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Quantitative microbial ecology: Future challenges and opportunities

William D. Orsi [1,2]

[1]Department of Earth and Environmental Sciences, Paleontology & Geobiology, Ludwig-Maximilians-Universität München, Munich, Germany

[2]GeoBio-Center[LMU], Ludwig-Maximilians-Universität München, Munich, Germany

Correspondence
William D. Orsi, Department of Earth and Environmental Sciences, Paleontology & Geobiology, Ludwig -Maximilians - Universität München, 80333 Munich, Germany.
Email: w.orsi@lrz.uni-muenchen.de

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Trying to figure out what a given microbe, or microbial group, is doing in a natural environmental sample is challenging. The overwhelming majority of microbial species have never been cultivated (Kuypers, 2007; Rappe & Giovannoni, 2003; Solden et al., 2016) and in many environments are dominated by 'microbial dark matter' (Rinke et al., 2013), which are groups that can be defined as those never isolated into a pure culture in a laboratory but rather are only known from their DNA sequences from environmental samples. That most microbes in from the environment have not been cultivated is not new, the 'great plate count anomaly' has been observed for decades whereby only a small fraction (0.01%–1%) microbes visible under a microscope from environmental samples will form colonies on petri dishes (Butkevich, 1932; Rappe & Giovannoni, 2003; Staley & Konopka, 1985). Several decades and hundreds of metagenomic studies later, an enormous number of new branches on the tree of life are now known to exist but correspond to microbial dark matter groups that have never been isolated in the laboratory (Hug et al., 2016; Rinke et al., 2013).

Novel cultivation strategies involving in situ incubations allowing for chemical exchange from the environment have succeeded in bringing some previously uncultivated groups into pure culture (Nichols et al., 2010). One notable example is the discovery of a novel bacterium isolated from the soil of someone's backyard that produces the novel antibiotic Teixobactin that is shown to be effective against many types of bacterial pathogens without detectable resistance (Ling et al., 2015). For the majority of dark matter groups, however, their functional ecology remains a mystery, because they have not yet been isolated into pure cultures and experimental evidence for their activities and role in ecological processes are lacking. This represents a challenge, but also an opportunity for discovery of new roles of uncultivated microbes in important biogeochemical processes.

Metagenomic techniques provide sign posts for potential ecological functions of microbial dark matter, but in the absence of experimental evidence the ecological relevance of the microbe from which the genome is derived remains speculative (Figure 1) and is based largely on metabolic models from predicted proteins from sequence data. Sometimes, the metagenomic data are correlated with geochemical parameters allowing for some conclusions based on indirect correlations. The application of metatranscriptomic methods is helpful to identify genes that are actively being expressed, but interpretations about functional ecology are still rather speculative if the translated proteins from the detected mRNA sequences are not quantified. Moreover, the possibility for constitutive expressed genes need to be considered, whereby mRNA is present in the cell but might not be translated into proteins. In the future, functional predictions from metagenomics and metatranscriptomics should be validated with controlled experiments to understand biogeochemical roles of uncultivated microbes in natural samples.

FIGURE 1 An iceberg model depicts the size of uncultivated microbial dark matter relative to cultivated microbes, that is typical for many types of environmental samples. Cultivated microbes yield invaluable information regarding ecological roles but often do not comprise the majority of microbes inhabiting a given ecosystem. The ecological role of many microbial dark matter groups is speculated from metagenome sequence data and therefore remains poorly understood. New quantitative experimental approaches will help to elucidate the ecological role of the lower part of the iceberg.

A common experimental approach of this nature typically involves spiking in of stable isotope labelled substrates (either with a ^{13}C or ^{15}N label) and then following the assimilation of those isotopes into the biomass of specific microbial groups (Hatzenpichler et al., 2020; Neufeld et al., 2007). Two popular approaches that provide a direct link between the identity of a microbe and its quantitative assimilation of the labelled substrates are NanoSIMS (Kitzinger et al., 2021; Orphan, 2009) and quantitative stable isotope probing (qSIP) (Hungate et al., 2015). With NanoSIMS, there is a substantial requirement in the form of a multi-million dollar investment for the instrument, not to mention resources required for full time technical staff. Therefore, access is limited to a small number of laboratory or centers with the required financial resources. It is possible for scientists from smaller laboratories to have their samples analysed at a NanoSIMS core facility, but this often involves an application process, user fees, and often long waiting times for a short time window to use the instrument. This makes trouble-shooting the experimental setup and the trial and error process cumbersome for those using NanoSIMS as an external service. In contrast, the qSIP method requires only a table top ultracentrifuge, fractionation system, and qPCR machine that can be acquired by an independent lab with a financial investment about 100-times less than NanoSIMS. The method (including trial and error, experimental trouble-shooting) can be performed start-to-finish by a PhD student. The results of a qSIP experiment provide an estimate of what percentage of carbon atoms in the 16S rRNA gene of a given operational taxonomic unit (OTU) are ^{13}C labelled (with a 90% confidence interval). One of the major advantages of the qSIP method is that the amount of ^{13}C incorporation is quantified for every individual OTU detected in the sample (Hungate et al., 2015; Sieradzki et al., 2020). Because of the smaller investment on part of the scientist to perform qSIP experiments (compared to NanoSIMS), this experimental approach can be more easily applied by relatively small laboratories independently. Furthermore, high-throughput laboratory protocols for processing qSIP experiments with the assistance of robots are now being developed (Nuccio et al., 2022). For these reasons, this Crystal Ball article will focus primarily on the application of the qSIP experimental method to future quantitative microbial ecology studies, with the understanding that other experimental methods such as NanoSIMS can provide similar answers to many of same types of questions whether sufficient access to those resources are available.

CHALLENGES FOR FUTURE QUANTITATIVE MICROBIAL ECOLOGY STUDIES

Experimental validation of metabolic potentials from (meta)genomic data associated with dark matter organisms is a major challenge. How should these experiments be designed? A key challenge for future qSIP experiments and studies is the need to carefully maintain the experimental conditions to best reflect the natural environment as close as possible (Neufeld et al., 2007). The kinetics of microbial growth and substrate assimilation will be effected by the concentration

of the substrate added. Substrates added at higher concentrations compared to their in situ concentrations will possibly 'tip the system' and result in anomalously high rates of microbial growth and will probably select for fast growing organisms that are adapted to use substrates at high concentrations quickly (Panikov, 1995). Therefore, the substrates should be added at a level that is proportional to the natural abundance of that substrate. This becomes challenging when one is working in a new environment where organic geochemical measurements have not yet been made. For environments that are high in organic matter content, it is also possible that the amount of added substrate is not enough to observe labelling and becomes diluted in the high concentration of naturally occurring organic matter. Ideally, the ^{13}C- or ^{15}N-labelled substrates should be added at different concentrations to identify the lowest concentration required at which labelling still becomes observable, without tipping the system away from the natural state. This will represent a challenge because it requires processing a relatively high number of qSIP experiments with different substrate concentrations to compare experimental treatments that received different substrate concentrations.

Additional aspects of experimental design should be monitoring of O_2 levels, since the availability of O_2 will control how much energy is available to the microbial ecosystem (Fenchel & Finlay, 1995) and therefore will control the kinetics of substrate assimilation. For example, gas tight ^{13}C incubations from lacustrine mud that went anoxic quickly had very low ^{13}C assimilation, compared to gas permeable ^{13}C incubations from the same mud that showed a large amount of ^{13}C assimilation (Coskun et al., 2018). Therefore, careful consideration should be given to the types of flasks used, whether plastic or glass, gas-tight or gas permeable. Moreover, in addition to O_2 measurements, the pH and redox of the incubations should also be monitored to understand further aspects of the environmental conditions (e.g. carbonate chemistry, potential electron acceptors) and interpret the results. Moreover, the possibility of an oxygen or redox gradient that forms within the incubation flask should also be considered and profiled, for example, using microsensors or non-invasive fibre-optic O_2 sensors. Ideally, biogeochemical information on the site sampled for the qSIP incubations will help to re-create the conditions inside the incubation flasks as accurately as possible. All of this requires a lot of different types of measurements, not even including the post-lab processing of the qSIP experiment itself, and therefore presents a challenge. However, if these goals can be met, it will certainly increase the accuracy of interpretations about why (and how) a particular OTU assimilated more or less ^{13}C label (and at what rate) under the particular experimental conditions. This would increase the chances for a convincing experimental validation of metabolic potentials from (meta)genomic data associated with a particular 'dark matter' organism.

Another challenge associated with the qSIP method is its potential application to low-biomass samples. I define low-biomass samples here as samples that have $<10^5$ total microbial cells per gram sample. Low-biomass samples of this nature are commonly found in the deep subsurface (Kallmeyer et al., 2012). In order to ensure success in a qSIP experiment from low-biomass samples, it is critical that enough DNA is extracted from the sample such that sufficient 16S rRNA genes can be quantified and sequenced from individual density fractions after ultracentrifugation and density gradient fractionation. The lowest-biomass sample whereby a qSIP experiment was successfully applied that I am aware of is from an endolithic (rock-dwelling) microbial community that had extractable DNA at a concentration of ca. 10^4 16S rRNA gene copies per gram (Coskun et al., 2021). A quantitative DNA and protein SIP study from a low-biomass deep groundwater habitat was also recently reported (Taubert et al., 2022). These examples show that qSIP experiments from low biomass samples are possible, but careful contamination measures are required and a highly efficient DNA extraction protocol.

Another challenge that may be associated with future qSIP studies is the potential application to the nitrogen cycle. The application of ^{15}N in traditional DNA-SIP has been applied to identify which microbes assimilate ^{15}N substrates (Buckley et al., 2007). However, I am not aware of a published qSIP experiment that quantified nitrogen assimilation from ^{15}N substrates by specific OTUs from an environmental sample. In contrast, the assimilation of ^{15}N (e.g. from ammonia) is a common measure of microbial activity in NanoSIMS studies (Dekas et al., 2016; Morono et al., 2011). Future qSIP experiments could also attempt to use ^{15}N-labelled substrates (such as ammonia or amino acids) to determine whether sufficient detectable labelling (defined as buoyant density shift) can be quantified in the 16S rRNA genes of OTUs. Because the amount of nitrogen that DNA contains is much less compared to carbon and therefore requires a much higher proportion of labelled N in the genome (Buckley et al., 2007), future ^{15}N-labelling studies for qSIP would most likely be operating close to the detection limit of ^{15}N-assimilation in the OTU 16S rRNA genes. This challenge could be overcome by increasing the number of density fractions after the ultracentrifugation (Neufeld et al., 2007) to increase sensitivity in terms of identifying relatively small statistically significant increases in DNA buoyant density from ^{15}N. This reduces the amount of DNA per fraction however and therefore would have highest chances for success in high biomass environments where relatively high concentrations of DNA can be extracted. For low-biomass samples, adding a carrier (such as glycogen) to DNA

precipitations from CsCl fractions helps to increase the detection limit (Neufeld et al., 2007). However, substantial amounts of contaminating DNA in glycogen have been found from commercial suppliers and therefore potential contamination in any glycogen carrier should be assessed before using it for precipitating low DNA samples. As a substitute, linear polyacrylamide has been found to be a better carrier for precipitating low DNA SIP samples with no detectable contaminating DNA (Bartram et al., 2018).

OPPORTUNITIES FOR FUTURE QUANTITATIVE MICROBIAL ECOLOGY STUDIES

There are opportunities associated with some of the challenges surrounding qSIP experiments, which might hold promise for developing a deeper understanding of the ecology for many microbial 'dark matter' groups. One example, is the application of ^{18}O-labelled water as a substrate for quantifying the growth rates of uncultivated microbes using qSIP. The oxygen atoms of water are assimilated into the DNA of actively growing microbes, primarily via phosphate molecules whereby an inorganic pyrophosphatase is responsible for exchanging oxygen atoms between water and phosphate (Schwartz, 2007). The assimilation of detectable ^{18}O from labelled water into microbial 16S rRNA genes with qSIP occurs only during genome replication and because microbes only replicate their genomes during vegetative growth the labelling of ^{18}O in OTUs is a measure of microbial growth rates (Coskun et al., 2019; Koch et al., 2018; Schwartz et al., 2016).

The ^{18}O-water method for qSIP has also been successfully applied to quantify growth rates of uncultivated soil bacteria (Hungate et al., 2015; Koch et al., 2018; Schwartz et al., 2016), uncultivated bacteria in anoxic coastal marine sediments (Coskun et al., 2019), and even microbial communities in million-year-old deep sea clay (Vuillemin et al., 2019). For the later study (Vuillemin et al., 2019), the qSIP with ^{18}O-water provided sufficient labelling even in a sample that had 10^4 16S rRNA gene copies per gram sediment—demonstrating that this method works for extremely low biomass samples. The high amount of ^{18}O-labelling of the ammonia oxidizing archaea (Vuillemin et al., 2019) also demonstrated that this method can also be used to quantify the growth of archaea (not only bacteria). In addition to the ammonia-oxidizing archaea, the currently uncultivated Woesearchaea (Castelle et al., 2015) were also found to be relatively high in ^{18}O-labelling from the million-year-old abyssal clay (Vuillemin et al., 2019). This showed the first potential growth rates of these uncultivated archaea. Therefore, the ^{18}O-water qSIP approach holds promise to measure growth rates of not only bacteria but also currently uncultivated groups of archaea, including those in low biomass environments.

The use of ^{18}O-water as a 'passive' tracer for microbial growth is attractive for several reasons. First, in comparison to ^{13}C or ^{15}N organic substrates, the addition of water should not overly stimulate fast growing organisms that are primed to use energy-rich labile organic matter. Second, ^{18}O-water could be added as a substrate together with very low levels of unlabelled organic matter (glucose, amino acids, etc) to reflect the in situ organic matter concentrations in organic matter limited environments. Similarly, electron acceptors (nitrate, sulfate, iron oxides, etc) can be added to ^{18}O-labelled water experiments to see which uncultivated 'dark matter' groups increase their growth in the presence of particular electron acceptors. This would provide quantitative experimental evidence to validate predictions from genome data regarding the metabolism of specific uncultivated groups known only by their DNA sequences.

Another potential future opportunity lies in the potential combining of qSIP with NanoSIMS approaches. To my knowledge, this has not yet been done. The qSIP method could act as a sign post, indicating which groups are most important for the assimilation of particular substrates in a given sample. Then, NanoSIMS could be used as a follow up tool to visualize cell-specific ^{13}C and ^{15}N assimilation by uncultivated clades implicated as important assimilators of these same compounds in qSIP experiments. Such a linkage with NanoSIMS could reveal cell-specific variabilities in substrate incorporation, an aspect that is not obtainable with the qSIP method because qSIP averages the ^{13}C assimilation across all cells within an OTU, whereas NanoSIMS gives cell-specific rate information (Hatzenpichler et al., 2020). Such a synergistic approach combining qSIP and NanoSIMS would have potential to provide strong experimental validations of metabolic predictions from metagenomic sequence data.

Another opportunity that the qSIP method presents is the ability to test hypotheses regarding the ecology of microbes implicated in key evolutionary events using experiments. For example, the Asgard archaea that have been proposed to be close living relatives to the last common eukaryotic ancestor (Spang et al., 2018), and establishment of the first pure culture of an Asgard archaeon provided exceptionally strong support for this hypothesis (Imachi et al., 2019). Understanding the ecology of these archaea could help to test hypotheses surrounding mechanisms of how eukaryogenesis occurred and what conditions might have promoted eukaryogenesis. For example, it is proposed that eukaryogenesis occurred via H_2 syntrophy involving a CO_2-fixing, H_2-oxidizing Asgard archaeal ancestor in an anoxic and sulfidic environment of the Proterozoic ocean (between 2 billion and 1 billion years ago) (Mills et al., 2022). A qSIP experiment from an analogue

environment in the modern ocean (the sulfidic Benguela Upwelling System in Namibia) showed that many Asgard archaea fix CO_2 and also can assimilate organic matter from diatoms (Orsi et al., 2020). This provided the first experimental evidence that supporting the hypothesis surrounding the ecology and metabolism of this group within the greater eukaryogenesis discussion (Mills et al., 2022). Since the ^{18}O-water qSIP method works to quantify growth rates of uncultivated archaea (Vuillemin et al., 2019), ^{18}O-water qSIP could also be used to investigate growth rates of Asgard archaea under different conditions such as the presence or absence (and varying concentrations) of particular electron acceptors and donors and or potential syntrophic partner organisms.

Ultimately, a comprehensive understanding of the ecological relevance and importance of a given novel microbe hinges upon establishment of a pure culture. Only then can the organism be carefully studied with in the laboratory with experiments that control most of the environmental variables. However, the rate at which novel microbes can be isolated in the lab is painstakingly slow. It requires a lot of time, patience, risk, and a bit of luck—and the financial freedom of the researcher to be able to take the time needed. Carefully designed qSIP experiments, combined with genomic and geochemical information, could provide a sign post for designing enrichments for specific groups uncultivated microbes. Targeted enrichments guided by qSIP experiments might help increase the rate of discovery of pure cultures from 'dark matter' groups.

I gave a talk recently to an Earth science faculty and received the comment by a colleague outside of the field afterwards (who studies earthquakes) who told me that if they could do their career over again they would have chosen environmental microbiology. When I asked this colleague why, they told me it was because they now realized that the possibility for new discoveries in the field of environmental microbiology is so exceptionally high. I thought this interaction was worth sharing here as an example for environmental microbiologists to be optimistic about the future of our research field. Within this future, quantitative microbial ecology experiments will play an important role by helping to validate predictions from (meta)genomic data on the ecological relevance of microbial 'dark matter'.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

DATA AVAILABILITY STATEMENT

This article contains no data.

ORCID

William D. Orsi https://orcid.org/0000-0002-4094-5637

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