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REVIEW

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Accessing the energy-limited and sparsely populated deep biosphere: achievements and ongoing challenges of available technologies

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Abstract

Microbes in marine sediments detected and counted by direct observation of membrane-filtered sediment samples stained with acridine orange. This technique can still be applied to high-biomass ($> 10^5$ cells/cm^3) sedimentary habitats, such as organic-rich sediments collected in shallow areas near the seafloor. However, to further explore the nutrients and energy turnover under extremely low energy flux conditions, or in habitats that are close to the lower limit of the biosphere, technological breakthroughs have been required to increase the detection sensitivity for microbial life at densities of a few cells/cm^3 of sediment. These technological developments contributed to increasing fundamental information on microbial life at the fringes of the subseafloor biosphere and led to the discovery of revivable microbes in sediments aged up to 101.5 million years old. More recently, chemical detection methods have revealed the existence of spores in the deep biosphere that are impermeable to conventional DNA stains. Previous applications of molecular biology-based approaches have been limited to relatively higher biomass samples, potentially because the cells surviving in these very low energy flux environments have less integrated genomes. Here, I review the contribution and importance of the technological developments that have been made in the study of microbes from the subseafloor biosphere, recent developments of alternative methods to microscopically detect microbial spores and their application to deep subseafloor sediments, and the challenges associated with applying molecular biological approaches to study low-biomass samples.

Keywords Subseafloor biosphere, Low energy habitat, Technical developments, Limits of life

1 Introduction

Our understanding of the distribution of microbial life on Earth has expanded continuously as research activities that focused on the discovery of life in new environments have progressed. The subseafloor environments are among the most explored natural habitats to date by numerous studies that have been conducted in recent decades. In 1955, Morita and ZoBell (1955) conducted a pioneering study of the subseafloor biosphere, in which they used cultivation-based techniques to suggest that the biosphere extends only several meters below the seafloor (mbsf). Several decades later, the Ocean Drilling Program (ODP: 1983–2003) significantly increased our understanding of the deep subseafloor biosphere (water depth from 151 to 3773 m, maximum sediment depth from 80.2 to 748.49 mbsf [meters below seafloor], age to 10 million years, and variety of physical/chemical environments) (Parkes et al. 1994, 2000b). The findings of the ODP accelerated attempts to

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explore the subseafloor biosphere and were followed by the Integrated Ocean Drilling Program (2003–2013) and the International Ocean Discovery Program (2013–2023) (e.g., Ciobanu et al. 2014; Inagaki et al. 2015; Roussel et al. 2008), which showed that the biosphere extended to more than 2.4 km below the seafloor. The accumulation of knowledge about subseafloor microbial abundance facilitated estimates of the global microbial biomass in the subseafloor biosphere, which is considered to account for 12–45% of the total microbial biomass on Earth, or 0.6–1.8% of the total living biomass on Earth (Bar-On et al. 2018; Kallmeyer et al. 2012; Magnabosco et al. 2018; Whitman et al. 1998).

Even though the entire population of subseafloor microbes is massive, volumetric estimates indicated that the microbial cells constitute only a minor component (<0.01% by volume) of the sediments, even at the seafloor where the highest microbial densities are typically found (Morono and Kallmeyer 2014). Consequently, the general distribution of microbial cells in the sediment matrix is considered to be relatively sparse. However, their real in situ distribution has not yet been directly observed. The majority (>90%) of marine sediments are composed of fine particles measuring less than 1 μm to several tens of micrometers (Diesing 2020; Dutkiewicz et al. 2015). This fine nature of subseafloor sediments restricts both active and passive transport of even small microbial cells and results in the formation of depth-stratified microbial communities (Inagaki et al. 2006). In addition, the fine nature of subseafloor sediments can complicate the analysis of subseafloor communities, such as identifying and enumerating microbial cells and performing DNA/RNA-based molecular analyses.

The accumulation of knowledge about the subseafloor biosphere has been achieved through extensive development of subseafloor-targeted technical and analytical approaches; using preexisting processes can produce variable (non-reproducible) and misleading results. The subseafloor environment, in which microbial communities constitute a very small and volumetrically minor component, contains numerous substances that can interfere with analysis and sampling, both of which are challenging using existing technologies. Consequently, extensive optimization of, or improvements in, analytical techniques has been necessary. This review focuses on technological aspects of subseafloor biosphere exploration, in particular, on effective solutions to previous challenges and areas that require further technological development.

2 Review

2.1 Fluorescence staining: Are those tiny blobs really the ones you are looking for?

Detection and enumeration of microbial life are the most fundamental steps for exploring the biosphere. Conventionally, these steps were conducted by cultivation techniques, such as by direct plating and counting colonies. A pioneering study on counting microbes from the subseafloor biosphere by Morita and ZoBell (1955) described the microbial life found in deep marine pelagic sediments of the Pacific Ocean. They conducted direct plating of diluted subseafloor sediments and succeeded in culturing colonies from several meters below the seafloor (mbsf). However, they did not detect any countable colonies from samples collected at 3.86–7.47 mbsf. Although they noted the potential for underestimation of microbial abundance due to the limitations of the culture media, the limit of the marine biosphere at that time was considered to be 7.47 mbsf. Beginning in the late 1980s, John Parkes' group began quantifying microbial biomass in deeper subseafloor sediments using fluorescence staining of intracellular DNA. They used the acridine orange direct count (AODC) method for their counts and biomass estimation (see review in Parkes et al. (2000a)). Acridine orange is a widely used DNA intercalator that efficiently stains the genomic DNA in microbial cells. Initially, all the cell counts of ODP core samples were performed by Parkes' research group, mainly by Barry Cragg (Cragg 1994; Cragg et al. 1992, 1997, 1998, 1999, 1990, 1995a, 1995b, 2000, 2002; Cragg and Kemp 1995; Cragg and Parkes 1993, 1994; Wellsbury et al. 2000). The fact that all of the measurements were performed by a single research group (and primarily by the same person) was of primary importance in creating a dataset of almost unrivaled consistency.

Although DNA-specific staining and fluorescence microscopy methods have been successfully applied to aquatic habitats (the most common examples include 4',6-diamidino-2-phenylindole (DAPI; Porter and Feig (1980)), acridine orange, (AO; Daley and Hobbie (1975)), and SYBR Green I (SYBR-I; Noble and Fuhrman (1998)), the application of these methods to marine subseafloor sediment analysis was not straightforward. These difficulties were primarily related to the presence of fine particles that constitute the majority (>90%) of marine sediments. For example, the native fluorescence of mineral grains, especially under UV excitation (Robbins 1994), caused problems with microbial cell detection using DAPI, one of the most widely used cell stains. As a result, DAPI is not commonly used for marine sediment analysis. Another problem is the non-specific adsorption of dyes to the fine particles that are present in marine sediments. Figure 1A shows an example of AO staining of marine sediments in which the fluorescence from many non-cellular particles was comparable to that of the microbial cells. The non-specific binding of the fluorescent dye to sediment particles made microbial cell detection difficult,

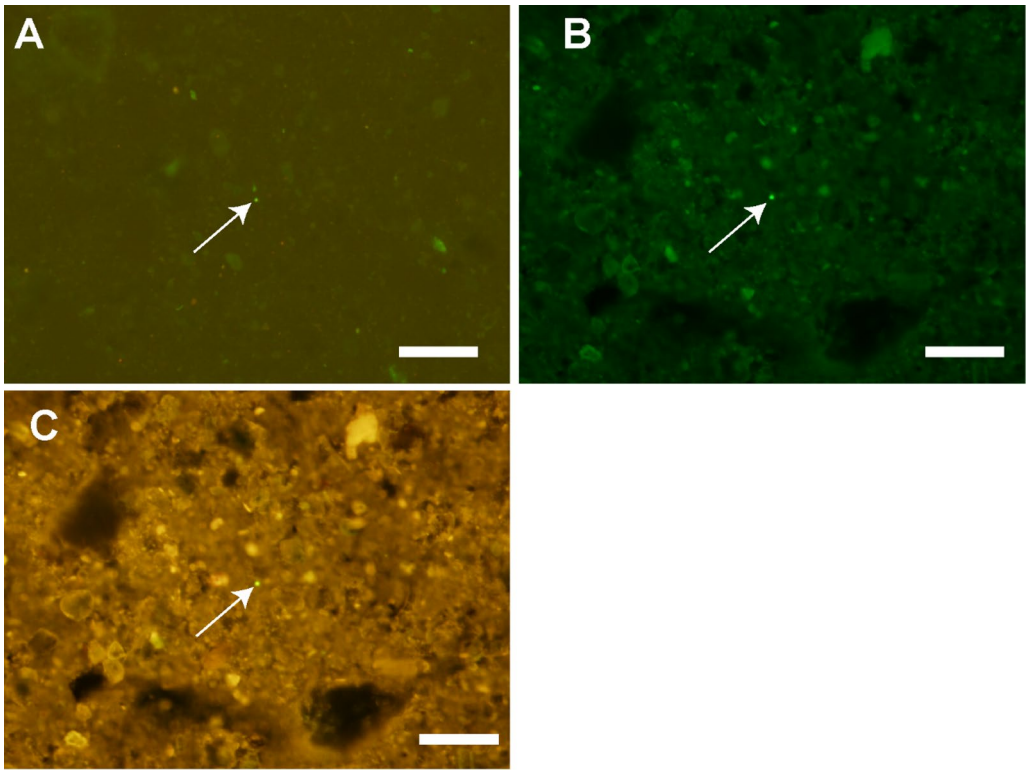

Fig. 1 Examples of fluorescence microscopy images. Sediment samples obtained by International Ocean Discovery Program (IODP) Expedition 370 (T-limit) at 207.5 mbsf (3F-3) was stained with AO (**A**) and SYBR-I (**B**, **C**). **B** and **C** show the same position of the prepared membrane observed with optical emission filter that passes only wavelength around 525 nm (**B**) and without emission filter (**C**). It is noted that only single cell (pointed by white arrows) is present in the area of each image. Bars represent 5 μm

and investigators had to be trained intensively to produce reliable and consistent data.

The problem was more significant when SYBR-I was used to stain microbes in sediments. The fluorescence intensity of SYBR-I has been reported to increase by ~ 1000-fold upon binding to DNA (Dragan et al. 2012). Since free SYBR-I dye has extremely faint fluorescence, it is often regarded as a DNA-specific stain that fluoresces brighter than other DNA stains. While this may be true for most samples, SYBR-I is preferentially bound by marine sediment particles (Fig. 1B), which means that the take-up of SYBR-I by microbes only occurs once sediment particle adsorption has reached saturation levels (Mori et al. 2021; Morono and Inagaki 2010; Morono et al. 2008). Even though the fluorescence intensity is enhanced after binding to DNA, the fluorescence intensity of the SYBR-I dye that accumulated on the sediments was similar to that taken up by the microbes; consequently, the problems associated with discriminating between microbes and sediments using SYBR-I were similar to those encountered with AO. Microscopes for fluorescence observations are equipped with optical filters, which allow only light at the excitation or emission wavelengths of the target fluorophore to pass through, and dichroic mirrors, which reflect the excitation wavelength and allow light at the emission wavelength to pass through. With the proper filter set, target fluorescence can be precisely observed; however, some specialized filter sets can hide important information. Conventionally, the green-fluorescent dye-stained (i.e., AO- or SYBR-stained) microbes are observed with an emission filter that only allows the green color of the fluorescence (generally around 525 nm) to pass through. However, problems can arise when identifying the signals of microbial cells from SYBR-I dye in situations where there is an overaccumulation of non-cellular particles (Fig. 1B). Morono et al. (2008) reported that the fluorescence from microbial cells and non-cellular particles can be distinguished by the fluorescence color when observations are performed without an emission filter (Fig. 1C). They proposed that even though high levels of SYBR-I adsorption to sediment particles results in the fluorescence intensity being comparable to that of the SYBR-I-DNA complex, the overaccumulation of SYBR-I may have induced a shift in the color of the fluorescence through intermolecular energy transfer. Based on their findings, a

computer-based recognition scheme was established to automatically detect "green" microbial cells by calculating the fluorescence of two images taken using emission filters through which only green (525 nm) and orange (605 nm) light could pass (SYBR-DiCE method; Morono and Inagaki 2010; Morono et al. 2009). According to their data, the "false-positive" counts from non-cellular particles were almost constant with subseafloor depth. However, the "actual" cell abundance decreased with an increase in depth; as a result, the scatter of two plots increased with sediment depth (Fig. 2). These findings provided a way to accurately distinguish between microbial cells and non-cellular particles in the sediment without intensive training of the investigator. Unfortunately, a similar shift in fluorescence color due to overaccumulation was also observed with several other green fluorescent dyes, but no shift due to overaccumulation was observed when using dyes of other colors.

There are currently more than several commercially available cell-permeable DNA stains on the market and SYBR-I is only one of them. However, there are certain types of microbial cells that do not take up many of these stains; spores, which are a type of microbial cells having almost impermeable cell wall structure. Microbial endospores, especially the spores of thermophilic microbes, or thermospores, are considered to be widely dispersed in the marine environment, and thus, in the subseafloor biosphere as well (Hubert et al. 2009). The abundance of spores in subseafloor sediments is thought to be enormous, and unlike their vegetative counterparts, the relative abundance of endospores is reported to increase with depth below the seafloor (Fichtel et al. 2007; Lomstein et al. 2012; Wörmer et al. 2019). Indeed, a spore-dominated horizon was discovered in the subseafloor sediments of the Nankai Trough (Heuer et al. 2020). Quantification of these microbial spores has been typically performed by chemical detection of the spore-specific compound, dipicolinic acid (DPA) (Fichtel et al. 2007; Lomstein et al. 2012) as well as estimates of microbial metabolic activity after germination (Hubert et al. 2009). However, to the author's knowledge, direct observations of microbial spores from marine sediments have not been reported to date. The reason for the lack of such observations is likely attributable to the impermeable nature of the spore coat. Heuer et al. (2020) reported that the SYBR-I stain was ineffective for staining cultured *Bacillus* spores. After SYBR-I staining, the *Bacillus* spores showed yellow fluorescence (Fig. 3), indicating that SYBR-I was adsorbed by the spore surface and unable to penetrate the outer surface of the spores. Although the abundance of spores is expected to be similar to their vegetative counterparts (Wörmer et al. 2019), they cannot be visualized using current technologies.

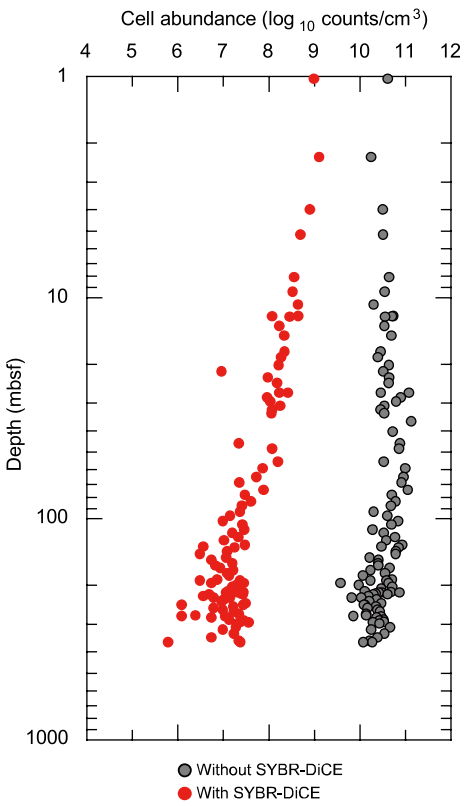

Fig. 2 Cell abundance plot for the samples obtained from offshore Shimokita Peninsula. Increased deviation of observed cell abundance was found between counts conducted without (gray) and with (red) SYBR-DiCE method along the depth of subseafloor sediments. Modified from Morono et al. (2009)

There is a large variety of fluorescence staining techniques, some of which use fairly complicated reactions and/or steps to detect microbial cells. Fluorescence in situ Hybridization (FISH) (Amann et al. 1990) is used to stain 16S rRNA molecules using fluorescent DNA probes that have been specifically designed to bind to taxon-specific sequences. There are also a variety of derivative techniques with enhanced detection sensitivity, such as horseradish peroxidase-catalyzed deposition of fluorescent molecules (catalyzed reporter deposition, CARD) (Pernthaler et al. 2002a) and cascade hybridization of fluorochrome-labeled DNA (hybridization chain reaction, HCR) (Yamaguchi et al. 2015). Schippers et al. (2005) successfully detected bacterial cells in subseafloor sediment samples using CARD-FISH. Their findings showed the existence of 16SrRNA molecules, which are easily degraded in dead cells, and indirectly showed that the microbes in subseafloor sediments were living cells and not remnants of dead cells. FISH-based microbial cell detection methods have several advantages over simple DNA stains; for example, they can be used to infer the phylogeny and physiology of microbes based on the

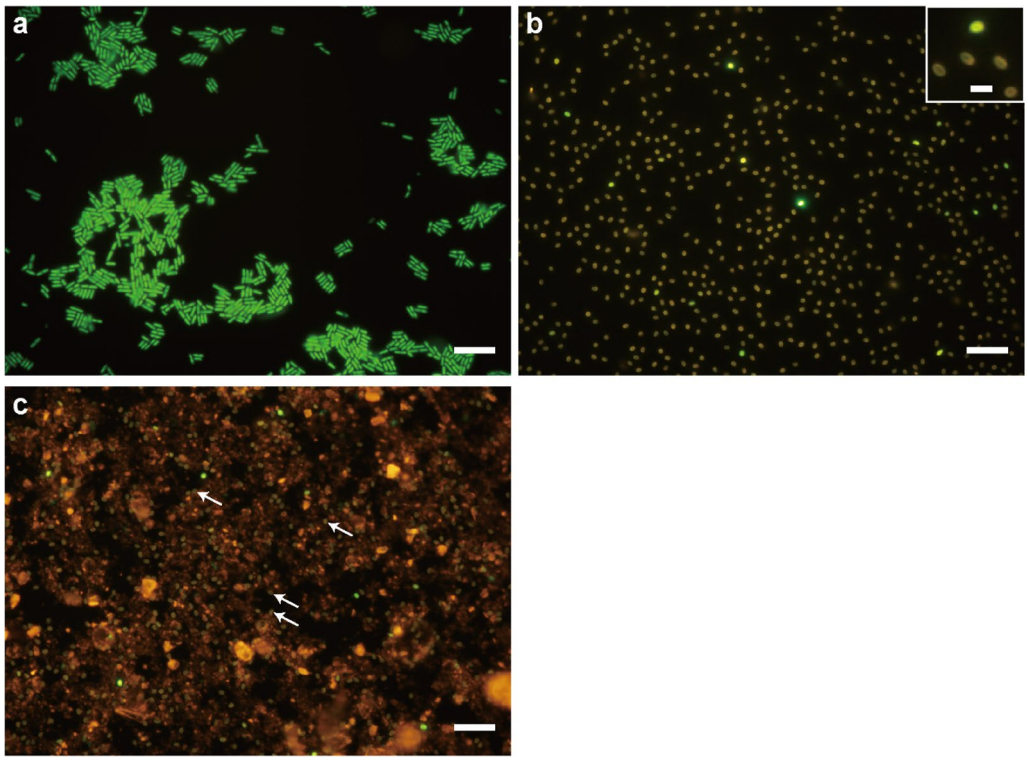

Fig. 3 SYBR Green I staining of vegetative cells (**a**) and endospores (**b**) of Bacillus subtilis NBRC13719. This figure illustrates the difficulty in identifying spores even of pure cultures of organisms that are mixed into sterilized sediments. In (**c**), endospores were mixed in sterilized sediment prior to staining by SYBR Green I. Several representative yellowish endospores, which are difficult to distinguish from sediment particles, are indicated by white arrows as examples. Bars represent 10 µm. Adopted from Heuer et al. (2020)

16S rRNA expression. However, because of difficulties in delivering probes that are larger than DNA dyes, the detection efficiency varies depending on the methods used to permeabilize the cells (Lloyd et al. 2013). In addition, the probes are also highly susceptible to non-specific adsorption to sediment particles, and this effect is exacerbated with CARD or HCR signal amplification. If it is the simple adsorption of DNA probes onto sediment particles, then green fluorochromes, such as FITC-labeled probes, can be discriminated by the fluorescence color shift induced by overaccumulation like SYBR-I (data not shown). However, in the case of signal amplification, the color shift does not occur, probably because the deposited tyramides or amplification probes dilute the local concentration of fluorochromes and thereby, prevent energy transfer. Therefore, preventing the adsorption of probes to sediment particles is extremely important for ensuring the efficacy of the FISH-based approach. The use of hybridization buffers containing a high concentration of EDTA has been found to be effective for reducing adsorption onto non-cellular particles (Morono et al. 2020). Jia et al. (2021) reported that the EDTA buffer was effective in reducing non-specific signals when using the HCR-FISH protocol.

As shown in the above examples, even the most straightforward DNA staining methods have pitfalls when they are applied to the analysis of marine sediments. It is clear that simply applying an existing technique can cause serious problems and lead to misleading results. There are many fluorescence staining techniques, each of which varies in its complexity and what it detects. In all of the techniques, obtaining either false positives or false negatives is possible, and a careful understanding of the detection principles and methodologies is required to correctly interpret the obtained results. Therefore, continuously asking whether the fluorescent blob that you are looking at really is the target or something different is one of the most basic strategies for avoiding mistakes and misleading results.

2.2 *Requirements/prerequisites* for cell detection and analysis: separation of microbial cells from the surrounding matrix and subsequent analysis

Given that microbial cells constitute only a minor fraction of marine subseafloor sediments (< 0.01% in volume), the ability to detect microbial cells is largely restricted to be insensitive. For fluorescence microscopic observations, subseafloor cells need to be trapped

onto a membrane; however, these membranes can easily become clogged, which restricts the volume of sediment samples that can be filtered/analyzed. Generally, the lower detection limit of subseafloor microbial cells is 10^4–10^5 cells per cm^3. However, the separation of microbial cells from other sedimentary particles is the simplest and most effective way to improve microbial cell detection. Kallmeyer et al. (2008) first reported a method for physically separating microbes and sedimentary particles in deep biosphere samples using differences in density. They first carefully tested a variety of approaches for detaching microbial cells from sedimentary particles and found that the combination of chemical and mechanical treatments yielded high and reproducible recovery of microbial cells with minimal lysis. They started with Percoll, a colloidal silica-based gradient agent that is widely used in medical and biochemical fields (Pertoft et al. 1978), to separate microbial cells from sediment samples. Even though it was effective for separating microbes from shallow or coastal sediments and anaerobic methane-oxidizing aggregates (Orphan et al. 2009), cell recovery after separation with Percoll was insufficient for deep subseafloor samples. Kallmeyer et al. subsequently used Nycodenz, a non-ionic density gradient agent (Rickwood et al. 1982), which increased the recovery of microbial cells from subseafloor sediments. At that time, separation was performed by layering the sediment suspension onto a cushion of 50% (w/v) Nycodenz. Later, Morono et al. (2013) modified this separation technique from a bilayer method to a multilayered method using different density agents and achieved higher recovery of microbial cells regardless of the sediment type (Fig. 4). They improved two points in bilayer separation: (i) co-precipitation of microbial cells with sediment particles, and (ii) precipitation of microbial cells with a higher density. The first point was considered to occur due to the turbulent flow caused by the precipitating particles as they moved through the density boundary and captured and co-precipitated with microbial cells floating on the density boundary. The second point was attributed to the decreased cell recovery observed in cell samples that had been fixed in formalin and stored for several months. After considering that the high viscosity of Nycodenz can cause turbulent flow, they used multiple layers of the Nycodenz density agent in combination with the less viscous density agent, sodium polytungstate. The combination of viscous Nycodenz and the less viscous sodium polytungstate resulted in recovery rates that were 8–46 times higher than the recovery rates obtained using the bilayer protocol. To further improve recovery, linear gradient density agents have been developed and applied to cell separation, which has facilitated the ultrasensitive detection and enumeration of microbial cells from a variety of subseafloor environments, including those near the fringe of the subseafloor biosphere (D'Hondt et al. 2015; Früh-Green et al. 2018; Heuer et al. 2020; Inagaki et al. 2015; Templeton et al. 2021).

2.3 Necessity of separation of microbial cells for obtaining reliable data on the single-cell-level analyses

Once the cell detection was optimized, further exploration of the subseafloor biosphere was possible. Indeed,

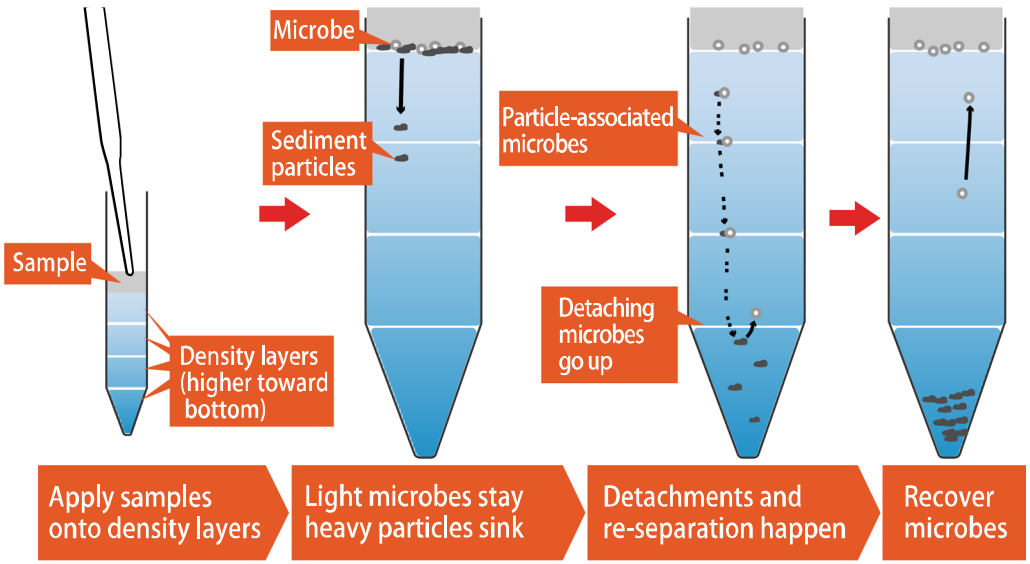

Fig. 4 Scheme of cell separation by multiple density layers. By using multiple density layers, better recovery of microbes was achieved than bilayer protocol

not only was ultrasensitive detection or enumeration possible, but the combination of fluorescence staining and cell separation was also effective for analyzing the communities found in subseafloor sediments. Determination of the living status of microbes from subseafloor environments by Schippers et al. (2005) further stimulated interest in the physiology of microbes in deeply buried, energy-starved sediments (comprehensive reviews can be found in Hoehler and Jørgensen (2013), Orcutt et al. (2011), and Orsi (2018)). Based on the availability of energy sources (organic matter), microbes were speculated to exist under extremely hostile and nutrient-deficient conditions, even though cell division under such conditions may only occur once over thousands of years after entrapment in the subseafloor environments (Jørgensen and Boetius 2007). Further modeling analyses suggested that the availability of energy sources for the microbial cells in subseafloor sediments was less than that was previously thought, considerably lower than the minimum requirements of any microbes that had been cultured to date (Bradley et al. 2020; LaRowe and Amend 2015). However, the detection of microbial life and geochemical evidence of very low, but detectable, respiration activity demonstrated that the subseafloor biosphere was indeed capable of sustaining biological life**,** even though the physiological and metabolic mechanisms employed by life under such extreme conditions were not yet fully understood (LaRowe and Amend 2015). In addition, many aspects related to the ecological physiology of microbes, such as what proportions of these microbes were alive, actively growing, just surviving, dormant, or dead fossils, were unknown. To address this fundamental question regarding the physiological status of subseafloor microbial life, single-cell-level probing of microbial metabolic activity by nanoscale secondary ion mass spectrometry (NanoSIMS) was used to clarify whether carbon and nitrogen substrates were being assimilated by microbes. The basic strategy for this approach is that the microbes in the subseafloor biosphere samples are supplied with substrates in which the carbon and/or nitrogen atoms have been replaced with stable isotopes (e.g., ^{2}H, ^{13}C, and ^{15}N). If the modified substrates are metabolized and assimilated into the microbial cells, then the relative abundance of the stable isotope(s) increases and becomes detectable by NanoSIMS analysis. Upon NanoSIMS analysis, a Cs^{+} primary ion beam is directed at the samples, producing secondary ions that are separated by mass spectroscopy, detected by an electron multiplier (in the case of NanoSIMS 50L, parallel acquisition of up to seven masses is possible), and then recorded as images of the selected ions. To detect stable isotope-labeled microbial cells, $^{12}C^{+}$, $^{13}C^{+}$, $^{12}C^{14}N^{+}$, and $^{12}C^{15}N^{+}$ ions are typically used (Morono et al. 2020, 2011), but $^{1}H^{+}$ and $^{2}H^{+}$ can also be included (Trembath-Reichert et al. 2017). The ability to trace stable isotope incorporation using NanoSIMS has opened up a vast new field of research in single-cell environmental microbiology (Kuypers and Jøgensen 2007; Morono et al. 2014; Musat et al. 2012). The application of NanoSIMS to the analysis of microbial ecophysiology was pioneered by McMahon et al. (2006). Although they were not the first to demonstrate analysis of microbes using a SIMS-based approach, the introduction of NanoSIMS was a technical breakthrough in terms of the ability to observe stable isotope incorporation at a high spatial resolution (50 nm), a level of resolution that is theoretically not possible using microscopic techniques. To date, more than 90 reports on the use of NanoSIMS in biology and microbiology have been published in high-profile journals, indicating that the introduction of NanoSIMS has been a groundbreaking development for bio-analysis and has led to numerous novel findings in previously unexplored natural systems.

Despite the extensive application of NanoSIMS to the analysis of metabolic activity in microbes, its application to microbes from the subseafloor biosphere has been limited, primarily because of the analytical problems associated with the sediment matrix. During NanoSIMS analysis, secondary ions generated by the bombardment of the primary ion are detected. By scanning with the primary ion, the produced secondary ions are used to generate an image of the scanned target area showing element distributions. The information that is obtained is the two-dimensional distribution of each element. Analyzing microbes in aquatic habitats is relatively straightforward, as microbial cells constitute the major source of solids with elemental compositions that can be distinguished with ease. However, subseafloor sediments contain large quantities of non-cellular particles, including particulate organic matter (POM), which contains carbon and sometimes nitrogen. In such cases, distinguishing between the organic matter in microbial cells and POM in images can be difficult using NanoSIMS. The sample area in NanoSIMS isotope imaging analysis typically encompasses an area of 30 μm × 30 μm. If samples are prepared by conventional filtration (i.e., suspending the sample in buffer and passing it through a membrane filter), the number of single cells per analysis area will be < 1 cell if the cell abundance in the sediment is $< 10^{7}$ cells per cm^{3}. Considering that the CCD view of microbial cells in the NanoSIMS system is obscured by the presence of large quantities of sediment grains, the analytical efficiency goes low since targeting microbial cells rarely found in low-abundance samples to capture in the field of analysis is almost impossible. To overcome these difficulties, identifying microbial cells after density cell

separation became more widely used. As described in the previous section, microbial cells and sediment particles can be recognized based on differences in their fluorescence characteristics after staining with SYBR-I. Further, a combination of density-based cell separation and selective sorting using the fluorescence-activated cell sorting (FACS) approach enabled the purification of microbial cells from subseafloor samples and concentrating them to a spot of ~ 200 μm on the membrane (Morono et al. 2013) (Fig. 5). Through these technical developments, substrate incorporation by subseafloor microbes, regardless of their abundance in the sediments, could be analyzed by NanoSIMS. In NanoSIMS analyses shown in Morono et al. (2011), in which no cell separation was conducted on the sediment incubation slurry of ~ 10^7 cells/mL, there were just a few cells per field of analysis. However, in another study, sorting and microbial substrate incorporation analysis successfully increased the number of microbes per field of analysis even using a sample containing 4×10^2 cells/gram of sedimentary rock collected from subseafloor shale and coal deposits sampled from as deep as 2 km below the seafloor (Trembath-Reichert et al. 2017). In the case of ultraoligotrophic subseafloor sediments, syringe-subsampled sediment mini-cores were incubated without preparing a slurry and drops of concentrated substrate solution were placed onto the mini-cores for incubation. Although the cell concentration varied (10^2–10^6 cells/cm^3 sediment), separation, purification, and concentration of cells were sufficient for NanoSIMS analysis (tens of cells per NanoSIMS analysis) (Morono et al. 2020). The findings of that study revealed the persistence of revivable aerobic microbial communities in sediments as old as 101.5 million years (Fig. 6).

The use of the cell sorting approach is not only for microbial cell separation from sediment particles, but the wider application is possible. Cell sorter utilizes laser irradiation, with a wavelength of typically 488 nm, but a number of lasers with other wavelengths are available to obtain optical information of laser-scattering and fluorescence. Approaches that are used to tag microbial cells, such as FISH and its derivatives (e.g., BioOrthogonal Non-Canonical Amino acid Tagging (BONCAT) (Hatzenpichler et al. 2014) and BrdU (Steward and Azam 1999)(Pernthaler et al. 2002b)/EdU (Yamakoshi et al. 2011)), can be incorporated into sample preparation for NanoSIMS analyses. However, it should be noted that the more processing the microbial cells have for tagging, the more exogenous carbon (and nitrogen and hydrogen) are introduced into the microbes, which dilutes the naturally abundant isotopes and alters the ratios of isotopes in the microbes (Meyer et al. 2021). Although the isotope incorporation itself can be detected, as the increase in the ratio of ^{13}C or ^{15}N following stable isotope incubation, quantitative analyses of isotope incorporation and rate analysis are compromised because of the uncertainty introduced by sample processing. Thus, minimizing the number of processing steps is highly recommended.

2.4 Remaining challenges for deciphering microbial dynamics in the subseafloor biosphere

The aforementioned technical developments have facilitated the exploration of areas close to the fringe of the deep subseafloor biosphere (D'Hondt et al. 2015; Früh-Green et al. 2018; Heuer et al. 2019; Inagaki et al. 2015;

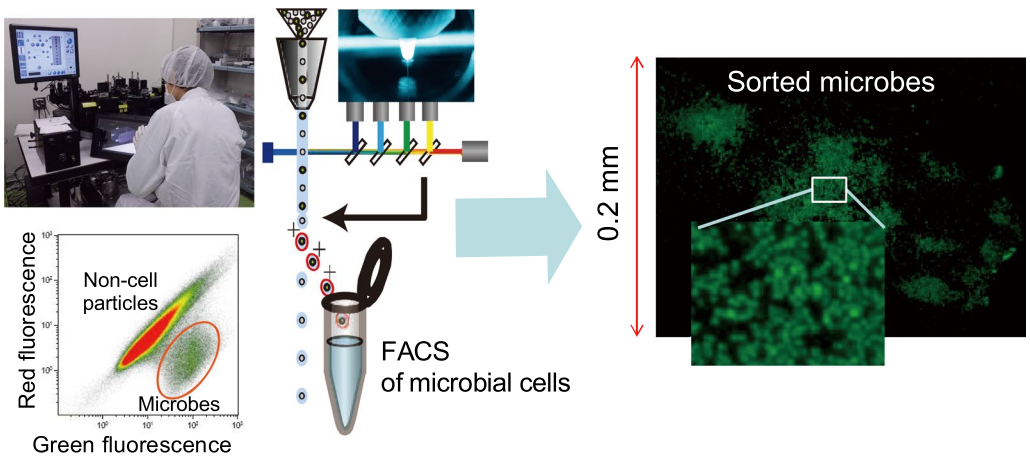

Fig. 5 Scheme of microbial cells purification for NanoSIMS analyses. Density-separated samples were applied to cell sorter after staining with SYBR-I (green). Then, the microbial cells (green) were specifically sorted onto polycarbonate membrane. The speed of sorting ranges few to hundreds of cells per second depending on the cell abundance in the original samples. Modified from Morono et al. (2014)

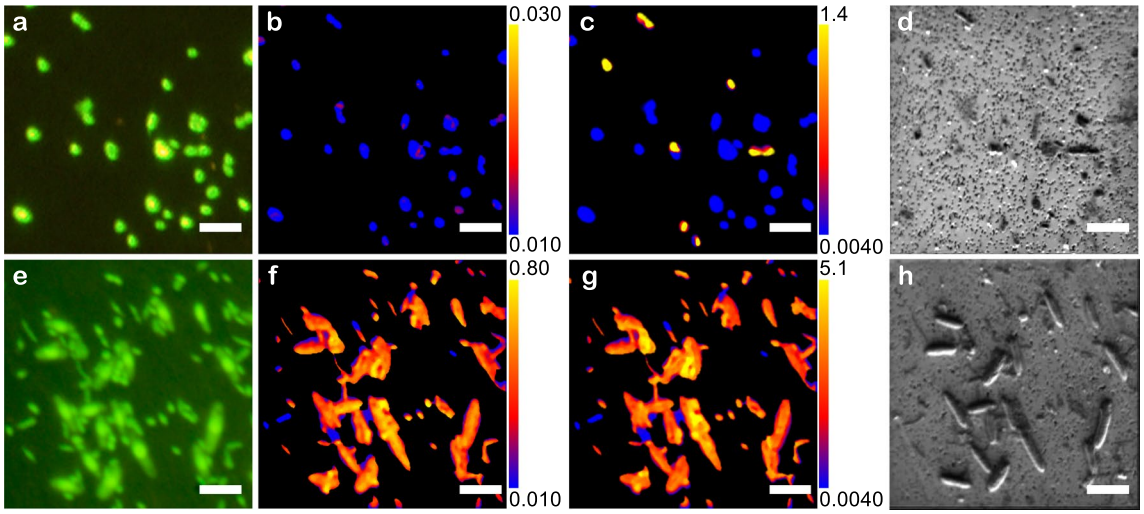

Fig. 6 Detection of substrate incorporation into microbial cells revived in 101.5 Ma sediment. Cells from incubations with ^{13}C-bicarbonate and ^{15}N ammonium (**a**, **b**, **c**, **d**) and ^{13}C,^{15}N-Amino acid mix (**e**, **f**, **g**, **h**) are shown. **a**, **e** SYBR Green I-stained cells under fluorescence microscopy. **b**, **c**, **f**, **g** Ratio images of ^{13}C/^{12}C (**b**, **f**) and ^{12}C^{15}N/^{12}C^{14}N (**c**, **g**) ratios of the same regions imaged in **a** and **e**, demonstrating locations of ^{13}C and ^{15}N incorporation. Color scale range of the ratios are shown as number appearing at top and bottom of the color bar. The background membrane region, which is identified by fluorescence images, is excluded from the ratio calculation and shown as black background. **d**, **h** Secondary electron (NanoSIMS) images of the same region in **a** and **e**. Bars represent 5 μm. Adopted from Morono et al. (2020)

Li et al. 2020). These areas are characterized by very low cell abundance and a partially undetectable region has been reported (Heuer et al. 2020). Studies have unveiled the extreme resilience and survivability of microbes in extreme environments, which were previously considered to be too hostile for life (e.g., areas with high temperatures and/or low energy fluxes). Nonetheless, environments, where life cannot survive, are yet to be discovered. "Defining the rules of microbial life" and "Exploring the limits of life" are among the most important future scientific goals of the global research community involved in scientific ocean drilling (Koppers and Coggon 2020). However, how these goals can be achieved is one of the remaining technical challenges in subseafloor research.

It has been said that the absence of evidence is not evidence of absence (Altman and Bland 1995; Sagan 1997). In the case of deep biosphere research, the existence of microbial life in subseafloor environments directly gives direct evidence of the presence of life, but in cases where such evidence is absent, researchers need to consider the possibility that the results of absence are from methodological limitation being below detection limits. The same is true for measurements of catabolic activity using radioisotopes as tracers; all carefully designed experiments have controls, which are used to provide statistical evidence that observations are beyond noise. Life is affected by a variety of environmental factors, including temperature, bioavailable energy flux, pressure, dissolved metal concentrations, water availability, and pH. Although identifying the limits of life cannot be ascertained with certainty when examining a limited number of samples, a series of samples can help to identify the trend of environmental factors and biological signatures and provide practical evidence of the limits of the biosphere. Given that subseafloor environments are saturated with water, they usually exhibit gradients of physical and chemical conditions; for example, temperature gradually increases with depth, and the compacted nature of subseafloor sediments restricts conductive flow and diffusion forms gradients of chemical species. The sampling of subseafloor substrates along these gradients should make it possible to identify the practical limits of the biosphere, although some inhabitable "spots" may exist close to the interfacial region. At around the depths where life appears to be absent, abiotic chemical reactions continue to take place. If the location of the drilling zone is appropriately selected, then the critical zone, i.e., the transition from habitable to inhabitable conditions, could be identified. In this regard, factors such as continuous sedimentation and plate movements which gradually change the physical environment and make it less habitable over geological time also need to be considered. The potential for chemical reactions involving leftover biochemicals occurring at different rates has been suggested (Orsi et al. 2020). For example, peptide hydrolysis, nucleic acid hydrolysis (RNA should decay faster than DNA), amino acid racemization, sugar decay, and lipid hydrolysis,

could potentially occur at different rates. Consequently, analyses that combine the detection of cells and biomolecules would provide a finer and clearer indication of the limits of the biosphere.

Despite the high sensitivity of microscopic methods for detecting microbial cells—down to several cells per cubic centimeter of sediment (Heuer et al. 2020)—the ability to accurately detect biomolecules such as DNA and/or RNA is partially left behind. Although there have been a number of reports describing subseafloor microbial community structure (e.g., Hoshino et al. 2020; Inagaki et al. 2006; Orsi 2018), only two studies have successfully described the composition of very deep (>1000 mbsf) subseafloor microbial communities (Ciobanu et al. 2014; Inagaki et al. 2015). The reason for these difficulties often involves DNA quantity and quality. In general, the approach for investigating microbial community structure employs 16S rRNA gene sequences amplified from extracted DNA pool by PCR amplification. There are numerous steps where contamination can be introduced from exogenous sources, such as during sample retrieval (shipboard sampling), subsampling at onshore laboratories, DNA extraction (from working environments and the kits used), and PCR amplification (from the environment and previously used PCR enzymes). Even though the working environments can be DNA-clean (Morono et al. 2018), the extreme sensitivity of PCR amplification means that even the slightest contamination can adversely affect results. The problem is exacerbated when using samples containing small amounts of DNA, such as the samples from deeper areas of the subseafloor environment where cell abundance is low. In order to resolve the problem and obtain molecular signals from indigenous microbial communities, intensive sequencing of various controls and probabilistic approaches are implemented to identify contaminating sequences (Hoshino et al. 2020; Inagaki et al. 2015; Morono et al. 2020). However, for omics approaches, such as metagenomics or metatranscriptomics, the low recovery of DNA/RNA is more problematic. Although there have been more than several attempts to conduct metagenomics studies using subseafloor samples, the samples are usually from shallower areas of the subseafloor biosphere. Nonetheless, there is a need for further technical developments that will facilitate investigations in deeper subseafloor regions. Particularly, the quality of the biomolecules in the retrieved samples should be a concern. Although not reported in the literature, there is a general feeling among some microbiologists that DNA amplification is more challenging for subseafloor microbes than it is for microbes in other habitats. Humic substances, which are abundant in subseafloor sediments, are generally considered to be the main cause of this problem, as they interfere with, or inhibit, enzymatic DNA amplification. However, even after density separation or selective sorting of microbial cells, when there are few humic substances, DNA amplification is often difficult. While this has not yet been empirically demonstrated, this belief seems to be shared among researchers in the field. Given the energy-limited conditions in subseafloor environments, microbes might not be able to meet the energetic requirements that are necessary to keep their DNA intact, causing problems associated with damaged DNA upon amplification.

3 Conclusions

Technological developments have driven and accelerated the biological exploration of the subseafloor biosphere. To date, a variety of subseafloor environments, such as ultradeep (~2 km below the seafloor (Trembath-Reichert et al. 2017)), extremely oligotrophic and old (~1 million years (Morono et al. 2020)), and hot (~120 °C (Heuer et al. 2020)) regions, have been investigated for microbial survival and living status. The findings of these studies have revealed that heterotrophic metabolism is dominant, regardless of the degree of availability of organic compounds in the surrounding environment. In addition to the biosphere regions mentioned above, there is also the rock biosphere. Despite the fact that relatively few studies have been conducted on the microbial abundance of this part of the biosphere (Früh-Green et al. 2018; Li et al. 2020; Suzuki et al. 2020; Wee et al. 2021) and on microbial activity in crustal fluids (Trembath-Reichert et al. 2021), further exploration will likely present investigators with yet additional challenges in future.

The unique environmental setting of the subseafloor biosphere provides critical information on the factors that constrain the extent of biosphere, the conditions that limit survival, and the evolution and adaptability of life. However, the limited amounts of biomass in samples necessitate the ability to sensitively detect biosignatures with appropriate techniques while minimizing contamination. In order to develop optimal techniques, it is of utmost importance to know the limitations and characteristics of the techniques employed. Based on an in-depth knowledge of the underlying principles, limiting factor(s), and sources of noise and interference, investigators can apply the most appropriate techniques to the analysis of particular samples and obtain reliable data as a result.

The accumulation of knowledge and development of optimal procedures to detect, characterize, and understand life below the seafloor will directly benefit the search for extraterrestrial life, where similar barriers to discovery exist. To overcome these challenges, the exchange of information, especially on "failures" and

negative results, is essential as failure often contains an abundance of important information.

Abbreviations

ODP	Ocean Drilling Program
AODC	Acridine orange direct counting
AO	Acridine orange
POM	Particulate organic matter
FISH	Fluorescence in situ hybridization

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Declarations

Competing interests
The author has no competing interests to declare.

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