organization and assist studies investigating and optimizing biofilms for environmental remediation applications	[69]
STXM & SEM	Suggested that EPS played a key role in metal capture and precipitation	Connects molecular-scale biogeochemical processes to those at the microorganism-level; Provides insight into how microbes influence larger, pore-scale biogeochemical reactions	[92]
	Bacterial consortium resistant to metal contamination produced EPS that bound metals	Study EPS formation and metal biosorption under differing environmental conditions	[44]
CLSM & SEM	*Micrococcus luteus* could be viewed as a microorganism capable of restoring environments polluted by lead and copper	Identification of bacterial species suitable for bioremediation studies	[43]
SEM-EDX & FTIR	Roles of EPS in metal biosorption	Implementation of metal biosorption technologies	[93]
CLSM & FTIR	Influx of water during biofilm growth results in significant changes in biofilm formation; Classification of various regions of the biofilm	Approach for describing complex natural biofilms; Understanding the heterogeneity of matrix components and correlating the functions of microbial species within complex natural biofilms	[97]
CLSM & RAMAN	Feasibility of Raman for nondestructive chemical characterization of multispecies biofilms	Raman with associated imaging technique allows chemical characterization of multispecies biofilms	[98]
OCT, CLSM & EPI	Structural and spatial differences in biofilm growth at the top and bottom of a sample	OCT has the potential to be used as a non-invasive, label-free, real-time, in-situ and/or in-vivo imaging modality for biofilm characterization	[99]

References

1. Decho AW (2000) Microbial biofilms in intertidal systems: an overview. Cont Shelf Res 20(10-11): 1257-1273.
2. Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8(9): 623−633.
3. Meyer-Reil LA (1994) Microbial life in sedimentary biofilms – the challenge to microbial ecologists. Mar Ecol Prog Ser 112: 303-311.
4. Costa A, Gonçalvez M, Mesquita L, Granato M (1995) Tratamento de efluentes para remoção de metais pesados utilizando uma resina biológica. Metalurgia & Materiais 51: 872–877.
5. Rao SR (2006) Biotechnological Processes. In: Resource Recovery and Recycling from Metallurgical Wastes. Elsevier Waste Management series, 5: Amsterdam 109-125.
6. Vijayaraghavan K, Yun YS (2008) Bacterial biosorbents and biosorption. Biotechnol Adv 26(3): 266–291.
7. Brito EMS, Duran R, Guyoneaud R, Goñi-Urriza M, Oteyza TG, et al. (2009) A case study of in situ oil contamination in a mangrove swamp (Rio De Janeiro, Brazil). Mar Poll Bull 58(3): 418–423.
8. Battin TJ, Besemer K, Bengtsson MM, Romani AM, Packmann AI (2016) The ecology and biogeochemistry of stream biofilms. Nat Rev Microbiol 14(4): 251-263.
9. Gaylarde CC, Bellinaso ML, Manfio GP (2005) Biorremediação – Aspectos biológicos e técnicos da biorremediação de xenobióticos. Biotecnolog Cienc Desenvolv 34: 36-43.
10. Urrutia MM (1997) General Bacterial Sorption Processes. In: Biosorbents for metal ions; Wase, J.; Forster, C., eds.; CRC Press: London 39–66.
11. Wang J, Chen C (2009) Biosorbents for heavy metals removal and their future. Biotechnol Adv 27(2): 195–226.
12. Pennafirme S, Lima I, Bitencourt JAP, Crapez MAC, Lopes RT (2015) Organic matter biodegradation by bacterial consortia under metal stress. In: Biodegradation; Intech: 2015. ISBN: 978-953-51-4271-3). DOI 10.5772/60980
13. Waite CCC, Silva GOA, Bitencourt JAP, Sabadini-Santos E, Crapez MAC (2016) Copper and lead removal from aqueous solutions by bacterial consortia acting as biosorbents. Mar Poll Bull 109(1): 386–392.
14. Naik MM, Dubey SK (2013) Lead resistant bacteria: Lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. Ecotoxicol Environ Saf 98: 1–7.
15. Mann H (1990) Removal and recovery of heavy metals by biosorption. In Biosorption of heavy metals; Volesky B, ed.; CRC press: Boca Raton 93-137.
16. Burns KA, Codi S, Pratt C, Duke NC (1999) Weathering of hydrocarbons in mangrove sediments: testing the effects of using dispersants to treat oil spills. Org Geochem 30(10): 1273–1286.
17. Duke NC, Burns KA, Swannell RJP, Dallahus O, Rupp RJ (2000) Dispersant use and a bioremediation strategy as alternate means of reducing impacts of large oil spills on Mangroves: the Gladstone field trials. Mar Poll Bull 41(7-12): 403–412.
18. Ramsay MA, Swannell RPJ, Shipton WA, Duke NC, Hill RT (2000) Effect of bioremediation on the microbial community in oiled Mangrove sediments. Mar Poll Bull 41(7-12): 413–419.
19. Tam NFY, Wong YS (2008) Effectiveness of bacterial inoculum and mangrove plants on remediation of sediment contaminated with polycyclic aromatic hydrocarbons. Mar Poll Bull 57(6-12): 716–726.
20. Wang YP, Shi JY, Wang H, Lin Q, Chen XC, et al. (2007) The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. Ecotoxicol Environ Saf 67(1): 75–81.
21. Moreira ITA, Oliveira OMC, Triguis JÁ, Santos AMP, Queiroz AFS, et al. (2011) Phytoremediation using *Rizophora mangle L*. in mangrove sediments contaminated by persistent total petroleum hydrocarbons (TPH's). Microchem J 99(2): 376–382.
22. Yenn R, Borah M, Deka Boruah HP, Sarma Roy A, Baruah R, et al. (2014) Phytoremediation of abandoned crude oil contaminated drill sites of Assam with the aid of a hydrocarbon-degrading bacterial formulation. Int J Phytoremediation 16(7-12): 909-925.
23. Krepsky N, Da Silva FS, Fontana LF, Crapez MAC (2007) Alternative methodology for isolation of biosurfactant-producing bacteria. Brazilian J Biol 67(1): 117-124.
24. Cruz GF, Angolini CFF, Neto EVS, Loha W, Marsaioli AJ (2010) Exopolymeric substances (EPS) produced by petroleum microbial consortia. J Braz Chem Soc 21: 1517-1523.
25. Abari AH, Emtiazi G, Ghasemi SM (2012) The role of exopolysaccharide, biosurfactant and peroxidase enzymes on toluene degradation by bacteria isolated from marine and wastewater environments. Jundishapur J Microbiol 5(3): 479-485.
26. Peele KA, Teja VRC, Kodali VP (2016) Emulsifying activity of a biosurfactant produced by a marine bacterium. Biotech 6(2): 177.
27. Baran S, Bielinska JE, Oleszczuk P (2004) Enzymatic activity in an airfield soil polluted with polycyclic aromatic hydrocarbons. Geoderma 118(3-4): 221–232.
28. Shen G, Lu Y, Zhou Q, Hong J (2005) Interaction of polycyclic aromatic hydrocarbons and heavy metals on soil enzyme. Chemosphere 61(8): 1175–1182.
29. Cheema SA, Khan MI, Tang X, Zhang C, Shen C, et al. (2009) Enhancement of phenanthrene and pyrene degradation in rhizosphere of tall fescue (*Festuca arundinacea*). J Hazard Mater 166(2-3): 1226–1231.
30. Fontana LF, Mendonça Filho JG, Netto ADP, Sabadini-Santos E, Figueiredo Jr AG, et al. (2010) Geomicrobiology of cores from Suruí Mangrove – Guanabara Bay – Brazil. Mar Poll Bull 60(10): 1674–1681.
31. Sabadini-Santos E, Silva TS, Lopes-Rosa T, Mendonça-Filho JC, Santelli RE, et al. (2014) Microbial activities and bioavailable concentrations of Cu, Zn, and Pb in sediments from a tropic and eutrophicated Bay. Water Air Soil Pollut 225: 1-11.
32. Wang J, Li Q, Li MM, Chen TH, Zhou YF, et al. (2014) Competitive adsorption of heavy metal by extracellular polymeric substances (EPS) extracted from sulfate reducing bacteria. Bioresour Technol 163: 374–376.

33. Wingender J et al. (1999) What are bacterial extracellular polymeric substances? In Microbial Extracellular Polymeric Substances. Germany 1-19.

34. Omoike A, Chorover J, Kwon KD, Kubicki JD (2004) Adhesion of bacterial exopolymers to α-FeOOH: Inner-sphere complexation of phosphodiester groups. Langmuir 20(25): 11108–11114.

35. González AG, Shirokova LS, Pokrovsky OS, Emnova EE, Martínez RE, et al. (2010) Adsorption of copper on *Pseudomonas aureofaciens*: Protective role of surface exopolysaccharides. J Colloid Interface Sci 350(1): 305-314.

36. Walker SL, Redman JA, Elimelech M (2004) Role of cell surface lipopolysaccharides in *Escherichia coli* K12 adhesion and transport. Langmuir 20(18): 7736–7746.

37. Perry TD, Klepac-Ceraj V, Zhang XV, McNamara CJ, Polz MF, et al. (2005) Binding of harvested bacterial exopolymers to the surface of calcite. Environ Sci Technol 39(22): 8770-8775.

38. Omoike A, Chorover J (2006) Adsorption to goethite of extracellular polymeric substances from *Bacillus subtilis*. Geochim Cosmochim Acta 70(4): 827–838.

39. Kim J, Choi H, Pachepsky Y (2010) Biofilm morphology as related to the porous media clogging. Water Res 44(4): 1193–1201.

40. Cammarota MC, Sant'Anna GL (1998) Metabolic blocking of exopolysaccharides synthesis: effects on microbial adhesion and biofilm accumulation. Biotechnol Lett 20(1): 1–4.

41. Laspidou CS, Rittmann BE (2002) A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Res 36(11): 2711–2720.

42. Neu TR, Manz B, Volke F, Dynes JJ, Hitchcock AP, et al. (2010) Advanced imaging techniques for assessment of structure, composition and function in biofilm systems. FEMS Microbiol Ecol 72(1): 1–21.

43. Puyen ZM, Villagrasa E, Maldonado J, Diestra E, Esteve I, et al. (2012) Biosorption of lead and copper by heavy-metal tolerant *Micrococcus luteus* DE2008. Bioresour Technol 126: 233–237.

44. Pennafirme S, Lima I, Bitencourt JAP, Crapez MAC, Lopes RT (2015) Microbial biofilm study by synchrotron X-ray microscopy. Radiat Phys Chem 116: 116-119. DOI 10.1016/j.radphyschem.2015.05.040

45. Williams DB (1996) Transmission Electron Microscopy. Springer Science + Business Media: New York 17.

46. Subudhi S, Batta N, Pathak M, Bisht V, Devi A, et al. (2014) Bioflocculant production and biosorption of zinc and lead by a novel bacterial species, *Achromobacter* sp. TERI-IASST N, isolated from oil refinery waste. Chemosphere 113: 116–124.

47. Xie GJ, Liu BF, Xing DF, Nan J, Ding J, et al. (2012) Photohydrogen production by *Rhodopseudomonas faecalis* RLD-53 immobilized on the surface of modified activated carbon fibers. Res Adv 2: 2225–2228.

48. Tourney J, Ngwenya BT (2014) The role of bacterial extracellular polymeric substances in geomicrobiology. Chem Geol 386:115–132.

49. Little B, Wagner P, Ray R, Pope R, Scheetz R (1991) Biofilms: an ESEM evaluation of artifacts introduced during SEM preparation. J Ind Microbiol 8(4): 213-222.

50. Pawley JB (1995) Handbook of Biological Confocal Microscopy (3 ed.) Springer: Berlin, 985 ISBN 0-387-25921-X.

51. Dexter SC, Xu K, Luther GL (2003) Mn cycling in marine biofilms: effect on the rate of localized corrosion. Biofouling 19: 139-149.

52. Xavier JB, White DC, Almeida JS (2003) Automated biofilm morphology quantification from confocal laser scanning microscopy imaging. Water Sci Technol 47(5): 31–37.

53. Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE (1991) Optical sectioning of microbial biofilms. J Bacteriol 173(20): 6558–6567.

54. Davit Y, Iltis G, Debenest G, Veran-Tissoires S, Wildenschild D, et al. (2010) Imaging biofilm in porous media using X-ray computed microtomography. J Microsc 242(1): 15-25.

55. Iltis GC, Armstrong RT, Jansik DP, Wood BD, Wildenschild D (2011) Imaging biofilm architecture within porous media using synchrotron-based X-ray computed microtomography. Water Resour Res 47(2): 1-5. DOI 10.1029/2010WR009410

56. Roscoat SR, Martins JMF, Séchet P, Vince E, Latil P, et al. (2014) Application of Synchrotron X-ray microtomography for visualizing bacterial biofilms 3D microstructure in porous media. Biotechnol Bioeng 111(6): 1265-1271.

57. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, et al. (2000) Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146(pt 10): 2395–2407.

58. Beyenal H, Donovan C, Lewandowski Z, Harkin G (2004) Three-dimensional biofilm structure quantification. J Microbiol Methods 59(3): 395 – 413.

59. Koley D, Ramsey MM, Bard AJ, Whiteley M (2011) Discovery of a biofilm electrocline using real-time 3D metabolite analysis. PNAS 108(50): 19996–20001.

60. Marx V (2014) Cell communication: Stop the microbial chatter. Nature 511: 493–497.

61. Zhi L, Satish KN (2012) Quorum sensing: How bacteria can coordinate activity and synchronize their response to external signals? Protein Sci 21(10): 1403-1417.

62. Liu X, Ramsey MM, Chena X, Koley D, Whiteley M, et al. (2011) Real-time mapping of a hydrogen peroxide concentration profile across a polymicrobial bacterial biofilm using scanning electrochemical microscopy. PNAS 108(7): 2668–2673.

63. Bard AJ, Mirkin MV (2001) Scanning Electrochemical Microscopy. (Marcel Dekker) New York 650.

64. Mueller LN, de Brouwer JFC, Almeida JS, Stal LJ, Xavier JB (2006) Analysis of a marine phototrophic biofilm by confocal laser scanning microscopy using the new image quantification software PHLIP. BMC Ecol 6(1): 1-15.

65. Kirz J, Jacobsen C, Howells M (1995) Soft X-ray microscopes and their biological applications. Q Rev Biophys 28: 33–130.

66. Ade H, Urquhart SG (2002) NEXAFS spectroscopy and microscopy of natural and synthetic polymers. Chemical Applications of Synchrotron Radiation (Sham TK, ed), World Scientific Publishing, Singapore 285–355.

67. Ade H, Hitchcock AP (2008) NEXAFS microscopy and resonant scattering: composition and orientation probed in real and reciprocal space. Polymer 49(3): 643–675.

68. Bluhm H, Andersson K, Araki T, Benzerara K, Brown GE, et al. (2006) Soft X-ray microscopy and spectroscopy at the molecular environmental science beamline at the advanced light source. J Electron Spectrosc 150(2-3): 86–104.

69. Lawrence JR, Swerhone GDW, Leppard GG, Araki T, Zhang X, et al. (2003) Scanning transmission X-ray, laser scanning, and transmission electron microscopy mapping of the exopolymeric matrix of microbial biofilms. Appl Environ Microb 69(9): 5543–5554.

70. Dynes JJ, Tyliszczak T, Araki T, Lawrence JR, Swerhone GDW, et al. (2006) Speciation and quantitative mapping of metal species in microbial biofilms using scanning transmission X-ray microscopy. Environ Sci Technol 40(5): 1556–1565.

71. Dynes JJ, Lawrence JR, Korber DR, Swerhone GDW, Leppard GG, et al. (2006) Quantitative mapping of chlorhexidine in natural river biofilms. Sci Total Environ 369(1-3): 369–383.

72. Yang Y, Heine R, Xu F, Suhonen H, Helfen L, et al. (2013) Correlative imaging of structural and elemental composition of bacterial biofilms. J Phys Conf Ser 463: 1-4.

73. Kepner Jr R, Pratt JR (1994) Use of fluorochromes for direct enumerations of total bacteria in environmental samples: past and present. Microbiol Rev 58(4): 603-615.

74. Vonesch C, Aguet F, Vonesch JL, Unser M (2006) The colored revolution of bioimaging. IEEE Signal Process Mag 20-31.

75. Bloem J, Bar-Gilissen MJ, Cappenberg T (1986) Fixation counting and manipulation of heterotrophic nanoflagellates. Appl Environ Microbiol 52(6): 1266-1272.

76. Medová H, Koblížek M, Elster J, Nedbalová L (2016) Abundance of aerobic anoxygenic bacteria in freshwater lakes on James Ross Island, Antarctic Peninsula. Antarct Sci 28(2): 101–102.

77. Crosby MP, Peele ER (1987) Detection of bacterial populations in oyster tissue homogenates using direct microscopic counts and thymidine incorporation. J Exp Mar Biol Ecol 108(1): 93-97.

78. Bitencourt JAP, Pereira DC, Silva Neto ID, Crapez MAC (2016) The toxic effect of copper on the association between ciliates *Euplotes vannus* and *Euplotes crassus* and their naturally associated bacteria isolated from a polluted tropical bay. Reg Stud Mar Sci 3: 25–32.

79. Harrison JJ, Ceri H, Turner RJ (2007) Multimetal resistance and tolerance in microbial biofilms. Nature 5(12): 928-938.

80. Carlucci AF, Craven DB, Robertson KJ, Williams PM (1986) Surface-film microbial populations: diel amino acid metabolism, carbon utilization, and growth rates. Mar Biol 92(2): 289-297.

81. Yue ZB, Li Q, Li CC, Chen TH, Wang J (2015) Component analysis and heavy metal adsorption ability of extracellular polymeric substances (EPS) from sulfate reducing bacteria. Bioresour Technol 194: 399–402.

82. D'Acunto B, Esposito G, Frunzo L, Mattei M, Pirozzi F (2015) Mathematical modeling of heavy metal biosorption in multispecies biofilms. J Environ Eng C4015020: 1-14.

83. Wong MF, Chua H, Lo W, Leung CK, Yu PHF (2001) Removal and recovery of copper (II) ions by bacterial biosorption. Appl Biochem Biotechnol 91: 447–457.

84. Choi A, Wang S, Lee M (2009) Biosorption of cadmium, copper, and lead ions from aqueous solutions by *Ralstonia* sp. and *Bacillus* sp. isolated from diesel and heavy metal contaminated soil. Geosciences Journal 13(4): 331–341.

85. Pereira S, Micheletti E, Zille A, Santos A, Moradas-Ferreira P, et al. (2011) Using extracellular polymeric substances (EPS) producing cyanobacteria for the bioremediation of heavy metals: do cations compete for the EPS functional groups and also accumulate inside the cell? Microbiology 157(pt 2): 451–458.

86. Chen CW, Kao CM, Chen CF, Dong CD (2007) Distribution and accumulation of heavy metals in the sediments of Kaohsiung Harbor, Taiwan. Chemosphere 66(8): 1431–1440.

87. Jean JS, Tsao CW, Chun MC (2004) Comparative endoscopic and SEM analyses and imaging for biofilm growth on porous quartz sand. Biogeochemistry 70(3): 427–445.

88. Nunan N, Ritz K, Rivers M, Feeney D, Young I (2006) Investigating microbial micro-habitat structure using X-ray computed tomography. Geoderma 133(3-4): 398-407. DOI 10.1016/j.geoderma.2005.08.004

89. Werth CJ, Zhang C, Brusseau ML, Oostrom M, Baumann T (2010) A review of non-invasive imaging methods and applications in contaminant hydrogeology research. J Contam Hydrol 113(104): 1–24. DOI 10.1016/j.conhyd.2010.01.001.

90. Wildenschild D, Vaz C, Rivers M, Rikard D, Christensen B (2002) Using X-ray computed tomography in hydrology: Systems, resolutions, and limitations. J Hydrol 267(3-4): 285–297. DOI 10.1016/S0022-1694(02)00157-9

91. Peszynska M, Trykozko A, Iltis G, Schlueter S, Wildenschild D (2016) Biofilm growth in porous media: Experiments, computational modeling at the porescale, and upscaling. Adv Water Resour 95: 288–301.

92. Marshall MJ, Cao B, Belchik SM, Hill EA, Kucek LA, et al. (2012) Correlative microscopy and chemical imaging to characterize the structure and biogeochemical function of biofilms. Microsc Microanal 18(2): 844-845.

93. Li WW, Yu HQ (2014) Insight into the roles of microbial extracellular polymer substances in metal biosorption. Bioresour Technol 160: 15–23.

94. Comte S, Guibaud G, Baudu M (2006) Biosorption properties of extracellular polymeric substances (EPS) resulting from activated sludge according to their type: soluble or bound. Process Biochem 41(4): 815–823.

95. Guibaud G, Bhatia D, d'Abzac P, Bourven I, Bordas F, et al. (2012) Cd(II) and Pb(II) sorption by extracellular polymeric substances (EPS) extracted from anaerobic granular biofilms: evidence of a pH sorption-edge. J Taiwan Inst Chem Eng 43(3): 444–449.

96. Liu Y, Xu H (2007) Equilibrium, thermodynamics and mechanisms of Ni^{2+} biosorption by aerobic granules. Biochem Eng J 35(2): 174–182.

97. Reuben S, Banas K, Banas A, Swarup S (2014) Combination of synchrotron radiation-based Fourier transforms infrared microspectroscopy and confocal laser scanning microscopy to understand spatial heterogeneity in aquatic multispecies biofilms. Water Res 64: 123–133.

98. Ivleva NP, Wagner M, Horn H, Niessner R, Haisch C (2009) Towards a nondestructive chemical characterization of biofilm matrix by Raman microscopy. Anal Bioanal Chem 393(1): 197–206.

99. Xi C, Marks D, Schlachter S, Luo W, Boppart SA (2006) High-resolution three-dimensional imaging of biofilm development using optical coherence tomography. J Biomed Opt 11(3): 1-6.

100. Armstrong RT, Wildenschild D, Bay BK (2015) The effect of pore morphology on microbial enhanced oil recovery. J Petrol Sci Eng 130: 16–25.

101. Pintelon TRR, Picioreanu C, van Loosdrecht MCM, Johns ML (2012) The Effect of Biofilm Permeability on Bio-Clogging of Porous Media. Biotechnol Bioeng 109(4): 1031-1042.

102. Karrabi M, Séchet P, Morra C, Cartellier A, Geindreau C, et al. (2011) Investigation of hydrodynamic/biomass growth coupling in a pilot scale granular bioreactor at low pore Reynolds number. Chem Eng Sci 66(8): 1765–1782.