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Corresponding Author:

Ayesha Muazzam

ashu2nice@gmail.com

Combined imaging and molecular techniques for evaluating microbial function and composition

[1]Javeria Sharif, [2]Uzma Bilal, [3]Hafiza Rida Fatima, [4]Abia Muazzam, [5]Aqsa Perveen, [6]Areesha Naveed, [7]Hafiza Arshi Saeed, [8]Ayesha Haidar, [9]Rameen Atique, [10*]Ayesha Muazzam

[1, 3, 5, 6,7,8,9] Department of Pathobiology and Biomedical Sciences, FVAS, MNS University of Agriculture, 25000, Multan Pakistan

[2]Department of Zoology, The women University Multan Pakistan

[4]Department of Biochemistry and Biotechnology, FVAS, MNS University of Agriculture, 25000, Multan Pakistan

[10]Department of Animal Science Gyeongsang National University 501, Jinju-daero, South Korea

[1]javeriasharif1024@gmail.com, [2]uzmabilal31@gmail.com, [3]ridafatimabaloch01@gmail.com, [4]abiamuazzam@gmail.com, [5]perveenaqsa4@gmail.com, [6]areesha.naveed237@gmail.com, [7]arshisaeed111@gmail.com, [8]ayeshahaidar3100@gmail.com, [9]rameenatiq5@gmail.com , [10]ashu2nice@gmail.com

Abstract

Microorganisms take key positions among ecological communities, supplying biogeochemical activities such as nutrient circulations and environmental pollution decomposition and ensuring ecosystem stability. The usual ways of studying microbial communities use cultivation techniques, which are a hindrance to our grasp of almost all microorganisms unable to grow in the controlled conditions of the laboratory. This review is meant to summarize the current development in imaging and molecular techniques for assessing microbe communities. The combined use of molecular and imaging techniques for microbial analysis was assessed through a review of literature reporting studies that employed this approach. The review suggested that there is a broad range of techniques that apply both imagery and molecular characteristics for microbial analysis which include fluorescence in situ hybridization (FISH), confocal laser scanning microscopy (CLSM), metagenomics, metatranscriptomics and metaproteomics. The applied combined imaging and molecular techniques represent an effective way of understanding microbial function and structure. By combining microscopic observation with molecular analysis, researchers can obtain significant information on the structure-function relationships of microbial communities and their interactions with the surroundings. These technologies are of great importance in solving the crucial issues of environmental surveillance, biodegradation, and human health. On the other hand, future technological progress and interdisciplinary cooperation are the key factors that will let us fully unleash the potential of these approaches and transform the findings of basic research into practical applications.

Keywords

Mass Spectrometry imaging, NanoSIMS, STXM, Atomic Force Microscopy, Magnetic Resonance Imaging (MRI), Scanning Electron Microscopy

Introduction

Bacterial and the earth's microbiome have been acknowledged as the chief mediators for the earth's processes and elemental cycles [1]. During thousands of years of development of the planet the number of bacterial taxa, estimated to amount to trillions, have managed to survive in both the extreme and normal environments including every corner of Earth. Most of them control the movements of the essential elements within the flat of global metabolic routes. In many cases, the existence of clearly defined redox gradients that are conditioned to chemical structure, several redox couples and microorganism communities involved is proved [2]. They are generally described in terms of what is supplied (like energy) with their resulting thermodynamic favorability. Both the increased level of chemical alteration and the presence of biological activity determine a relative redox condition via global and local effects as well. Redox reactions participating in the biogeochemical processes are varied and pertinent for different kinds of aqueous systems.

Frequently, these mechanisms are of microscopic scale they affect not only molecular diffusion, and substance distribution throughout the environmental gradient but also substrate and product distribution [3]. In the majority of instances, redox zonation may occur vertically in sediments, water-unsaturated soils, or water columns with meromictic activity. A shift of vertical redox state commonly means that electron acceptors become limited (i.e. oxygen, nitrate, manganese, etc.) and the microbial environment moves towards a more reduced state as depletion of electron acceptors progresses. Due to this unexpected event, the microbial populations will encounter ongoing stress which will force the microbial community to search for a way to survive. This is likely to increase the switches from on to off states and vice versa in the course of chemical responses which will impact the timing and distribution of essential biogeochemical transformations.

Here, microorganisms are dominant players in the mobilization and toxicity of contaminants. To expand the knowledge about microbial ecology and the role it plays in elemental cycles, we need to apply tools which take into account the specific functional microbial groups in a spacious

temporal context. The capability of observing how chemical speciation varies at the microscale will provide information on the bacterial metabolic exchange within complex communities. Along with the development of the HFMSI (High-Resolution Mass Spectrometry Imaging) technique, researchers can identify detailed structures of systems and single molecules of microorganisms. "Molecular techniques" developments that have been provided as well have also immensely contributed to unsembling the mystery of some complex bacteria/ mineral interface systems [4]. This fluorescent in-situ hybridization (FISH) technology has been proven effective along its enzymatic variation – catalyzed reporter deposition fluorescent in-situ hybridization (CARD-FISH) – which is perfect for quantifying specific bacterial taxa in a complex assemblage and for imaging spatial associations of bacteria with mineral phases. Technological advances in the

Domain of nucleic acids research, namely sequencing of both genomic DNA and the RNA of the cells, prompted the emergence of cheap methods to examine the entire microbial communities concerning their genetic construct and their functions. Either way, whether culture or culture-independent approach is settled on, it is their abilities to work with either controlled microbial communities or multi-species arrays that shine the most. Gene-dedicated taxonomy surveys such as 16S rRNA and metagenomics are suitable for determining the presence of any type of microbe in any given kind of sample. In contrast, the RNA-sequencing technique allows for the identification of the activities metabolic carried out by the microbial consortia. Countless mixed-modal research including SEM and TEM with the molecular tools have produced significant knowledge [5].

Microbial communities carried the operational responsibilities of metals removal in a newly built AMD bioreactor. In this instance, the utilization of SEM enabled the imaging of the biofilms and microbial communities. The investigation would have greatly benefited from additional and novel MSI techniques for characterizing the intricate microbe-mineral interactions in such systems. The addition of molecular techniques with these new imaging and isotopic methods such as nanoscale secondary ion mass spectrometry (nanoSIMS) and scanning transmission X-ray microscopy

(STXM) can give better fundamental comprehension of microbial community dynamics, interactions, and functional activity when coupled with previously mentioned methods of hybridization (e.g. FISH-SIMS; MSI). Besides this, the mentioned methods serve to introduce new perspectives on controlling the dynamics of the uptake of nutrients, redox processes and transformations generated by interaction in the community of microbes with minerals or with other bacteria. This review focuses on the most recent development in high-resolution in situ imaging of microbial communities and the characterization of molecule-based components of microbial communities in natural systems [6].

Mass spectrometry imaging

The advantage of MSI techniques is their ability to collect discrete chemical information from biological matrices. Most of today's analytical approaches focus on the collection of chemical information, and these include vibrational spectroscopy, nuclear magnetic resonance, and in some cases coupled techniques based on chromatographic methods. The principle behind MSI involves a sample interaction with a focused incident beam of ions or microprobe. The interaction induces desorption of chemical compounds from the surface of the matrix into an inert gas phase which is then ionized and resolved based on their mass-to-charge ratio (m/z). There is a large selection of MSI-based techniques; however, only a few have been suitably adapted for microbial studies. Some of these include focused ion probes such as matrix-assisted laser desorption/ionization (MALDI), NanoSIMS, and STXM [7].

Matrix-Assisted Laser Desorption Ionization (MALDI)

MALDI is a favourite ion source now that has highly penetrated the field of protein sequencing and proteomics, and it is rarely used independently of ESI technology [8]. MALDI is based on the ionization desorbed from the solid surface. A sample is next solved in a proper solvent, and an excessive amount is added to it from the same group as the analyte. The MALDI plate where the sample will be loaded is located and air dried (or under a stream of nitrogen gas) next. The next step is to introduce the sample to the matrix so that they are grown together (the sample is cocrystallized with the matrix). Within the mixture of compositions, the sample is heated vaporized and desorbed by the use of a laser beam (typically a nitrogen laser type at a wavelength

of 337nm) that hits the sample-matrix crystal, the matrix absorbs the laser energy and the analytes in the sample are then ionized and released in the gas formation. In the ion formation the atoms break and form ions.

As MALDI was originally designed for use under vacuum, its modified version, also known as AP MALDI was developed a few years later [9]. This novel improvement has helped to decrease costs, operate with ease, and commercially produce the various spectrometers and MALDI and ESI sources which readily can be interchanged. Indeed, the question of how ions are produced in the ion source in MADLI remains completely resolved for this method and the selection of a particular target material is instrumental. Furthermore, the decision to use the neutral matrix or an ionizing matrix should be based on the nature of the analytes as well. These can be either, a positive mode ionization or negative mode ionization. By a system of basic factors, the latter type is made more productive while in the case of the former (i.e., proton donor) acids usually are used to organize facilitation [10].

NanoSIMS

NanoSIMS is distinguished by the fact that it can analyze a sample with a resolved scale that is very small [11]. However, having in mind the intensities of these beams make the fragments sometimes a problem, and as well as the surface damage, the whole range of mass may be shortened. However, the ions presented with some troubles of isotopic ratio interference and some instruments solve this issue by using primary source ions such as larger polyatomic ions (for example, Bi_3^+ or C_{60}^+) or even gas clusters such as argon or water. The extensions of mass ranges observed by mass spectrometers towards 2000 m/z units and the interest in introducing 3D imaging have now been realized. Even if the method avoids any uncertainties in the mass range, it is still limited regarding the number of species that can be measured simultaneously: 5 -7 at a given occasion It is a challenge to perform effective site assessments when dealing with difficult biological samples such as microbial communities (e.g biofilm), minerals and natural organic matter (e.g., humic substances) which can be found in sediments. Moreover, the problem arises in measuring the quantity that is in agreement with different isotopes and elemental contents in the samples. Matrix effects

Inherently are the foremost area of concern and usually, these are based on SIMS techniques, corresponding to the reference Standards - to eliminate them or in the case of the isotopes, where the mass ratios can be correlated. Generally speaking, the two methods mentioned above - quick Freezing or chemical fixation, can reduce complications in the organic and chemical levels of the sample [12].

On the contrary, if this technique is coupled with isotopic and or elemental labelling methods (for example, MAR-FISH; GoldFISH), this tool becomes a strong visualization feature and provides a chance to detect cells and structures of interest specifically. Since 1999, SIMS-based methods using chemicals radioisotope-labelled substrates have been used to study microbial mitigation pathways of hydrogen, carbon, sulfur, and phosphorus uptake. The application of nanoSIMS imaging either alone or in combination with specific isotope-labelling experiments has been quite successful in the elucidation of metabolic functions of microbes in various environments or has further been applied in the understanding of bacterial controls on metals in mine wastes. For example, the examinations on the stable isotopes of oxygen, iron, copper and sulfur as well as low-weight organic acids provide the most crucial data about bio-chemical element circulation, of which prediction also helps to identify metal release in AMD formation [13].

STXM

STXM utilization is more particular offering not only chemical speciation but also quantity determination with different spatial regions: 10 to 30 nm, depending on the accessory optics. This is because the ability of STXM to generate contiguous, microscale quantitative maps of redox-sensitive states, for example, As (III/V) and Fe (II/III), are some advantages that allow STXM to probe the location and identity of selenite/selenate, polysaccharides and proteins. STXM can take advantage of both soft (75–2400 eV) and hard X-rays (>2600eV) as its energy spans are KE across the two regions. If the majority of the peaks in the spectrum belong to elemental lines of the major elements (C, N, O, Na, Mg, Al, S, P, Cl, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, and Zn), both L-edges and K-edges are discovered in most cases. In particular, this technique's capacity to gather properly the only available infrastructure exposes montage differences between the two cultures.

This is of utmost benefit due to their ability to pass through water (like in hydrated samples) and therefore being highly suited for the investigation of such samples. However, succeeding research took advantage of its biomolecule mapping, metallic species, and complex sediment quantification in microbiology, biogeochemistry, and soil science [14]. The imaging that is demonstrated using STXM is done through a series of dimensional pixels wherein the appropriate focus on the sample is achieved by utilization of a mechanical raster scanning ring made by a zone plate assembly. This method indeed has limitations, for example, the damage occurred from the irradiation which involves special sample preparation as it usually requires only thin samples (near the thickness of 200nm).

Although sample preparation is efficient like HTEM, the sample tends to delicately be broken. Even though the volumes of articles on MSI techniques are quickly developing, some revolutionizing advancements in complementary alternatives are also igniting. Due to the concomitant development of new isotope imaging methods, which apply advanced genomic techniques, there is an encouraging possibility of tracing the complicated interaction between microbial communities and the natural systems we inhabit. The latest accomplishment via using labelled gold oligonucleotides as well as nanoSIMS not only powered up the sample stability, but also this work brought the spatial resolution up to 0.8 nm. This process meets the cell wall criteria for microbes and also deposits less carbon and nitrogen veils in the microbial mechanism [15].

Fluorescence in situ hybridisation:

To define FISH procedures, nucleotide probes, such as DNA or RNA, (labelled with a reporter molecule such as fluorochromes, ^14^C, ^3^H, isotopic or enzyme-linked enzymes) were designed to hybridize to a specific target nucleic acid sequence in cellular compartments. These techniques emerged from non-fluorescent in situ Reverse hybridization of multiplex probes with distinct ribosomal RNA sequences from the same bacterial and mycobacterial pathogens have hence been applied to diagnosis. In the 1990s, histopathological detection of the Mycobacterium leprae on tissue and the Helicobacter pylori in tissue was described. Moreover, ISH found the acknowledges in human papillomaviruses and viruses of the herpes family, including varicella-

zoster virus, cytomegalovirus Epstein-–Barr virus as well as HHV 6 and 8, hepatitis B & C virus, human immunodeficiency virus 1, JC virus, adenoviruses and human parvovirus [16].

The first FISH application was mentioned in the year 1980 and specifically involved DNA-binding using a 3′-fluorescence-labeled RNA probe. In one year, involving amino-allyl modification instead of the bases of nucleic acids probes, arbitrary attachment of varied haptens or fluorophores was provided, implying a new molecular basis of conjugation. It was unfortunately found that the hybridization of the long-chain interrogating probes caused specific adhesion of such, which makes the background fluorescence one of the early problems with FISH [17]. The "suppression hybridization" by the pre-treatment with denatured blocked binding sites which caused the non-specific binding was worked out in 1987 as a solution to competing for the luciferase signals. The decrease in probe size further improved the "signal-to-noise ratio". Intracyptre diaphanoscopes (FISH) were initially described in 1980 as a method of detecting DNA sequences in animal cells with a single fluorescent-coloured probe.

This discovery was followed by two-colour detections in 1986 that illustrated chromosomal patterns in metaphase and interphase nucleus of a human-mouse somatic cell hybrid and by three- From 1990 to 2000 the frequency of citations FISH in the PubMed database went up from digits which were less than one hundred to more than two thousand a year. The advancements in FISH technology as well as the furthering innovation and the increase in mechanization of diagnostic FISH have further increased the reliability and reduced the chances of operator-to-operator variability thus making it dependable and accurate [18]. A technique using PNA (peptide nucleic acid) that processes permeabilization steps and increases specificity has been improved. The standardization process of PNA-FISH techniques has been accomplished at quite a high level and the FDA and EMEA approval in clinical analysis of microorganisms has been provided by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). Moreover, the technology-based techniques for instance beaconing FISH expedite the steps of diagnosis and raise the probed point of the tests. Thus, thanks to these recent advances, FISH can be a routine technique to be used alongside other modern molecular diagnostic

Methods of clinical microbiology [19]. Sometimes you would encounter an article published even in recent years that does not include PNA-FISH in the Modern review articles on clinical microbiology in which suggests that PNA-FISH is no more applicable in routine diagnostics compared to the mass MALDI–TOF and high-throughput sequencing. Unlike the other writers who state that FISH helps to precisely determine bacteria in the samples of infection, the researchers claim that this approach is fast. The current issue they encounter is that different experts have different perspectives, therefore, outlining the benefits of FISH, which is a method for rapid identification of microbial pathogens and the issues with the the testing method in human infections [20].

Principle and procedure of FISH:

The fluorescence in situ hybridization (FISH) technique is used to identify microbial target organisms (bacteria, yeasts and protozoa) down to the genus or species level by using short, usually 18 - 25 base-pair oligonucleotide probes, which are labelled with fluorochromes and their specific atomic resistances and is bound to the rR. The main thing is the FISH probes are quarried for different targets and afterwards collected into "probeBase" database [21]. Following the targeting of labelling with conventional samples on standard glass slides followed by the prerequisite target-specific permeabilization steps, the labelled probe hybridizes with the complementary ribosomal RNAs. The probes-excess probes are removed in the washing step.

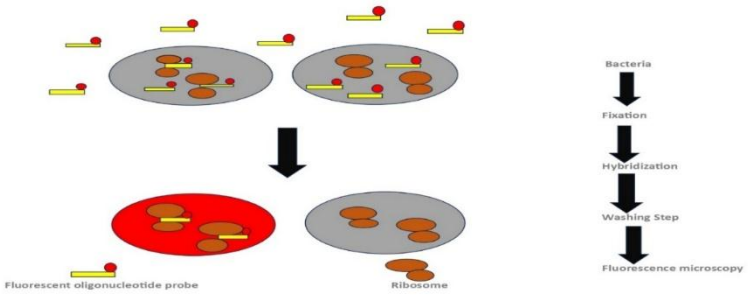

Figure 1. Sequential procedures of FISH. Big ellipses: bacteria; small double ellipses: ribosomes; bars with dots: fluorescent oligonucleotide probes [17].

Atomic Force Microscopy

The AFM technology releases tremendous opportunities for in-situ examination of the nanoscale construction of the surfaces and their local properties. The invention of this probing surface imaging technique, in 1986, involves a probe that is sharp and possesses abilities to measure the forces when scanning across the surface of a sample. Under the former modality, scanning the probe against the sample is the usual mode and the force is kept constant at all times. The opposite approach can also be used involving the recording of the probe deflection while the latter scans system goes along with a fixed height. Thus, it means the surface topography is seen at such a resolution that (sub) nanometer features can be resolved [22]. Another important aspect of AFM is surface topographic imaging, which can be obtained by monitoring vertical forces (adhesion). The technique can also be utilized in determining a variety of local surface properties including lateral forces (friction), mechanical properties and surface forces. The literature beyond these basics of AFM theory and different modes of operations are well studied, which will provide further information. As opposed to most surface analysis and electron microscopy techniques, AFM can be operated in aqueous solutions, which makes it possible to investigate biological samples under physiological conditions. A large variety of biological specimens have been examined, from biomolecules [23].

Magnetic Resonance Imaging (MRI)

Different professional approaches that can be used to detect the chemical composition, as well as the physicochemical properties of the cell walls of microorganisms, include surface X-ray photoelectron spectroscopy, infrared spectroscopy, contact angle measurement and electrophoretic mobility. Also, the surface force techniques, like the osmotic stress method, the surface force apparatus and the micropipette method are widely used methods to determine the forces at the aqueous-solid interfaces. As same as skilful surgeons, these tools can range from electromagnetic scans to angiography, but still cannot present direct information at the order of nanometer-scale resolution. In the past three decades, magnetic resonance imaging (MRI), has evolved into the most sensitive, precise and commonly used diagnostic tool for clinical diagnosis [24].

MRI is one of the essential clinical diagnostic tools in acute trauma when dealing with musculoskeletal disease, brain-related pathologies such as stroke or neurodegenerative diseases, cancer detection and staging, and cardiac imaging [25]. In contrast, the effectiveness of ultrasound in the diagnosis of infectious disease is minor, and it usually is used just for the detection of inflammation, effusion formation, and other signs of immune reaction, but not for the direct detection of the pathogenic organism nor the spread of secondary infection. Such absence of application is incredible because infectious disease is still the major disease problem in healthcare worldwide. Infections commonly result in life-threatening conditions such as endocarditis, osteomyelitis, and encephalopathies among others, and patients often die as a result. This is especially true following early or chronic infections as they develop in a scarcely detectable way. To exemplify, in osteomyelitis, a non-infected bone tissue can never be differentiated from an infected one intra-operatively [26].

A diagnostic imaging technique that would be non-invasive and discriminating in differentiating between bacterial infections and other causes of inflammation would be a great breakthrough to improving diagnosis and treatment and, therefore, effective management of these diseases. To achieve this goal various imaging techniques have been developed and applied to both infected patients and working animal models. These imaging techniques include bioluminescence (BLI) and fluorescence imaging, positron emission tomography (PET) single photon emission computed tomography (SPECT) and MRI. PET methods generally manifest such indirect changes in cell behaviour by monitoring for alterations in the processes of cellular signalling and metabolic changes [27].

Among these leaders of physicians to come up with several particularly specific radiotracer molecules based on antibiotics, antimicrobial peptides, cytokines, or monoclonal antibodies that would be able to specifically label bacterial infections. Unlike other types of imaging techniques, MRI often gives low-resolution images and better imaging modalities will help improve this. By the way of choice, the BLI technique for preclinical or basic microbiological research purposes, FRI (Fluorescence Reflectance Imaging) and FMT (Fluorescence-Mediated Tomography) might be promising alternatives. Uncovering the course of viral or bacterial infection is done via BLI by

generating recombinant pathogens that are modified to take on the task of luciferase enzyme expression [28].

Bacterial biosensors' low resilience to a single bacterium, unfortunately, limited BLI progress. However, taking a fluorescence approach which utilises green or red fluorescent proteins allows in vivo detection of single cells. In the latter case, more invasive techniques are used. In some cases, autofluorescence and scattering do not cause any significant problems, but when autofluorescence and scattering are used for non-invasive imaging, these issues turn out to be the most troublesome. To meet this requirement, new near-infrared fluorescent probes have been advanced that are good for use in imaging because they have a longer penetration depth, which is permissive over problems existing by green autofluorescence of tissue. In the last few years, the ability to monitor murine lung inflammation by FMT has been demonstrated as well. On the other hand, lack of spatial resolution intrinsic contrast is one of the deficiencies of FMT that can be overcome through co-registration with other modalities such as MRI [29].

Scanning Electron Microscopy:

Scanning Electron Microscope (SEM) technique is used to understand plans of the surface at different folds and magnifications [30]. Nevertheless, an extremely high vacuum is compulsory for the samples evaluation and also the fact that the biological samples do not have conductive qualities predetermines such features as fixation, dehydration as well as coating with the conductive material. Besides that, methods need to be invented that do not ruin the structure of the samples or cause anomalous results. Once the sample is glued on the grid with

Aldehydes in phosphate or cacodylate buffer, dehydration takes place using a gradual ascending concentration of acetone or ethanol. This method solubilizes the molecules of water by deterioration which is brought by the organic solvents. Then, the samples undergo the stage of microwave-assisted drying to maintain the integrity of the intricate structures. The artefact could be avoided because no structure of solid would be formed except for the critical point drying. Given that ethanol can create obstacles in the prediction and observation of human brain pathology, it is replaced by a transitional solvent which comprises carbon dioxide. Also, these samples could undergo freeze-dry treatment. Lastly, sequentially, the specimens must be coated

with a conductive material, such as sputtering with gold. SEM is not able to be used in searching for diverse species of bacteria due to a lack of spectrometry techniques. On the other side, identification of the type of bacteria, for example, cocci, rods and filaments is sometimes difficult, as it depends on the shape of the bacteria. Although SEM analysis is not applicable in this context for providing insights into the formation of complex biomimetic layers, SEM can surely be used for the investigative study of the conditioning films and proteinaceous layers like the acquired dental pellicle. Consequently, a combination of SEM with gold-immunolabelling notes can be utilized for the evaluation and numerical determination of given proteins [31].

Transmission electron Microscopy:

The viral particle evaluation is mostly done via an electron microscopic technique called transmission electron microscopy (TEM) [24]. TEM is a benefit that is associated with its high-resolution power of down to 0.1 nm glass. The fixation that is necessary for further investigation is performed with glutaraldehyde or osmium tetroxide both of which show up lipid stains. Another option could include cryo-fixation at −135 °CA to help with artefacts. Nevertheless, the very thing which gears the intriguer is the low contrast. Moreover, complementary dehydration spent in an ascending concentration of ethanol into acetone is demanded, and embedding into acrylic resin as an element before ultramicrotomy processing is indispensable. Last but not least, the presence of dyeing with heavy materials like lead citrate or uranyl acetate for the sake of contrast is often common during staining. To conclude, this method (TEM) is very suitable for studying the structure of bacteria when interacting with the extracellular matrix or the conditioning film (dental pellicle) but this technique is complex and time-consuming. At the same time, because it provides very high resolution, TEM is also regarded as the leading technique of electron microscopy [32].

Transmission X-ray microscopy:

A fully developed biofilm is a multi-layered matrix made of exopolysaccharides, proteins, and nucleic acids. The features of high-resolution transmission X-ray microscopy lead to the employment of this technique for mapping the localization of macromolecules within microbial

Biofilms [26]. STXM pose advantages over full-field TEM because the STXM instruments are installed in synchrotron beamlines of a better resolution and produce spectra of

Higher quality. The analytical skills of STXM may be attributed not only to resolving a few controversial questions about proteins, carbohydrates, lipid and nucleic acids in biofilms but also their distribution and functions, with greater attention given to the extracellular matrix. At present, the roles of fibrils of polysaccharide (polysaccharide-coated protein), and other polymers (extracellular nucleic acids, extracellular proteins and associated fibril-lipid structures) are limited. The recent research on a DNAse enzyme focused on the extracellular DNA presumably having a structural role in the formation of biofilms that give stability. On this note, visualisation of macromolecular distribution is highly valuable for revealing mechanisms responsible for the formation of biofilms, and for the modification and even the control of these phenomena [33].

Positron Emission Tomography

PET (positron emission tomography) is an excellent diagnostic method, which is not intrusive, and can be used in research and the clinic in the exploration of the pathophysiology of many kinds of brain disorders, such as neurodegenerative disorders, epilepsy, seizing disorders, psychiatric conditions, and brain tumours. Furthermore, it aids in the understanding of the brain physiology of the healthy brain PET is a most sensitive imaging method that allows for the spotting-out of positron-emitting radiopharmaceuticals so that scanning can be done in 3D for miniscule (µg) quantities without incurring notable physiological and pharmacological effects. PET, therefore, is often--times referred to as a functional or molecular and diagnostic imaging tool demonstrating the study of biological functions in health and disease, respectively [34].

Metagenomics

In the beginning of the ‘20-ies of our century’, the term ‘metagenomics’, designating an amazing way to simultaneously study intersecting branches of biology and chemistry was for the first time used. The most important factor in all these was the fact that metagenomics in soil was the first application and later on it became a driving force in different fields like microbial ecology and

biotechnology. In addition, it has become very helpful in the journey of exploring the hidden world of invisible creatures. Being a part of the biotechnological developments, sequencing technologies have become cheaper, faster, simpler to use, and multi-dimensional. As a result, we can explore which microbes exist in any environment and what their functions are with increases in speed and accuracy. The ability of the metagenomic approaches to solve complex problems is highly dependent upon the algorithms and arithmetical sophistications they are running [35]. The employment of metagenomics, directly sequencing and analyzing DNA, from microbe's assemblages, has come to be a routine method for characterizing the functional diversity of microbial communities. DNA sample processing involves isolation, arrangement into libraries, and high-throughput sequencing platforms with short-read or long-read requirements.

Met genomics analysis begins with quality filtering of the sequence reads, to reduce sequence bias/artefacts by removing adapter sequences, low-quality calls, and non-source pollution that is not the environment. The earlier utilization of metagenomics depended more on a gene-centric approach, and it focused on the estimation of gene abundance of the gene of interest and its function within a metagenome by gene detection and annotation of short reads. Apathy towards the relatively new science of metagenomics came to an end as these tasks immediately doubled the number of genetic markers in the database and stimulated the appetite for new applications. Through the use of this diverse set of tools, very little is now known about >50% of the types of genes contained in environmental microbiomes. One of them is that the amount of protein is similar to one-third of protein-coding genes of the isolate genomes. So, the extent of our identification of functional genes holds a direct relationship with the completeness of gene databases and further work that is done aimed at improving our understanding of gene functions [36].

Metaproteomics

In the realm of functional genomics, metatranscriptomics, and metaproteomics help determine the abundance, and variety of gene expression revealing the genetic makeup of microbial communities [37]. One speciality is sequencing mRNA transcriptome (a molecular entity that is referred to as RNAseq) which can be done using the same sequencing machines that are used to

sequence DNA genomes. Transcriptome sequences are commonly mapped onto assembled genomic sequences and further exploited for quantification purposes or re-annotation of the genome (e.g., detection of operons, gene length and transcription start site). In essence, rather than only coding for protein, metaproteomics seeks to provide information and the nature of post-translational modification and provision of localization information which is not present in the transcriptome. A particular labelling technique can give information about the quantities of proteins. These techniques can be qualitative from a practical label-free perspective, metabolic labelling and isotope-labelled/cod isobaric chemical labelling approach can determine the relative abundances of proteins [38].

Next-generation-Sequencing (NGS)

Naming and grouping of microscopic organisms that result in infections should be a prime concern for the successful treatment and cure of the patients, especially when it comes to their safety. Cultural methods in the diagnostic laboratory may sometimes miss not every bacterial species, while more developed molecular tests of pathogens are still unable to detect emerging genetic changes in the line of spread in people, animals, and the environment. Easy transmission of hospital pathogens that evade patients' recognition and might cause an outbreak in a hospital can sometimes be a hazard for patients admitted during their stay in the hospital. The last 10 years have witnessed a boom in the development of molecular diagnostic methods which are now used extensively in medical laboratories working in the microbiology area.

Currently, multicultured media and molecular-based methods are used that have significantly reduced the turnaround time. Also, it is now possible to detect non-cultivable pathogens. Therefore is the NGS method a Technology that performs whole-genome sequencing of various strains from all patients at the same time either from bacterial isolates or from all bacteria present in one patient material (metagenomics) [39]. Both the one-time cost of NGS and its recurring costs for operation and staffing have been in stark decrease through the last decade. One very important NGS benefit is the fact that sample processing for all pathogen identification and typing tasks can be performed with a single protocol. This, thus, indicates that the technology is applicable in medical microbiology labs while also assisting in infection prevention

measures. There are no target-specific primers that you need for NGS, instead of you adding them to Sanger sequencing as you had needed before. In the course of a single night, an unknown virus happens to solve the riddle of its entire genome sequence simultaneously. Fragmentation of the genome is made before the sequencing since the maximum length a bench-top sequencer can sequence is from 100 to 1000 bases, which is an insufficient size to perform the sequencing of the genome in one piece [40].

Functional Gene Microarrays

To obtain specific and sensitive information on the whole microbial communities as well as the quantitative and permissible type of metagenomic, functional gene arrays (FGAs) are considered [41]. For instance, the most profound FGAs, namely, GeoChips, are used to survey the functional diversity, the structure, and the composition of the metabolic capabilities of a great variety of habitats, including water ecosystems, soils, contaminated sites, and extremely hostile environments, as well as the functioning of bioreactors. FGAs are represented as a tool kit, which could be used to answer the important questions of global change, land use, bio-remediation, human health, ecology etc. It is also important to link microbial community structure to environmental properties and ecosystem functioning to get a clear picture. FGA is a particular type of microarray consisting of probes for essential genes which carry out the fundamental functions of microbes like carbon (C), nitrogen (N), sulfur (S), phosphorus (P) metals cycling, antibiotic resistance, environmental contaminants biodegradation, and stress responses [42].

Stable Isotope Probing

Stable isotope probing (SIP) is a technology for the traceability of microorganisms in environmental samples based on their usage of a particular nutrient substrate that they use. To check the authenticity of this technique in the study of the methanol utilizers of the bacteria soil, it was found that 1 gram soil comprised two phylogenetically different groups of bacteria, the α - proteobacteria and acidobacterium lineages. Stable-isotope probing thus, generates a great new tool for the discovery of the inhabitants that are not only active but that also are engaged in the

Same metabolic functions as in the natural habitat. Isotopes in biochemistry have been counted among the most powerful tools for almost one century. At first, the core constituents were merely the radioactive isotopes, either 14C-propionate by Escherichia coli in 1927 or the incorporation of 14C-glycine into proteins of yeast [43].

Conclusion:

Over the last decade, microbial ecologists and microbiologists have made the most benefits from the advances in the MSI technique as well as the emerging molecular methods. The novel methods are of crucial importance for identifying microbial diversity and how it is linked to environmental substrate. Despite that, the exponentially intricate nature of the microbial domain will ultimately play a major role in sparking science and technology to broaden constantly the capacity of the tools even more. Therefore, where do we head from now? With the increasing number of new microbial-microbial and microbial-environment interactions, the prospect of uncovering the motivators of these cooperative relationships should be explored systematically. These new techniques and approaches are today enabling researchers to watch the microbial societies performing in the environment in an impartial way that is uncultivated in a laboratory. The microscopic and genomic worlds are now getting close to each other because these kinds of advances keep taking place in the form of new collaborations, which in turn expand our understanding of the mind-blowing complexity represented by microorganisms governed biogeochemistry.

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