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# Targeted analysis of metagenomes: divide and conquer

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ABSTRACT

The rapidly developing field of targeted analysis of metagenomes focuses on retrieving information about specific genes and/or genome(s) from environmental DNA. The traditional shotgun sequencing methods overemphasise dominant microorganisms and often fail to confidently assign the entirety of the analysed genetic material to specific species, genomovars, or strains. The ultimate goal of the targeted methods is to overcome this limitation of throughput and precision of current shotgun metagenomics when analysing complex microbial communities in the quest of refined information. Here, we discuss recent technological advances that are designed to focus the analytical power of diagnostic tools like sequencing, towards phylogenetically or functionally distinct and rare microbial groups and enhance e.g. the confidence in the assignment of genetic elements to their respective owning organisms. We specifically showcase the capabilities of these technological advances for targeted analysis of metagenomes, identify suitable related applications, discuss methodological limitations, and propose solutions for addressing these limitations. This review aspires to inspire highly relevant experimental designs in the future that will unlock unknown and important aspects of microbial ecology, and the yet-uncultivated microbial majority.

## 1. Introduction

Since the emergence of the field of microbiology, microscopic observation, microbial isolation, growth studies and biochemical profiling have led to the characterization and documentation of more than 575,000 of microorganisms to date, in 154 culture collections globally (Fan et al., 2025). This has allowed to contextualise microbial behaviour in the framework of Microbial Ecology as proposed by Sergei Winogradsky (Escobar-Zepeda et al., 2015). The large body of generated knowledge, along with early molecular biology approaches, have advanced the fields of Microbiology, Environmental Microbiology and Microbial Ecology, however, they failed to capture the bulk, yet uncultivated, microbial majority. Metagenomics, the study of collective genomes of an environmental sample and their functional analysis (Handelsman et al., 1998), has led to the rapid exploration of previously uncharted areas of microbial phylogenetic and functional diversity. It further increased our awareness of the magnitude of current gaps in our knowledge of uncultivated microorganisms. Metagenomic-based modelling efforts provided estimates of the expected phylogenetic diversity of microorganisms inhabiting our planet, ranging from millions to a trillion species (Larsen et al., 2017; Locey and Lennon, 2016; Louca et al., 2019). A range breadth demonstrating that, despite current technological advents, we are far from being able to accurately capture or even predict the extant of global microbial phylogenetic and functional diversity.

Shotgun metagenome analysis has largely contributed to our understanding of this extant microbial diversity, due to its capacity to capture both phylogenetic and functional information of microbiomes (Bharti and Grimm, 2019; Escobar-Zepeda et al., 2015). This is best demonstrated by the global initiatives like the Human Microbiome

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Project, the Human Gut Microbiome Atlas, or the Earth Microbiome Project and the Earth Microbiome Project 500 (Bharti and Grimm, 2019; Lee et al., 2024b; Shaffer et al., 2022; Thompson et al., 2017; Turnbaugh et al., 2007). Sequencing costs have dropped and methods have improved over the past years, yet, a plateau seems to have been reached (Wetterstrand, 2025), requiring more technological advents for its breach.

Currently, prevalent sequencing methods in shotgun metagenomics are accompanied by several advantages and disadvantages which were reviewed elsewhere (Bharti and Grimm, 2019; Escobar-Zepeda et al., 2015; Meslier et al., 2022; Quince et al., 2017). Briefly, short reads generated by the current gold standard technique of Illumina chemistry instruments and alike methods can provide cheap and accessible sequence information with relatively low error rates.Yet, their limitation in DNA fragment length hampers sequence assembly due to genomic repeats and homologies in the functionally redundant environmental samples. Third generation sequencing technologies, providing long reads, thanks to late improvements reducing error rates to values below 1 %, have led to their recent establishment in metagenomic studies, but still suffer from low sequencing depth (Agustinho et al., 2024; Kim et al., 2024).

Sequencing depth is crucial in shotgun metagenomics (Rodriguez-R and Konstantinidis, 2014a). For example, a genetic information coverage of 60 % has been proposed as a threshold for deciding between direct database mapping of reads vs de novo assembling the metagenome prior annotation, where short read technology is used for sequencing (Rodriguez-R and Konstantinidis, 2014b). By contrast, although long read sequencing approaches in shotgun metagenomics follow similar strategies as compared to short read technology (mapping/assembly), they enable assembly at even low coverage levels (Bharti and Grimm, 2019; Ciuffreda et al., 2021). Nevertheless, their throughput is still far inferior to that of the short-read sequencing methods. A combination of short and long reads has been demonstrated to result in higher quality metagenome assembled genomes (MAGs) than that of each approach alone, particularly in the case of well characterized environments like the human stool microbiome (Jia et al., 2023), or small microbial consortia derived from environmental enrichments (Quince et al., 2017; Vasileiadis et al., 2022).

Shotgun sequencing approaches are very informative and are able to report on the functional diversity within samples and environments, but they suffer from two major drawbacks as also illustrated in Fig. 1 (Hillmann et al., 2018): (i) even in the cases of deep sequencing, it is not easy to retrieve sufficient genetic information of ecologically relevant microorganisms that occur at low frequencies (Shade et al., 2014) in highly complex environments (Lindner et al., 2024); and (ii) without any pre-processing of the samples, shotgun metagenomics lack the confidence of properly assigning horizontally transferred genetic material (such as antibiotic resistance genes or integrative conjugative elements) to their genomes of origin (Rice et al., 2020). Several approaches have been developed to address these major drawbacks and to further refine metagenomic information to inform about keystone functional microbial groups in their respective micro-ecosystems.

Approaches used to combine phylogeny of sequenced organisms with function, are grouped into the two major categories: enrichment of relevant DNA from the metagenome or by enriching the organisms prior to DNA extraction. Enrichment methods include substrate, taxon, genetic loci, microbial guild and individual cells-based approaches, with some of them existing since some time ago (Quince et al., 2017; Rice et al., 2020) and others being recently developed. Here, we provide an overview of the current literature on this rapidly evolving field and discuss the key questions each method answers, its limitations, and possible solutions. Brief conceptual descriptions are provided in Fig. 2, while Tables 1 and 2 further summarize the information of this review about their applications commonly faced issues and literature derived solutions. This review lays the foundations for inspiring new research in the field of targeted metagenome mining for microorganisms and functions, with prospects in revolutionising Microbial Ecology, and unlocking knowledge about the vast, yet uncultivated, microbial majority.

## 2. DNA enrichment methods

DNA enrichment methods provide the capacity to study specific DNA loci out of the context of environmental microbial genomes. In several cases, DNA enrichment methods, aim at studying DNA loci that sufficiently capture microbial genomic identity and functional information for assigning functions to taxonomic groups. In other cases, they focus on the functional aspects of the flanking genetic information of the target locus towards gaining understanding about cooccurrence of functions or gain mechanistic insights about their distribution among microbial community members. The methods described here include those of probe capture (probe-based enrichment of target DNA), the DNA stable isotope probing (enrichment of DNA based on the levels of incorporation of e.g. labelled substrates), the Emulsion, Paired Isolation and Concatenation PCR (epic-PCR; intracellular amplification and fusion of functional and phylogenetic markers), intracellular linkage and enrichment of cellular genetic elements (the Hi-C connection). Their principles, applications, challenges and the way the challenges may be addressed are summarised in Tables 1 and 2. As described later, all of these methods are highly flexible, and can be tailored to explore both the

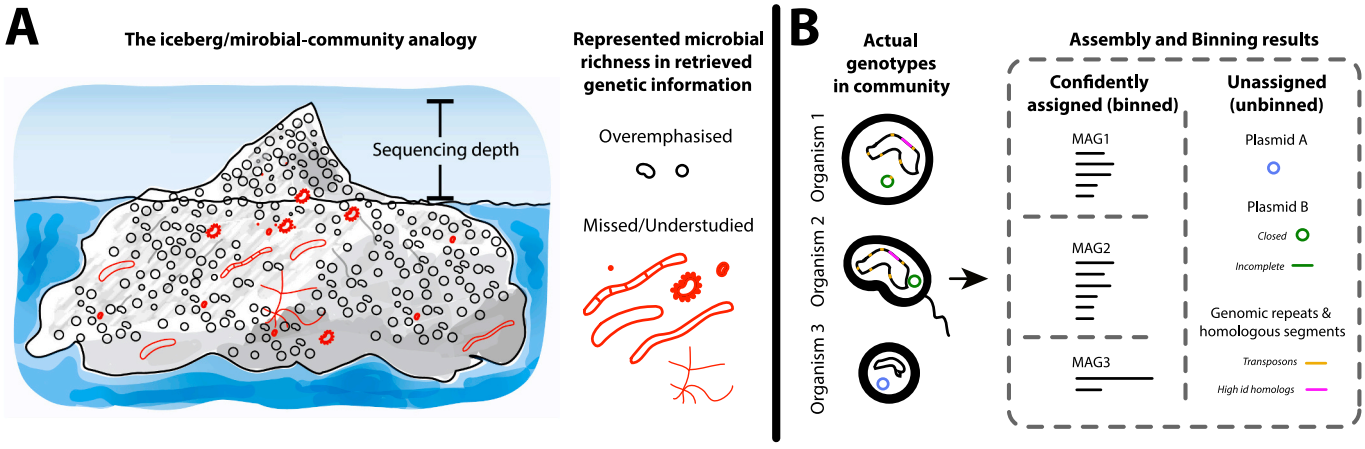


**Fig. 1.** Illustration of contemporary shotgun sequencing challenges: reduced coverage of under-represented, but possibly important taxa (A); reduced capacity to link cellular genetic elements (B).

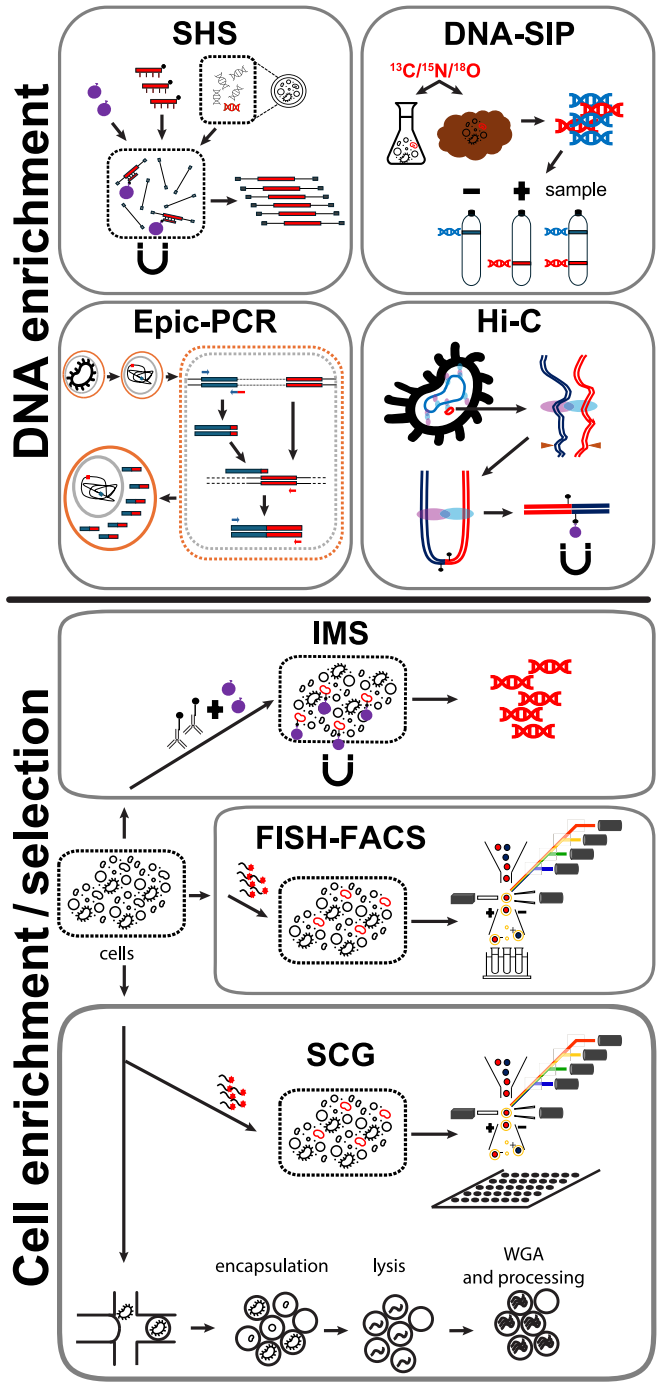


**Fig. 2.** Conceptual representation of the core workflows of the approaches described in this review. All these methods are detailed in the review text and presented in brief in Tables 1 and 2.

identity and function from owners of the targeted loci.

### 2.1. Probe capture, or solution-phase hybrid selection (SHS)

#### 2.1.1. Methodological principle and recent advances

Hybridization probe capture uses recoverable biotinylated probes (can be fished out with streptavidin coated magnetic beads) to selectively isolate complementary DNA to the target sequences and their flanking regions (Quek and Ng, 2024). It is performed in solution (in contrast to the solid-support microarray assays), and is frequently termed solution-phase hybrid selection (SHS) (Gasc et al., 2016). Captured DNA may be amplified if necessary and is sequenced (with 2nd or 3rd generation sequencing), assembled, and annotated. SHS was first introduced for human exome sequencing, where biotinylated RNA probes (RNA "baits") were used for exon capture from human whole-genome libraries (Gnirke et al., 2009). Since then, its use was expanded in targeted metagenomics and has been used for simultaneously analysing microbial diversity and function through suitable markers (Gasc et al., 2016). Along its implementation, appropriate probe designing strategies (Gasc and Peyret, 2017) and portable 3rd generation sequencing advances (Slizovskiy et al., 2022) have facilitated on-site and nearly complete genome population genomics (Hu et al., 2021) as described below.

#### 2.1.2. Distinct advantages

Even though probe designing utilizes known sequence information, SHS, like other probe-based methods, has been demonstrated to be less biased by database knowledge compared e.g. with PCR methods (Denonfoux et al., 2013). This is due to reduced hybridization specificity which can be further achieved on demand, through adjustment of hybridization conditions (temperature, duration, denaturant content, ionic strength etc). Moreover, given its ability to select sequences out of the bulk extracted nucleic acids, it can uncover much more relevant genetic information compared with the non-targeted shotgun metagenomics. The SHS ability to focus the sequencing diagnostic effort on a particular genetic locus was clearly demonstrated in the early environmental study of Denonfoux et al. (2013). Specifically, sequencing of the *mcrA* targeting probe-set recovered DNA resulted in 41.32 % *mcrA* containing DNA sequences compared with the shotgun sequencing approach that contained only 0.003 % ($\sim 10^5$ more target related genetic information). In the same study, SHS resulted in many more novel translated variants of *mcrA* open reading frames (ORFs) and associated phylogenetic affiliations compared with their more biased *mcrA* PCR product sequencing approach (Denonfoux et al., 2013).

Another inherent advent of SHS is its ability to uncover the genetic information of the flanking regions of the target gene. This largely depends on the size of the captured DNA fragment, and, in turn, on the size of the probes employed. The step-wise probe size increase, starting from smaller (i.e. targeting highly conserved sequences), to larger, sample/genome specific probes, can be achieved by serial recapturing and biotinylating of probes (Gasc and Peyret, 2017). For example, a set of 80 bp degenerate non-overlapping probes, targeting the hexachlorocyclohexane (pesticide) degradation associated *linA* gene, were used to capture gene fragment variants, which in turn were used for the co-capture of larger fragments. As a result, large genomic regions were reconstructed, containing phylogenetic markers next to functional and horizontal gene transfer (HGT) markers. Furthermore, a large probe-size strategy in resistome analysis combined with the enriched target long-read sequencing (TELSeq) (Slizovskiy et al., 2022), led to the revelation of rare and novel ARGs along with their flanking mobilome genes. The capacity to capture genetic information can, in several cases, expand to complete genomes. For example, ViroCap (Wylie et al., 2015) and VirCapSeq-VERT (Briese et al., 2015), two probe panels designed to capture viral genomic information of vertebrate hosts, besides addressing difficulties arising by viral low abundance (Fernandez-Cassi and Kohn, 2024), led to the study of near complete environmental viral genomes. This was further enhanced with the integration of long read 3rd generation sequencing technologies in ViroCap, improving viral strain identification capacity (Schuele et al., 2020), and making whole-genome sequencing easier (Hu et al., 2021). On top of that, on-site diagnosis of DNA/RNA human pathogenic viruses with the ViroCap panel was made possible due to the portability of Oxford Nanopore sequencing technology (ONT) (Hu et al., 2021). Similarly to ViroCap, BacCapSeq was designed to capture 307 complete bacterial pathogen genomes using 75 bp long probes with 121 bp space intervals of coding sequences paving the way for multiple cellular organism genome-wide DNA recovery from environmental samples (Allicock et al., 2018).

**Table 1**
Cheat sheet for method selection in experimental designs of targeted analysis of metagenomes via DNA enrichment. Each challenge faced (column "Challenges") is provided with a possible remedy (corresponding number in column "Possible solutions"). Cases with yet unresolved challenges exist where no corresponding numbers can be found in the "Possible solutions" column.

| Method[a] | Principle | Applications | Challenges | Possible solutions |
|---|---|---|---|---|
| SHS | Capture of target sequences through biotinylated probes and streptavidin coated magnetic beads | • Reduced bias and exploratory analysis of phylogenetic and/or functional diversity with emphasis in novelty (due to low specificity)<br>• Analysis of flanking DNA regions of target loci<br>• Analysis of (near) complete viral/bacterial genomes<br>• In-depth analysis of low abundance sequences or genomes | 1. Probe design<br>2. Capture biases due to probe design/length<br>3. Library preparation biases, due to low yields<br>4. Protocol biases due to target sequences, hybridization time and temperature, rounds of enrichment, sample type and sample characteristics (e.g. DNA quality and quantity), and the sequencing method | 1. Use of dedicated software accounting for biases<br>2. Hybridization benchmarking against reference genomes/databases<br>3. Correction with UMIs[b] ligation<br>4. Protocol validation with low-cost experiments (e.g. hybridization time and temperature, or rounds of enrichment), and careful selection of sequencing methods |
| DNA-SIP | Incorporation of labelled compounds in microbial DNA and enrichment of the labelled DNA through ultra-centrifuge density-gradient fractionation | • Identification of key and/or novel functional microbial groups<br>• Monitoring of microbial dynamics and functions, and their metabolic activity (e.g. $^{18}$O-water SIP)<br>• Study of microbial interactions<br>• Study of environments for bioprospecting | 1. Fractionation biases due to GC content<br>2. Reduced yields of 'heavy' (isotope enriched) DNA<br>3. Cross-feeding might lead to false-positively labelled DNA<br>4. Evaporation and pre-existing heavy isotope in the sample ($^{18}$O-water)<br>5. Low throughput due to expensive equipment and compounds<br>6. Only suitable for assimilation studies | 1. Analysis of multiple fractions and non-labelled controls, extra centrifugation, or fractionation simulation<br>2. Amplification of recovered DNA<br>3. Testing of different incubation/sampling times; time-series for identifying primary incorporators and cross-feeders<br>4. Incorporation of $^{18}$O-water in soil (as in $^{18}$O-water vapor equilibration and vapor-qSIP[c])<br>5. Semi-automated HT-SIP[c] method |
| epicPCR | Single cell encapsulation and lysis, followed by fusion PCR linking functional and phylogenetic gene markers | • Identification of microbial hosts for functional genes (e.g. ARGs) in environmental matrices<br>• Study of the host range of mobile elements<br>• Study of parasitic microbial interactions | 1. Primer biases<br>2. Cell separation/disaggregation issues<br>3. Underrepresentation of rare taxa dictated by the Poisson model governing the cell encapsulation process<br>4. Cell lysis biases<br>5. Low taxonomic resolution of small amplicons<br>6. Spurious off-cell fusion events leading to erroneous linkages<br>7. Inability to infer gene mobility | 1. Careful primer selection/design<br>2. Protocol optimization: enzymatic, chemical, or mechanical protocol modifications for disaggregation and separation of cells; staining protocols to detect mixed aggregates<br>3. qPCR pre-screening of target gene abundance to optimize cell density in the encapsulation process<br>4. Additional lysis steps (including enzymatic and/or chemical lysis)<br>5. Amplify longer 16S rRNA gene regions and apply 3rd generation sequencing<br>6. Blocking primers and replicate validation (rare taxa loss possible) |
| Hi-C | Cell fixation, followed by cross-linked DNA digestion, ends biotinylation, blunt-end ligation and capture through streptavidin coated beads | • Study of the microbial communities, for strain-level genome deconvolution<br>• Study of microbial genome structure and organization<br>• Linkage of mobilome to host microorganisms<br>• Linkage of endosymbiont/virus-host association | 1. Biases due to restriction site frequencies and GC contents<br>2. Erroneous binning and assembly due to spurious inter-cellular and/or inter-species cross-links, caused by shared-MGE/homologous/repetitive sequences and the capacity of deconvolution/methods and algorithms | 1. Preparation of multiple and multi restriction enzyme libraries, and/or utilization of tools for restriction sites frequency and binning prediction<br>2. Utilization of spike-in controls, 3rd generation or hybrid sequencing and assembly, and improvement of binning algorithms |

[a] *SHS*: solution-phase hybridization selection, *DNA-SIP*: DNA stable isotope probing; *epicPCR*: emulsion, paired isolation and concatenation PCR; *Hi-C*: high-throughput chromosome conformation capture.
[b] *UMIs*: unique molecular identifiers.
[c] *qSIP*: quantitative SIP; *HT-SIP*: high-throughput SIP.

### 2.1.3. Example applications

One of the initial SHS metagenomic applications concerned the diversity analysis of the methanogenic communities of a lacustrine (aka lake associated) anoxic depositional environment, by focusing their analysis around the, central to methanogenic life, methyl coenzyme M reductase subunit A (*mcrA*) gene (Denonfoux et al., 2013). Since then, the analysis of further functional and phylogenetic marker targets, including carbohydrate metabolism genes and the complete 16S rRNA gene, have been studied in soil, stool and clinical samples (Beaudry et al., 2021b; Gasc and Peyret, 2018; Manoharan et al., 2017; Manoharan et al., 2015; Rassoulian Barrett et al., 2020).

Resistome and virome SHS research has generated probe panels useful for environmental epidemiology studies, where relevant functional/phylogenetic markers, or even genomes, exist in low abundances. The MEGaRICH 120-mer probe-set (Noyes et al., 2017) has captured resistome and virome elements in retail food (Doster et al., 2020) and milk samples (Warder et al., 2021). ResCap targeted antimicrobial resistance genes (ARGs) and relaxases (participating in plasmid transfer) in gut (Guitor et al., 2022; Guitor et al., 2019; Lanza et al., 2018; Stege et al., 2023; Stege et al., 2022), manure-fertilized soil (Macedo et al., 2021) and retail food (Flint et al., 2023; Shay et al., 2023) metagenomes. In addition to the resistome panels, the viral genetic material targeting panels,ViroCap or VirCapSeq-VERT,have been applied to faecal (Jansen et al., 2020; Tokarz et al., 2019), clinical (Carbo et al., 2020; Williams et al., 2018) and environmental samples (Bonny et al., 2021b), for the identification of known variants and novel viruses or strains (Gogarten et al., 2019; Zhang et al., 2021a). ViroCap also allowed the detection of epidemiologically relevant genes like antiviral resistance genes (Wylie et al., 2018a) and virulence determinants (Wylie et al., 2018b). As expected, the SARS-CoV-2 pandemic has stimulated the development of a

**Table 2**
Cheat sheet for method selection in experimental designs of targeted analysis of metagenomes via cell enrichment. Each challenge faced (column "Challenges") is provided with a possible remedy (corresponding number in column "Possible solutions").

| Method[a] | Principle | Applications | Challenges | Possible solutions |
|---|---|---|---|---|
| IMS | Capture of microorganisms through biotinylated antibodies and streptavidin coated magnetic beads | • Phylogeny linked population genomics analysis<br>• Function driven metagenomic analysis<br>• Host interaction linked metagenomics (e.g. fishing IgA and linked microorganisms with IgA targeting antibodies) | 1. Difficult cell separation due to aggregates<br>2. Low-quality of resulting DNA, due to low target cell content, sample complexity, high antibody specificity<br>3. Antibody specificity issues and loss of taxonomic groups due to antibody selection and epitope conservation<br>4. Outcome also dependent on other protocol parameters (e.g. beads or antibody concentration, and incubation time) | 1. Protocol optimization of enzymatic, chemical, or mechanical processing<br>2. Whole genome amplification (WGA) for sufficient nucleic acids, culture-media assisted enrichment (quasi-metagenomics), suitable cell extraction methods and antibody selection<br>3. Careful antibody choice or use of polyclonal antibodies if broader target taxon range is desired<br>4. Optimisation of protocol parameters |
| FISH-FACS | Cell extraction, cell fixation, labelling through hybridization of fluorescent oligonucleotide probes, and sorting of positive/negative events | • Targeted cell population analysis using abundant probe targets (e.g. loci of the small ribosomal subunit)<br>• Analysis of low copy mRNA or genes (e.g. RING-FISH[b], or CARD-FISH[b])<br>• Analysis of identity and functions (e.g. MAR-FISH[b], geneFISH, or direct-geneFISH) | 1. Difficult cell separation due to aggregates<br>2. Highly complex environments reduce MAG deconvolution capacity<br>3. Environmental background fluorescence or cellular autofluorescence<br>4. Difficulty to distinguish some taxonomic groups due to low target-sequence variability<br>5. Low accessibility of probe target due to cell walls, or cellular component cross-linking<br>6. Detector-range-dictated restriction to screening of low number of microbial groups<br>7. Sorter-detector issues, like low signal, or non-single cell events | 1. Protocol optimization concerning enzymatic, chemical, or mechanical methods<br>2. Mini/Midi-metagenomics based cell population fractionation for complexity reduction<br>3. Gradient density centrifugation (e.g. with Nycodenz or Ficoll) for cell extraction/purification, and prior target cell autofluorescence evaluation where possible<br>4. Finetuning of hybridization conditions/sites and/or utilization of PNAs[c] or LNAs[c]<br>5. Utilization of enzymes for enhancing target accessibility, and optimization of fixation protocols<br>6. Utilization of multiple and multi-labelled probes (as in DOPE-FISH[b], MiL-FISH[b], or CLASI-FISH[b])<br>7. Utilization of strong dyes, optimised instrument settings, and enhanced signals (e.g. CARD-FISH) |
| SCG | Cell extraction, disaggregation and sorting/separation, followed by single-cell genome extraction and amplification | • Phylogeny and function-driven strain-level analysis<br>• Linkage of genes and mobile elements to host strains | 1. Difficult cell separation due to aggregates<br>2. Sorter-detector issues, like low signal, non-single cell events<br>3. Low cell number encapsulation in hydrogel approaches leads to low-positive counts during sorting methods, and understudy of rare taxonomic groups<br>4. Lysis biases<br>5. Whole genome amplification biases<br>6. Non-specific amplification products due to primer interactions or contamination (e.g. microbial nucleic acids in samples or in not properly purified regents)<br>7. Costly cell separation equipment | 1. Protocol optimization concerning enzymatic, chemical, or mechanical methods<br>2. Utilization of microscopy-based micromanipulation methods, or the use of strong dyes and optimal instrument settings<br>3. Machine learning supported imaging methods for event sorting validation<br>4. Utilization of novel chemical (e.g. ozone-based), or physical/physical-electric (e.g. piezoelectric, sonication and others) technologies<br>5. Utilization of thermostable polymerases, or of novel amplification methods (e.g. PTA-MDA[d], LIANTI[d])<br>6. Control reactions with e.g. primers lacking cross-hybridization capacity and check for assembly contamination<br>7. Exploration for the applicability of low-cost library preparation methods (e.g. combinatorial/split-pool barcoding). |

[a] *IMS*: immunomagnetic separation; *FISH-FACS*: fluorescence in situ hybridization fluorescence activated cell sorting; *SCG*: single cell genomics.
[b] *RING-FISH*: recognition of individual genes FISH; *CARD-FISH*: catalyzed reporter deposition FISH; *MAR-FISH*: micro-autoradiography FISH; *DOPE-FISH*: double labeling of oligonucleotide probes FISH; *MiL-FISH*: multilabelled FISH; *CLASI-FISH*: combinatorial labelling and spectral imaging FISH.
[c] *PNA*: peptide nucleic acid; *LNA*: locked nucleic acid.
[d] *PTA-MDA*: primary template-directed multiple displacement amplification; *LIANTI*: linear amplification via transposon insertion.

large number of virus and AMR marker targeting probe-sets (Nagy-Szakal et al., 2021; Paskey et al., 2019; Piantadosi et al., 2021; Rehn et al., 2021; Wylezich et al., 2021). Finally, an array of cellular microorganisms whole genome capture prob-sets were developed. These included the: BacCapSeq (Boruah et al., 2023; McLaren et al., 2023) targeting an array of bacterial taxa; strain specific probe sets targeting, for example, various *Chlamydia* (Bowden et al., 2021; Joseph et al., 2023; Taylor-Brown et al., 2018), or *Mycobacterium tuberculosis* (Brown et al., 2015; Doyle et al., 2018; Lozano et al., 2021; Nimmo et al., 2017) strains, or the plant pathogen *Xylella fastidiosa* (Boutigny et al., 2023); eukaryotic microbial specific probe-sets, such as the protozoan parasite *Plasmodium falciparum* in clinical samples (Melnikov et al., 2011); EctoBaits and similar, capturing ectoparasites (e.g. tick-borne) and host DNA, facilitating their epidemiological analysis (Campana et al., 2016; Jain et al., 2021; Sanchez-Vicente et al., 2022).

### 2.1.4. Challenges and possible solutions

Points of caution in SHS are factors like database status, sequencing library preparation process, and the hybridization approach (duration and conditions). Tools for probe design are integral to probe capture approaches. Several such tools exist to date, like MetCap (Kushwaha et al., 2015), BaitFisher (Mayer et al., 2016), BaitTools (Campana,

2018), MrBait (Chafin et al., 2018), CATCH (Viral Hemorrhagic Fever Consortium et al., 2019), AnthOligo (Jayaraman et al., 2020), HUBDesign (Dickson et al., 2021), Syotti (Alanko et al., 2022) and ProbeTools (Kuchinski et al., 2022). More existing tools are described elsewhere (Gasc et al., 2016). All of these rely on databases and, hence, limitations exist due to database biases concerning reduced sequence representation and database update status, affecting probe design and capture performance (Beaudry et al., 2021a; Manoharan et al., 2015; Noyes et al., 2017). Computational tools for assessing the ability of the probe-sets to keep up-to-date with contemporary databases were provided by Beaudry et al. (2021a). In the latter study, for resistome analysis, the authors assessed the hybridization capacity of probe-sets against reference genomes following examples derived from whole-exome sequencing, such as CapSim (Cao et al., 2018) and Wessim (Kim et al., 2013).

Moreover, library preparation biases associated with the need of PCR amplification during sequencing library preparation (Noyes et al., 2017) have also been reported. A normalization approach using unique molecular identifier (UMI) ligation to the extracted DNA fragments (hence, UMI deduplication accounts for any downstream PCR induced duplication events) has been implemented in the strategy "MEGaRICH" to address this issue (Noyes et al., 2017). As far as hybridization performance optimization is concerned, in some cases, longer hybridization times led to higher enrichment (Manoharan et al., 2015). In other cases where the ViroCap platform was used, hybridization efficiency (e.g. fragments captured) and sensitivity (richness of captured fragments) varied with hybridization time and read length (aka sequencing generation) used (Schuele et al., 2022; Schuele et al., 2020). Higher sensitivity concerning DNA fragment richness was observed in implementation of shorter hybridization times and the use of long, 3rd generation sequencing, reads. Moreover, enrichment rounds also affect the hybridization efficiency with multiple rounds being more efficient for small DNA content samples (Bragalini et al., 2014). Finally, different hybridization temperatures could affect the method sensitivity. Hawkins et al. (2016) proposed lower temperatures for more relaxed hybridization conditions, facilitating the identification of novel variants.

### 2.2. DNA-based stable isotope probing (DNA-SIP)

#### 2.2.1. Methodological principle and recent advances

DNA stable isotope probing (DNA-SIP) is a technique based on the separation of "heavy" and "light" DNA fractions formed by the incorporation to DNA of isotopic elements present in a given substrate assimilated by the microbial community. This allows the efficient separation and study of the DNA of microorganisms that assimilated the substrate and hence incorporated the isotopic elements in their DNA as described further below. In general, substrates labelled with heavier elemental stable isotopes (usually $^{13}C$, $^{15}N$, $^{18}O$) are provided to the microbiota in situ or in vitro and are integrated to the DNA of microorganisms with capacity to assimilate them (Neufeld et al., 2007). Following DNA extraction, DNA fractionation into buoyant density (BD) gradients (using e.g. CsCl solutions and ultracentrifuging) roughly distinguishes into two major fraction clusters of "heavy" and "light" DNA, that could be subject to downstream analysis using a range of methods to identify microorganisms that actively utilize the labelled substrate. Fingerprinting, phylogenetic microarrays and amplicon sequencing approaches have been traditionally used in combination with SIP to identify the taxonomic composition of "heavy" and "light" DNA fractions, while shotgun sequencing of the total DNA of each fraction has also been performed (Chen and Murrell, 2010; Coyotzi et al., 2016; Dumont and Murrell, 2005; Friedrich, 2006; Uhlik et al., 2013; Uhlík et al., 2009; Vasileiadis et al., 2022).

A more recent SIP variation is that of quantitative SIP (qSIP) that monitors population fluctuations between label free substrate controls and isotope labelled substrate treatments in every recovered DNA fraction (Hungate et al., 2015). Quantitative real-time PCR (qPCR) is performed for each recovered fraction with broad taxon level primers (e.g. targeting total bacteria), while the diversity of this taxon or its broader functional potential is assessed with metabarcoding (i.e. PCR amplicon sequencing of phylogenetic markers) or shotgun sequencing respectively (Greenlon et al., 2022; Lai et al., 2023; Sieradzki et al., 2021; Starr et al., 2021). The quantitative nature of qSIP allows modelling of the incorporation rates of the labelled compound part and, in combination with metabarcoding, it can provide information about labelled atom fluxes of the substrate among analysed microbial community members, or even microbial activity if the substrate is suitable (e.g. $^{18}O$-water).

Some advancements that support SIP include the integration of automation and the computational tools developed around it. One of these is high-throughput SIP (HT-SIP) (Nuccio et al., 2022) that relies on the use of robotic systems and wet lab optimisations, and results in high reproducibility, and good-quality of outputs once properly set (high 'heavy' DNA recovery, and good quality of MAGs). Next to wet lab advancements, DNA-SIP dedicated computational tools have been developed as well. A purpose-built R programming language package called HTSSIP, is able to facilitate data analysis for qSIP, qPCR free high resolution (HR) SIP, and multiple window (MW) HR-SIP variations (Youngblut et al., 2018a). Moreover, SIPSim and MetaSIPSim (metagenomic-SIPSim) were developed for simulating amplicon and shotgun metagenomic sequencing results of DNA-SIP respectively, with the ultimate goal to improve associated experimental designs (Barnett and Buckley, 2020; Youngblut et al., 2018b).

#### 2.2.2. Distinct advantages

DNA-SIP has been commonly used to link microbial functions with identities in situ for microorganisms which are active with respect to a labelled substate of choice (Dumont and Murrell, 2005; Friedrich, 2006; Uhlík et al., 2009). The substrate labelling approach, partial or complete (regarding the number of labelled atoms per compound for an element), or dual/multiple if two or more elements instead of one are labelled at the employed substrate(s), could dictate the results provided by the DNA-SIP analysis. For example, in an aquatic assay Liu et al. (2022) used $^{13}C$-dimethyl-sulfoniopropionate ($^{13}C$-DMSP) that was labelled in the propionate moiety (not at dimethylsulfide – DMS). This resulted in the identification of DMSP degraders/assimilators and their enzymes (in the heavy fraction assimilating at least the labelled propionate), while the authors speculated about the presence of possible degraders/assimilators of DMS according to taxa identified based on the light DNA fraction. Dual labelling (e.g. both $^{13}C$ and $^{15}N$) has been employed for assessing the assimilation of N, or C, or both by the various microbial guilds (Kim et al., 2023), and also for assessing chemolithoautotrophic growth (Deng et al., 2023).

One of the advantages of DNA-SIP is that it enables the study of low-abundance/rare taxa that carry a specific function (Chen and Murrell, 2010; Coyotzi et al., 2016). Moreover, it may facilitate high quality genome reconstruction of such taxa out of their environmental samples if combined with shotgun metagenome sequencing (Vasileiadis et al., 2022). The fractionation-based selection of the DNA of actively involved microbes in the assimilation of a labelled substrate, reduces the chances of chimeric metagenomic assemblies, resulting in high quality retrieved MAGs.

#### 2.2.3. Example applications

Fields of research where SIP was used extensively thus far include: (i) identification of key microbial players and associated roles in the degradation of common organic pollutants (e.g. hydrocarbon contaminants and pesticides) and contaminants of emerging concern (e.g. antibiotics, personal care products, hormones and others), a flagship DNA-SIP application (Dai et al., 2021; Gieg et al., 2014; Kim et al., 2023; Ouyang et al., 2019; Uhlik et al., 2013; Vasileiadis et al., 2022; Zhang et al., 2021b); (ii) monitoring of the dynamics and function of soil microbiomes by analysing nutrient fluxes through temporal sampling and in combination with analysis of the labelled metabolites (Kim et al., 2023; Nkongolo and Narendrula-Kotha, 2020); (iii) the assessment of

metabolic activity via the DNA incorporation of $^{18}O$ of labelled water (Schwartz et al., 2016); (iv) the study of less common or extreme environments ideal for bioprospecting (Coskun et al., 2019; Hayer et al., 2016; Propster et al., 2023; Purcell et al., 2022; Ruan et al., 2023; Xu et al., 2024); (v) the study of within/cross-kingdom interactions including syntrophic degradation in anaerobic systems along the process of methanogenesis (Gieg et al., 2014; Jin and Lu, 2023), or the $^{18}O$-water-based study of microbe-microbe or plant-microbe interactions (Nuccio et al., 2022); (vi) the study of microbial growth under drought with e.g. $^{18}O$-water vapor (Canarini et al., 2020); (vii) the determination of the activity of the role of the different groups of chemolithoautotrophic ammonia oxidizing microorganisms in soil supplied with $^{13}C$-$CO_2$ (Nicol and Prosser, 2011); (viii) the identification of active virus–host interactions and specifically of phages infecting methanotrophs in soil supplied with $^{13}C$-$CH_4$ (Lee et al., 2021).

#### 2.2.4. Challenges and possible solutions

DNA-SIP suffers from false positives or negatives due to extreme GC contents (besides elemental labelling) that tend to affect the output of DNA fractionation (Buckley et al., 2007; Youngblut and Buckley, 2014). This could be resolved through the inclusion in the analysis of same amplicons/amplicon-clusters through multiple fractions and non-labelled controls (Barnett et al., 2019; Youngblut and Buckley, 2014), while a second ultracentrifugation step with bis-benzimide has been also proposed (Buckley et al., 2007). Simulation tools can also be used for prognosis of BD fractionation and related issues (Barnett and Buckley, 2020; Youngblut et al., 2018b), yet, one of the issues of such approaches is that of underrepresentation of phylogenetic/functional markers in databases (Chen and Murrell, 2010; Uhlik et al., 2013). Another issue of SIP-based approaches is the low DNA recovery at the "heavy" fractions (Chen and Murrell, 2010). Sensitive, e.g. PCR-based, screening methods or multiple displacement amplification (MDA) with isothermal amplification (e.g. phi29 polymerase) in the case of shotgun analysis might offer a solution. Yet, MDA-introduced biases (e.g. GC content dependent amplification and chimeras; see more detailed discussion in the "Single cell genomics" section) may negatively impact the qualitative and, primarily, the quantitative potential of the method (Sobol and Kaster, 2023). Further important experimental design aspects that need careful consideration, have to do with the labelled substrate concentrations, and incubation times of the microbial communities with these substrates prior to sampling. These should be carefully selected, as cross-feeding effects and associated labelled atom enrichment could occur, which can complicate the interpretation of experimental outcomes (Uhlik et al., 2013). Careful design is also required in $^{18}O$-water experiments. Despite the ability of $^{18}O$-water to lead to important discoveries regarding microbial activity, issues like its evaporation during incubation, or the pre-existing sample content in un-labelled water are not trivial to resolve (Schwartz et al., 2016). Techniques developed, like $^{18}O$-water vapor equilibration (Canarini et al., 2020) and vapor-qSIP (Metze et al., 2023) can assist to partly overcome the latter problem in certain experimental designs. Another issue is that DNA-SIP is suitable for assimilatory, but not for dissimilatory processes, detecting only labelled atom assimilating microorganisms and leaving other participants in the studied activity, undetected (Uhlik et al., 2013). Finally, DNA-SIP methods are currently low-throughput, partly due to the costs of the equipment and the labelled compounds (Chen and Murrell, 2010; Schwartz et al., 2016), which, may not always be available (Chen and Murrell, 2010). HT-SIP partly addresses the throughput issues (Nuccio et al., 2022), however, it also relies on expensive equipment, like e.g. high precision/throughput robotic liquid handlers.

### 2.3. Emulsion, paired isolation and concatenation PCR (epicPCR)

#### 2.3.1. Methodological principle and recent advances

EpicPCR is a PCR amplicon sequencing method that intentionally fuses a functional gene of interest with a phylogenetic marker (16S rRNA gene) of the same host genome (Spencer et al., 2016). This makes it possible to directly determine which organisms carry a target functional gene of interest. Briefly, single cells are encapsulated in emulsion droplets containing acrylamide monomers, which polymerize into polyacrylamide beads, trapping the cells inside. After cell lysis, the released genomes remain in the polyacrylamide matrix and are re-encapsulated in new emulsion droplets, where fusion PCR takes place to fuse a functional and a phylogenetic marker. The fusion PCR includes three primers: F1 - a forward primer for the functional gene of interest, R2 - a reverse primer for e.g. the 16S rRNA-coding phylogenetic marker gene, and R1-F2′ - a bridge primer, present in a limited concentration, that merges the functional gene and the 16S rRNA gene. First, the F1 and R1-F2′ amplify the functional gene that has the overhang of 16S rRNA gene homology. Once the R1-F2′ primer is consumed, the amplified functional gene (with the 16S rRNA gene overhang) servers as a primer, annealing to the 16S rRNA gene and extending into it, thus creating a fused product. Finally, the R2 primer amplifies the fused product. The fused PCR products form only if the functional gene is present. A nested PCR is then performed to (i) amplify the fused products while incorporating the Illumina multiplex sequencing adapters with nested primers; and (ii) block the amplification of unfused products with blocking primers. These primers contain a 3′ carbon spacer that prevents DNA extension and a 5′ oligo-T extension, which includes A tail formation of unfused PCR products, ensuring they do not generate unwanted sequences after polyacrylamide bead degradation. In this way, functional genes are linked to phylogenetic markers and thus to host identities.

Recently, epicPCR 2.0 was developed standardizing the original method (Roman et al., 2021b). First, the bead loading was optimised using Poisson distribution to ensure that 90 % of the beads remained empty. This maximized the proportion of non-empty beads containing single cells (~85 %) and minimized the probability of multiple cell loading. This distribution was confirmed using SYBR Green I staining and microscopy. Next, to increase true positive amplification, the PCR step with the blocking primers (BP) was performed after the fusion PCR and before the nested PCR, either before or after the destruction of polyacrylamide beads. Finally, true OTUs were selected based on their presence in biological replicates, increasing confidence in OTU identification, though rarer sequences may be excluded.

One-step Isolation and Lysis PCR (OIL-PCR) was also recently developed as an upgrade to epicPCR. In OIL-PCR, cell lysis and fusion PCR occur in a single emulsion step using non-toxic, commercially available reagents, and it does not rely on microfluidics or specialized equipment (Diebold et al., 2021). Most importantly, OIL-PCR can be multiplexed, enabling the amplification of e.g. multiple target ARGs. Additional improvements to epicPCR include sequencing multiple functional marker genes alongside the 16S rRNA marker gene (Qi et al., 2023, 2024). This approach successfully identified bacterial hosts of class 1 integrons carrying gene cassettes in water samples. Finally, epicPCR combined with long read sequencing (ONT) has been applied to link ARGs to their hosts in wastewater samples, showing better performance in detecting ARG hosts compared to short/long-read shotgun sequencing (Lou et al., 2024).

#### 2.3.2. Distinct advantages

As briefly described above, epicPCR enables the linkage of functional genes to their unculturable host bacteria by combining an emulsion-based technique with multiple PCR steps (Spencer et al., 2016). A major advantage of epicPCR is that it bypasses the need for cultivation, which is particularly important as most environmental bacteria cannot be cultivated under laboratory conditions. In doing so, it addresses two major limitations of non-selective shotgun metagenomic approach: its relatively high detection limit and its limited ability to associate functional genes with specific phylogenetic identities. Another notable advantage of epicPCR is its ability to capture microbial interactions by encapsulating cell clusters and replacing functional marker primers with phylogenetic marker primers, as demonstrated in the study of Florenza

et al. (2019). In this study, microbial clusters were encapsulated, followed by the fusion of the 18S rRNA gene (for eukaryotes) with the 16S rRNA gene (for prokaryotes), enabling the identification of inter-domain microbial interactions.

#### 2.3.3. Example applications

EpicPCR was first applied in lake water samples to fuse the dissimilatory sulphate reductase gene (*dsrB*) and the 16S rRNA gene, detecting sulphate-reducing bacteria, including some novel sequences related to the *Deltaproteobacteria* class (Spencer et al., 2016). In a similar study, sulphate-reducing prokaryotes (SRP), including bacteria and archaea along with novel SRP taxa were detected (Qin et al., 2019).

Given the method's capacity to connect functions with taxa, it was not long before epicPCR was used to study the host range of ARGs and other horizontally transferred genes. For example, hosts for *tetM* (tetracycline resistance), $bla_{OXA}$ (β-lactam resistance), *qacE* (disinfectant resistance), and *int1* (integron 1 integrase, a marker for horizontal gene transfer – HGT) or $bla_{TEM}$, $bla_{VIM}$ and $bla_{OXA\text{-}48\text{-}like}$ (β-lactamase genes) were identified in wastewater treatment plants (WWTPs) influents and effluents, by fusing ARG amplicons with the 16S rRNA gene amplicon (Dekić Rozman et al., 2024; Hultman et al., 2018). Similar assays were conducted in activated sludge (Wei et al., 2021), soils after organic fertilization (Wei et al., 2022) and anaerobic digestion systems (Dai et al., 2023). In parallel, OIL-PCR has also been employed to identify ARG hosts by fusing plasmid-borne β-lactamase genes with 16S rRNA gene amplicons from various samples (Diebold et al., 2021).

In addition to studies on AMR, the versatility of epicPCR has also proven its worth in researching microbial interactions in various biological areas. Building on the work of Florenza et al. (2019) on eukaryote-prokaryote interactions, epicPCR was also used to study virus-host relationships. Sakowski et al. (2021) achieved this by fusing the ribonucleotide reductase (*RNR*) gene (a phage marker) to the 16S rRNA gene, which enabled the identification of viral host by sequencing the fused amplicons. Furthermore, epicPCR has been successfully integrated into a targeted cultivation approach to resolve in situ episymbiotic relationships (Xie et al., 2022). In this study, epicPCR was used to determine the bacterial hosts of the *Saccharibacteria* TM7i episymbiont so that the hosts could be cultured and the TM7i isolated for further characterization of its adhesion mechanisms.

#### 2.3.4. Challenges and possible solutions

While epicPCR is a powerful, culture independent method for linking hosts with specific functions and studying microbial interactions, it still has room for optimization. An inherent limitation of epicPCR is its reliance on PCR, which introduces primer biases (Sipos et al., 2007; Suzuki and Giovannoni, 1996), leading to a loss of microbial diversity (Hong et al., 2009; Jeon et al., 2008). This issue could be partly addressed using degenerate primers (Spencer et al., 2016; Wei et al., 2022, Wei et al., 2021). Cell lysis also poses a challenge, particularly when cells are treated individually (see the "single cell genomics" section for detailed discussion). Additional lysis steps could help reveal more hosts and genes (Spencer et al., 2016). Furthermore, epicPCR targets specific gene regions that may lack strong phylogenetic signals for accurate taxonomic resolution (Spencer et al., 2016). Comparisons with methods like SHS which interrogate larger genetic loci, show the benefits of amplifying longer gene segments (Cariou et al., 2018; Denonfoux et al., 2013; Gasc and Peyret, 2018). Amplifying a longer 16S rRNA gene segment combined with long-read 3rd generation sequencing can improve taxonomic resolution and enable species-level host identification (Hu et al., 2021; Liu et al., 2025). Such approach also supports analysing multi-functional marker gene loci, such as those found in *int1* gene cassettes (Lou et al., 2024; Qi et al., 2024; Qi et al., 2023; Sakowski et al., 2021).

Besides issues with taxonomic precision and coverage, epicPCR also faces challenges with cell disaggregation (e.g., in soil and manure) and low single-cell encapsulation rates. If cells are not properly separated from the aggregates, cell clusters can end up in the beads, making it difficult to produce beads with single cells. This is critical for epicPCR, as one cell per bead is necessary for accurate results, with each cell representing one bacterial species. When multiple cells are encapsulated in a bead, the 16S rRNA gene and target gene from different cells can fuse, leading to false positives where the 16S rRNA gene does not correspond to the actual carrier of the amplified target gene. In addition, multiple cells inside the beads can interfere with and inhibit PCR reactions, potentially preventing amplification. When cell disaggregation is not an issue, 90 % of beads must remain empty to achieve satisfactory proportions of single-cell encapsulation, as predicted by the Poisson distribution (Roman et al., 2021b). Such issues affect the method's ability to capture rare events and restricts its use to the detection of moderately rare but relatively abundant functional genes (Roman et al., 2021b; Spencer et al., 2016). To improve accuracy, qPCR has been suggested as a preliminary step to support the epicPCR protocol optimisation (Dai et al., 2023; Roman et al., 2021a; Wei et al., 2022).

Beyond challenges with single-cell encapsulation, another crucial issue in epicPCR is false fusion events that occur after DNA extraction, where genetic material from different cells leaks and mistakenly fuses, leading to incorrect associations (Spencer et al., 2016). EpicPCR 2.0 addressed this by: (i) adding an extra blocking PCR step before the nested PCR thus improving the blocking of the unfused amplicons; (ii) and through biological replication based PCR product validation, even at the cost of losing rare positives (Roman et al., 2021b).

Finally, in most cases successful cell-derived fused amplicons do not reveal the nature of the origin (chromosomal or mobile) of the functional marker, as flanking sequences of the functional target are not necessarily interrogated (Rice et al., 2020). The mobility of certain genes/sequences can be inferred based on database knowledge and taxonomic prevalence (Hall et al., 2020; Hultman et al., 2018; Qi et al., 2023; Roman et al., 2021a). Alternatively, the genetic environment of ARGs can be further assessed using techniques such as inverse PCR (Ochman et al., 1988; Pärnänen et al., 2016) to investigate their mobility. By combining these data with host bacterial profiles from epicPCR, we can identify key elements and genera involved in the spread of AMR.

### 2.4. The Hi-C connection

#### 2.4.1. Methodological principle and recent advances

Hi-C is a method where chemically induced cross linking provides the ability to link intracellularly derived DNA fragments. Hi-C use in metagenomics was based on the chromosome conformation capture (3C) and 3C-based techniques, developed for uncovering genome structure and organization, and chromatin interactions (Dekker et al., 2013; Schmitt et al., 2016). In 3C-based methods, cells are fixed via formalin solutions resulting in chromatin cross-links, involving nucleic acids and proteins. Following, DNA digestion and ligation of the cross-linked fragments (proximity ligation) leads to intracellularly derived chimeric DNA fragments. Finally, samples are analysed through PCR amplification and sequencing according to target locus knowledge (e.g. primers designed on a target locus – belonging in the studied chromatin – basis in such a manner that the amplified product contains the interacting ligated cross-linked chromatin). Therefore, in parallel with the chromatin reconstruction via shotgun sequencing, chromatin proximity and interactions are inferred via 3C. Hi-C is a 3C derivative where: (i) cross-links get digested; (ii) post digestion, ends are biotinylated and filled to become blunt; (iii) afterwards, blunt-end ligation mostly occurs among cross-linked fragments; (iv) these biotinylated fragments are enriched via streptavidin coated magnetic beads and shotgun sequencing of the enriched fragments is performed (Lieberman-Aiden et al., 2009; Van Berkum et al., 2010) as opposed to target locus-specific PCR enrichment and sequencing employed in 3C-based methods (Dekker et al., 2013, Dekker et al., 2002; Schmitt et al., 2016). When applied in complex microbial cell samples Hi-C provides physical

evidence of linkages between chromosomal and extrachromosomal DNA, a daunting task for resolution through computational evidence of shotgun metagenomics. To this end, Hi-C is heavily reliant on sophisticated computational tools for data analysis. ProxiMeta (Press et al., 2017), bin3C (DeMaere and Darling, 2019) and MetaTOR (Baudry et al., 2019), HiCBin (Du and Sun, 2022a), MetaCC (Du and Sun, 2023), hicSPAdes (Ivanova et al., 2022) have been developed for DNA assembly and linking of the resulting contigs based on cross-links and/or contig traits (e.g. phylogenetic markers, tetranucleotide fingerprints and relative abundances).

#### 2.4.2. Distinct advantages

Hi-C has the inherent capacity to detect the genomic context of metagenome assembled contigs. Its purpose in genome deconvolution became apparent since its initial applications in metagenomics. As a proof of concept, it was used to identify plasmid hosts and demonstrate its use in strain-level genome deconvolution in a synthetic bacterial community (Beitel et al., 2014). Moreover, in another study published in the same year, Hi-C was used for deconvoluting a synthetic community parted by both eukaryotes and prokaryotes, facilitating both chromosomal interaction dentification, and plasmid host identification (Burton et al., 2014).

#### 2.4.3. Example applications

The 3C advancement was first demonstrated in *Saccharomyces cerevisiae* cells, for the analysis of the chromosome III spatial organization (Dekker et al., 2002) and other eukaryotes (Dekker et al., 2013; Schmitt et al., 2016), and far less frequently for the genome conformation exploration of bacteria (Le et al., 2013; Umbarger et al., 2011). The first studies that demonstrated the utility of Hi-C and 3C in metagenomics attempted to link mobile genetic elements with their hosts as described in the previous section (Beitel et al., 2014). Hi-C was further applied in culture-enriched river sediments, in gut microbiomes and others, for analysing microbial diversity, chromosomal organization, and for linking extrachromosomal MGEs and ARGs with their host strains (Liu and Darling, 2015; Marbouty et al., 2014; Marbouty and Koszul, 2015; Rice et al., 2020; Saak et al., 2020; Yaffe and Relman, 2019).

Several computational tools followed the method evolution with different foci. Out of these MetaCC can utilize long-read sequencing data and detect multi-copy plasmids (Du and Sun, 2023). ViralCC was developed for viral binning from Hi-C data, facilitating detection of viruses and virus-host associations (Du et al., 2023). HAM-ART, another Hi-C dedicated pipeline, performs tasks like genome recovery and taxonomic identification, association of MGEs to hosts, and ARG annotation (Kalmar et al., 2022). HAM-ART provided enhanced evidence of AMR dispersal through the Hi-C protocol (Tams et al., 2023). HiFine (Du and Sun, 2022b) and the self-supervised learning algorithm of metaBAT-LR, have emphasized in strain level deconvolution with the help of both the shotgun and Hi-C data (Ho et al., 2023). Two more accessory, yet important tools in such experiments are that of the quality control associated qc3C (DeMaere and Darling, 2021) and the Sim3C, a Hi-C experiment simulation software, that can assist in proper Hi-C experimental design (DeMaere and Darling, 2018).

#### 2.4.4. Challenges and possible solutions

Hi-C faces shortcomings associated with biases in the frequency of restriction digestion sites utilized for DNA shearing that varies among microbial taxa (Du et al., 2022), and is also affected by the genomic GC content (Marbouty and Koszul, 2015). A possible solution has been proposed, employing the generation of multiple Hi-C libraries with a corresponding number of restriction digestion enzymes (Marbouty et al., 2014; Marbouty and Koszul, 2015) and the use of purpose built bioinformatic tools for proper restriction site frequency prediction and contig binning (Baudry et al., 2019; DeMaere and Darling, 2019; Du and Sun, 2022a, 2023). Another source of bias is associated with the spurious inter-cellular/species cross-links (Du et al., 2022). Such were identified in several Hi-C studies at low relative abundances (Beitel et al., 2014; Burton et al., 2014; Marbouty et al., 2014; Marbouty and Koszul, 2015; Stalder et al., 2019) with algorithms such as those included in HiCBin and MetaCC (Du and Sun, 2022a, 2023). Despite the physical evidence provided by Hi-C that help in resolving HGT events, common elements between genomes, such as MGEs, homologous sequences or repeats, can still lead in spurious links and make contig binning tedious (Liu and Darling, 2015; Marbouty et al., 2014; Marbouty and Koszul, 2015; Stalder et al., 2019). The combination of spike-in abundance controls and coverage information (considering rare events to putatively be spurious) has been used for resolving HGT events (McCallum et al., 2023). Besides MGE associated difficulties, high identities of homologous loci of closely related strains can also lead to chimeric assemblies and erroneous Hi-C outputs (Liu and Darling, 2015; Marbouty and Koszul, 2015) making strain-deconvolution difficult (Baudry et al., 2019; Burton et al., 2014; DeMaere and Darling, 2019; Du and Sun, 2022a, 2023). Next to the physical evidence provided by Hi-C that assist in the identification of intespecifically hybridised yeasts (Smukowski Heil et al., 2018) and achieve species deconvolution (DeMaere and Darling, 2016), 3rd generation sequencing technologies can assist with resolving contig mis-binning caused by taxonomically dispersed homologous regions like e.g. repetitive and mobile elements (Hu et al., 2021). A use of long-read sequencing and Hi-C led to improved assemblies (Du and Sun, 2023) and confident detection of MGEs (Cuscó et al., 2022), while further improvement was achieved with the additional combination of high quality short Illumina reads, error correcting the long-read assemblies (Gounot et al., 2022; Jia et al., 2023; Kumar et al., 2019).

## 3. Cell enrichment methods

As opposed to DNA enrichment methods, cell enrichment aims at the reduction of the total metagenome to a handful of genomes, that can sufficiently be interrogated by current sequencing technologies, based on their identities, or their functions, or pure chance. Each of the three main methodological fields described below, can provide different selection modes with varying levels of resolution, from coarse to fine grain. Their main common trait is, in most cases, their dependence on a type labelling, e.g. with antibodies, fluorescence, radioactivity etc. (Sturm et al., 2023). Immunomagnetic precipitation (IMS) relies on the presence of target cell specific epitopes, that can support their phylogenetic and/or functional identification, and its resolution capacity is defined by the antibody specificity. Fluorescence in situ hybridization (FISH) based methods rely on the presence of target specific phylogenetic or functional nucleic acid markers, and guarantee, in the least, population level resolution. Single cell genomics aspire to achieve cell level resolution and can be either targeted, or random. In all aforementioned cases, the provided genomic context can deepen our understanding of ecological aspects and functional roles of strains, species, genomovars, and beyond. Essential information about these methods which is detailed in the text, is also summarised in Table 2.

### 3.1. Immunomagnetic separation (IMS)

#### 3.1.1. Methodological principle and recent advances

Immunomagnetic separation (IMS) is a method for microbial cell enrichment, based on the interaction between antibodies and cells carrying the target epitope (Wang et al., 2020). Briefly, biotinylated antibodies bind on the target extracellular epitopes, the cell-carrying biotinylated antibodies non-covalently interact with streptavidin coated magnetic beads (Chivers et al., 2011), and the whole complex is retrieved through application of magnetic fields. The antibody/epitope complex and the target cells are then separated from the rest of the sample. IMS can be combined with downstream analysis methods for microorganism detection, like nucleic acid amplification (e.g. PCR), fluorescence, immunological assays (e.g. ELISA), and biosensors. IMS

enriched cells have also been subjected to DNA extraction for sequencing analysis with the assistance of whole cell amplification methods for obtaining sufficient amounts of DNA for sequencing. Such an approach has been applied in population genomic analysis of *Chlamidia trachomatis* (Putman et al., 2013; Seth-Smith et al., 2013b; Taylor-Brown et al., 2018), *Cryptosporidium* (Andersson et al., 2015; Hadfield et al., 2015), and *Giardia lamblia* (Hanevik et al., 2015) from clinical and faecal samples.

#### 3.1.2. Distinct advantages

An important advent of IMS is its capacity to take advantage of an array of epitopes of varying conservation levels for achieving the desired assay specificity. Given the high specificity potential of antibody use (e.g. in the case of monoclonal antibodies), one of its most frequent uses is that of species resolution level microorganism retrieval and subtyping. As representative examples, past studies include the identification and typing of: shiga-toxing producing *Escherichia coli* (STEC) (De Boer and Heuvelink, 2000); waterborne parasites (Quintero-Betancourt et al., 2002); *Salmonella* species (Lee et al., 2015); and *Legionella pneumophila* (Walker and McDermott, 2021). Such approaches allow to exploit the full analytical power of downstream methods to the high-resolution taxa of interest.

#### 3.1.3. Example applications

IMS has been employed in microorganism detection in clinical samples (Olsvik et al., 1994), foodborne bacteria (Benoit and Donahue, 2003; Stevens and Jaykus, 2004; Wang et al., 2020), or waterborne enteric viruses (Hamza et al., 2011). IMS comprises a promising tool for protist research due to the recalcitrance of protists to axenic cultivation (Hadfield et al., 2015; Hanevik et al., 2015). IMS can enrich complete cells of a microbial population, providing the ability to go beyond the detection, to mapping the complete functional potential of the studied population via shogun sequencing (Hanevik et al., 2015; Noh et al., 2023). Besides bacteria and protozoa, IMS has been employed for studying influenza subtypes, using multiplex PCR amplifying eight marker gene segments for amplification instead of MDA, followed by sequencing (Noh et al., 2023). Finally, microbe-host interaction associated IgA magnetic immunoprecipitation has allowed to enrich IgA interacting intestinal microorganisms, prior a fluorescently activated cell sorting (FACS) enrichment, and perform metagenomic analysis of the final community subset populations (Olm et al., 2025). IgA staining demonstrated 6-fold or more enrichment of the IgA-bound cells in the case of the IgA immunoprecipitated cells, speeding up the downstream FACS based enrichment by orders of magnitude compared with previous approaches (Palm et al., 2014) not including the immunomagnetic separation step.

#### 3.1.4. Challenges and possible solutions

Like any other cell enrichment method, IMS requires unicellular non aggregated events, separated from environmental particles that may hamper downstream analytical methods. Cell extraction, purification, and dispersion comprise potentially challenging tasks preceding any sorting approach (either in population metagenomics, or in single cell genomics approaches). One of the most challenging matrices is that of soil (El Mujtar et al., 2022). Given soil heterogeneity and cell absorbance onto the complex soil matrix, cell extraction may employ an array of methods for achieving the cell detachment and their separation from particles of such matrices (El Mujtar et al., 2022; Khalili et al., 2019). Diluents utilized range from water to saline, tween, buffered saline, sodium cholate and others, at diluent to soil ratios of 12–44 %. The diluent composition aims at either protecting the cell structure from osmotic stress, or at achieving cell dispersion or both. To further enhance cell dispersion, physical methods (shaking, vortexing, sonication) can be combined with the chemical methods mentioned above. Finally, filtration or centrifugation with density gradient generating agents like Nycodenz, Percoll, Sucroze, Metrizamide, Ludox, or Ficoll facilitates cell purification.

In complex samples IMS does not always lead to high quality genomes, depending on the sample target cell content, sample type, the specificity of antibodies, the cell morphology and size (Putman et al., 2013; Seth-Smith et al., 2013b). Low cell counts result in low target DNA recoveries. Total DNA amplification with methods like MDA partly address this issue facilitating shotgun sequencing (Seth-Smith et al., 2013b). Even with MDA, some samples may not reach the necessary DNA mass for achieving satisfactory genome coverage via sequencing, due to very low initial material and MDA or sample purity associated biases (detailed description of total DNA amplification methods and associated biases is provided in the single-cell genomics analysis section) (Hadfield et al., 2015; Kirchner et al., 2019). PCR/qPCR diagnostics can assist in deeper understanding quantitative issues (Hadfield et al., 2015; Seth-Smith et al., 2013a, 2013b). Another approach for addressing the low cell count issue is that of the addition of an extra step of enrichment of target populations using taxon-specific nutrient media where possible (Hyeon et al., 2018a, 2018b). This approach, coined as quasi-metagenomics assisted IMS, succeeded in the reduction of diagnostics time requirements from 10 to 14 days to 1–2 days for *Salmonella enterica* when combined with ONT (Hyeon et al., 2018a). Variations of IMS-based quasimetagenomics included a, post IMS, secondary enrichment after spread plating on solid media, with colonies being used in downstream further analysis of *Listeria monocytogenes*, abolishing the need of the MDA step for their experimental needs (Azinheiro et al., 2022). In another study, chromogenic media in the post IMS step were employed for the characterization of *stx* (coding for shiga-toxin) gene carrying *E. coli* – STEC (Egervärn and Flink, 2024). It has to be noted though, that competitive exclusion for resources applies also to strains of the same species depending on the species niche breadth (Segura Munoz et al., 2022), enhancing the possibility of missing naturally occurring subtypes after successive cultures.

Depending on the type of antibodies and conservation level of the epitopes, specificity issues might arise. IMS was demonstrated to reach specificity $\geq$90 % at species level (Andersson et al., 2015), $\geq$ 60 % at strain level (Kirchner et al., 2019), or even $\geq$95 % in other occasions (Putman et al., 2013). However, lack of appropriate epitopes humper the enrichment of some species (Kirchner et al., 2019), with examples observed in several cases dealing with complex samples (Putman et al., 2013; Seth-Smith et al., 2013b). Furthermore, antibody efficiency varies in different sample types that may have properties that affect the antibodies or the epitopes (Putman et al., 2013). Other factors that may affect IMS are the number of beads and the incubation time, next to the choice of appropriate antibody/cell elution buffer (King et al., 2013). Technical points of concern (e.g. poor mixing/homogenization, insufficient amplification, low sample integrity, incompatible target bacterial content) and associated troubleshooting have been reported in previously published protocols (Seth-Smith et al., 2013a).

### 3.2. Fluorescence in situ hybridization (FISH) population genomics

#### 3.2.1. Methodological principle and recent advances

An established method for dissecting environmental microbiomes and collect individual cells or populations of microbial populations of interest, is fluorescence in situ hybridization (FISH). FISH allows for phylogenetic identification and sorting of cells, e.g. by means of fluorescent activated cell sorting – FACS (Davey and Kell, 1996; Haroon et al., 2013). During FISH, fluorescently labelled oligonucleotide probes are introduced into, usually, in situ fixed (aka dead) cells, where they hybridize with target nucleic acids. Common fixatives, i.e. agents causing biomolecular cross-linking include formalin (4 % paraformaldehyde in aqueous solution), ethanol, methanol, glutarhaldehyde, and glyoxal (Channathodiyil and Houseley, 2021; Richter et al., 2018). Usually, taxonomically conserved regions of ribosomal RNAs are targeted during FISH (Amann and Fuchs, 2008). Ribosomal RNA is abundant in most cells and thereby leads to strong whole-cell

fluorescent signals that are easily detectable. By contrast, when low copy number nucleic acid targets are targeted, such as chromosomal genes, additional strategies are needed to produce detectable signals. Sample preparation may be performed on microscopy slides, or in-solution, depending on the intended downstream method of detection. If cells were processed in solution, the samples can be analysed using fluorescence activated cell sorting (FACS) flow cytometry. FACS allows to sort and collect populations, or individual cells from complex community samples by exciting them using one or more lasers (Haroon et al., 2013; Kaster and Sobol, 2020). Their properties are measured in several ways: scattered light is analysed by detectors opposite to the cell (forward scatter, indicative of particle size), perpendicularly scattering light (indicative of the particle density), and intensity and wavelength of the perpendicularly scattered light, i.e. emitted fluorescence of the particle. The resulting property fingerprint allows to select and sort bacterial populations of interest by gating them based on conditional decisions such as fluorescence intensity and light scatter. Sorting of positive/negative events and downstream analysis include phylogenetic or functional marker genes and population/single-cell genomics (Amann and Fuchs, 2008; Jia et al., 2021; Zand et al., 2021).

#### 3.2.2. Distinct advantages

FISH allows for the detection of a wide range of targets, either by targeting taxonomically conserved regions of the phylogenetic ribosomal RNA markers or the commonly of functional screening value but less abundant DNA or mRNA targets (Amann and Fuchs, 2008). Such low copy targets require signal amplification methods, including: target amplification with e.g. isothermal amplification or affiliate methods; use of long polynucleotide probes with multiple labels (e.g. “recognition of individual genes” FISH – RING-FISH) (Amann and Fuchs, 2008; Moraru et al., 2010); tyramide-labelled fluorescent dyes interacting with horseradish peroxidase (HRP) tagged probes in catalyzed reporter deposition FISH (CARD-FISH) (Pernthaler et al., 2002); hybridization chain reaction FISH (HCR-FISH) with probes carrying overhangs that serve as hybridization chain facilitators for multiple labelled probes (Grieb et al., 2020; Jia et al., 2021; Yamaguchi et al., 2015). Finally, cell activity can be measured using resourceful approaches as briefly described in the following section (Amann and Fuchs, 2008; Okabe et al., 2004).

#### 3.2.3. Example applications

Originally developed to paint eukaryotic chromosomes (Langer-Safer et al., 1982), FISH is a powerful tool that has been introduced to microbial ecology in the late 1980s. FISH is able to provide both quantitative and spatial distribution information of taxa and functions of microbiota depending on the downstream diagnostic method (Arrigucci et al., 2017; Pernthaler et al., 2002; Pernthaler et al., 2001; Pernthaler and Amann, 2004; Remus-Emsermann et al., 2014; Zwirglmaier, 2005). For example a combination of targeting phylogenetic (e.g. targeting the abundant ribosomes) and functional markers (using short polynucleotide probes carrying multiple labelling dye molecules like in geneFISH or direct-geneFISH) can facilitate a combined phylogeny/function-based sorting of microorganisms of interest (Barrero-Canosa and Moraru, 2021; Moraru et al., 2010) for downstream population genomics analysis.

Micro-autoradiography “MAR”-FISH uses radiolabelled substrates prior to phylogenetic marker targeting FISH has been used to classify substrate degraders (Amann and Fuchs, 2008; Okabe et al., 2004). Radiolabelled substrates included contaminants of emerging concern like triclosan (Lolas et al., 2012). Moreover, redox sensing cell dying compounds like 5-cyano-2,3-tolyl-tetrazolium chloride (CTC) have been combined with FISH to identify metabolically active taxa (Kalyuzhnaya et al., 2008; Nielsen et al., 2003). Bioorthogonal non-canonical amino acid tagging (BONCAT) FISH has been used to determine translationally active cells in complex environments (Hatzenpichler et al., 2014).

Focusing on active microorganisms, transcript-annealing molecular beacons FISH (FISH-TAMB) allows to determine the activity of live cells by tracking the accumulation of polyarginine cell penetrating peptides that deliver molecular beacons across prokaryotic cell walls and membranes (Harris et al., 2022). Molecular beacons are quenching themselves if they are not annealed to target nucleic acid sequences, while, upon annealing, they start fluorescing, resulting in living cell labelling.

#### 3.2.4. Challenges and possible solutions

Issues regarding cell dispersion and separation from background environmental particles as described in the IMS section above apply for FISH based methods too. In fact, methods like those arising from the concepts of mini/midi-metagenomics (Vollmers et al., 2023; Yu et al., 2017) have been applied in FACS or random cell separation in order to better resolve metagenomic binning in a multi-cell level fashion. In mini metagenomics cells are separated into cell groups, with the number of cells in each group varying from 5 to 10 (Yu et al., 2017) to up to 100 (Schulz et al., 2018) or more (Geesink et al., 2020). The small-scale population genomics of mini-metagenomics have led to the exploration and discovery of rare bacterial taxa in complex environments (Alteio et al., 2020; Yu et al., 2017). Just like mini-, midi-metagenomics share the same workflow with FACS sorting, but in this case several hundred thousand to million cells are sorted in groups (Vollmers et al., 2023). Both of these methods are oriented towards genome deconvolution by reducing metagenomic complexity within the broader context of population genomics principles.

A known source of noise in fluorescence measurements originates from environmental and cellular autofluorescence (Davey and Guyot, 2020; Eickhorst and Tippkotter, 2008). Due to particle carryover, this issue is particularly problematic for soil/sediment/sludge environment-derived cells (Arrigucci et al., 2017; El Mujtar et al., 2022). Centrifugation density cell extraction and purification like Nycodenz may significantly remedy this issue (Bertaux et al., 2007). However, autofluorescence also arises form cellular constituents, and, therefore, requires associated knowledge.

Using FACS for retrieving specific taxonomic groups from complex environments with FISH is a serious challenge for probe designing (Haroon et al., 2013). Several taxa lack marker sequence variability with close relatives, leading to false positive labelling. Established probes can be found in databases like probeBase (Greuter et al., 2016; Loy et al., 2007). Next to hybridization conditions testing (i.e. denaturant concentration, and hybridization temperature), probe types that can partly improve hybridization stability and specificity are those of peptide nucleic and locked nucleic acid (PNA and LNA respectively) oligos (Guimarães et al., 2007; Kubota et al., 2006; Worden et al., 2000). PNA or LNA oligos can achieve the additional stability and specificity due to their lack of the negative charges compared to nucleic acid phosphate backbones, responsible for the electrostatic repulsion with the complementary target DNA strand.

Another inherent issue of FISH is the accessibility of probe target sites that can be restricted due to cell walls and cross-linking caused by fixation. Lysozyme has been used to increase cell wall permeability allowing e.g. HRP labelled probe access (Bidnenko et al., 1998), at the cost, however, of cell integrity (Chen et al., 2000; Derde et al., 2013). The cross-linking nature of fixatives like paraformaldehyde and glutaraldehyde, hamper access of DNA for polymerases and other enzymes during downstream procedures (Channathodiyil and Houseley, 2021; Richter et al., 2018). Alternatives such as methanol and ethanol may malform the cells and provide relatively poor fixation. Glyoxal has shown reduced cross-linking of nucleic acids with the rest cell biomolecules. Nevertheless, no universal behaviour has been shown possible and testing of multiple taxonomical representative isolates is required for the best compromise (Grieb et al., 2020). Fixation-free FISH protocols based on brief-ethanol-exposure have been proposed (Yilmaz et al., 2010), with the compromise of sample storability.

Furthermore, the limited number of distinct emission/excitation spectra of fluorophores and diagnostic instrumentation filters limits

FISH by limiting the diversity of organisms that can be targeted in a sample. Currently, mono-labelled FISH probes are limited to maximally 7 different probes (theoretically up to 8) different probes that can be used simultaneously for unambiguous detection of taxa (Lukumbuzya et al., 2019). Dual (DOPE-FISH) or higher (multi-labelled FISH – MiL-FISH) probe labelling can assist in expanding the simultaneously analysed probe numbers (Behnam et al., 2012; Schimak et al., 2016). Finally, mixtures of mono-labelled probes per taxon have been used for higher level combinatorial labelling and spectral imaging-FISH (CLASI-FISH) for the simultaneous detection of up to 15 different taxa (Lukumbuzya et al., 2019).

FACS may face issues with detection limits and background signal interference. Prokaryotic cells often have very low signals (sometimes indistinguishable from cellular autofluorescence or background fluorescence, or non-specific signals) (Davey and Kell, 1996; Marrone, 2009). To overcome this limitation several strategies have been used: (i) dyes with stronger fluorescence; (ii) enhanced signals with multi-labelled probes or CARD-FISH; (iii) the use of suitable instrumentation for small-sized events and signal deconvolution (e.g. detectors with increased sensitivity, imaging FACS instruments with high resolution cameras, and spectral detectors – as opposed to monochromatic detectors) (McKinnon, 2018; Robinson et al., 2023).

Cell aggregation may lead to the perception of several cells as a single event and causing issues with downstream analysis (Ou et al., 2017). In parts, this issue can be reduced by using surfactants and mechanical forces. The cell sorting may damage and break weak cells, e.g. after cell wall permeabilization, if performed with high sheer force fluidics setups (Kaster and Sobol, 2020). Combinations of cell/taxon/function preselection methods can reduce sample complexity which allows for FACS with smaller shear forces. Finally, a solution ensuring selection of intact single cells is image activated cell sorting (Kuhn et al., 2024; Nitta et al., 2018; Riba et al., 2016; Wiegand et al., 2021).

### 3.3. Single cell genomics

#### 3.3.1. Methodological principle and recent advances

Many traditional targeted metagenomics methods focus on population scale responses. Those methods have difficulties to confidently capture the population heterogeneity at fine-grained species, genomovar (i.e. intra-species subgroup), or strain level (Gross et al., 2015; Kaster and Sobol, 2020; Keating et al., 2024; Keloth et al., 2018). By contrast, single cell genomics methods allow to analyse each cell individually to determine differences in genes or whole genomes. In single cell analysis, cells are extracted, purified and disaggregated/dispersed, and then sorted individually in a targeted or a random manner (Kaster and Sobol, 2020). Extracted cell sorting may be performed with FACS, (micro)-fluidics cell sorters, limiting dilution, and micromanipulation including combinations with optical traps/tweezers (Keating et al., 2024; Keloth et al., 2018). After cell-sorting, the genomic material of the single-cells is amplified to single amplified genomes (SAG) and then sequenced.

#### 3.3.2. Distinct advantages

Single cell analysis, when successful, has the unique advantage of interrogating each cell for its complete replicon set, both chromosomal and or plasmids (Hosokawa and Nishikawa, 2024; Lan et al., 2017). Several studies have used sorted cells for single-cell transcriptomics and proteomics (Ahmad and Budnik, 2023; Bennett et al., 2023; Conchouso et al., 2021; Moon et al., 2023). Moreover, some microfluidics devices, when combined with suitable monitoring equipment, also provide physiological information at the single cell resolution (Kaster and Sobol, 2020; Keating et al., 2024; Song et al., 2024; Yu et al., 2022).

#### 3.3.3. Example applications

FACS has, thus far been, the most commonly applied method for cell sorting in single-cell genomics approaches, due to its relative consistency and its capacity to sort cells based on cell traits and fluorescent tags, allowing for phylogenetic or functional marker filtering (Also see section 2.2.2 above) (Kaster and Sobol, 2020). Next to FACS, microscopy-based single cell isolation methods include micromanipulation and optofluidics. Micromanipulation cell sorting is based on the use of microcapillaries in combination with pumps and inverted microscopes for single cell picking and retrieval in a microchamber, a relatively straightforward, but labour intensive, approach in single cell analysis (Ishoey et al., 2008; Keloth et al., 2018; Woyke et al., 2010). Optical trapping/tweezers cell selection (also called optofluidics) has been long used in the microscopical observations of living bacteria (Ashkin et al., 1987) and in conjunction with microfluidics devices (Zhang et al., 2019). Optical tweezers take advantage of the difference in refractive indexes of cells and their surrounding medium, which allows to use focused high energy light (Landry et al., 2013; Neuman and Block, 2004) to collect single cells in individual microfluidic chambers. This allows downstream genome sequencing of those cells.

As reviewed elsewhere, to facilitate single-cell analysis, several different, dedicated microfluidics devices have been designed (Kaster and Sobol, 2020). So-called lab-on-a-chip devices are able to perform cell sorting, DNA extraction, PCR amplification, and sequencing library preparation on one device (Yu et al., 2022). Generally, microfluidics sorting methods may rely on hydrodynamic trapping, micro-valves, or mineral oil encapsulation droplets (Zhou et al., 2021). Due to the small cell size of prokaryotic cells, the latter method is most commonly used for the investigation of microbiomes (Hosokawa and Nishikawa, 2024; Luan et al., 2020; Yu et al., 2022). To that end, monodispersed hydrogel-in-oil droplets are generated in high throughput, by shearing a molten agarose cell suspension with carrier oil in a microfluidic channel. The cell concentration in the molten agarose is such that only one cell or less per droplet are produced (Hosokawa and Nishikawa, 2024). The droplets are then solidified and the mineral oil is removed. Afterwards, the beads are exposed to a sequence of reagents that process the cells including typical steps such as cell lysis, whole genome amplification, and DNA purification. By performing these steps in beads, it is possible to massively parallelize the reactions and upscale the throughput to millions of beads. Afterwards the beads can be qualified and sorted (e.g. using FISH) and their DNA can be indexed for SAG analysis.

Single-cell genomics can be also performed in a random manner using limiting dilutions. Limiting dilution is a method where the cellular concentration of a medium along with the Poisson probability density function are used for estimating the chances of retrieving individual cells from a population of cells (Gross et al., 2015). For example, if an environmental sample has a density of 50 cells $ml^{-1}$, the chances of retrieving 0–1 cells per 10 μl is 91 % (with 61 % for 0 cells and 30 % for 1 cell) (Gross et al., 2015; Zhang et al., 2006). Validation of cell density in the picked samples can be qualitatively performed via qPCR.

A critical step of single-cell methods is whole genome amplification (WGA) (Gawad et al., 2016; Sobol and Kaster, 2023). WGA is either based on PCR, isothermal amplification, or hybrid approaches of both. Non-isothermal genome amplification methods have not been implemented in microbial single-cell genomics, probably due to very low genome coverage, site specific biases, DNA polymerase error rates (Gawad et al., 2016; Sobol and Kaster, 2023), and also the effect of high temperature during PCR on fixed cell and the bead integrity. The most common WGA method is that of MDA, using random primers and the φ29 isothermal DNA polymerase. The φ29 DNA polymerase has the advantages of rolling cycle amplification of DNA, low error rates ($10^{-6}$) and generates fragments longer than 10 kb.

#### 3.3.4. Challenges and possible solutions

Most of the challenges mentioned for FACS in the population genomics section (e.g. cell extraction, purification, dispersal, fragile cells and sheer force of fluidics) are also valid for single cell sequencing. For single cell genomics though, next to the possible solutions provided in the previous section, methods with smaller sheer forces, like those of optofluidics, can be used if high throughput screening is not necessary

(Landry et al., 2013). This also reduces bad signal to noise ratios that lead to false positives, and the amount of consumables such as costly MDA reagents.

Limiting dilution and microdroplet methods suffer from Poisson distribution-based cell recovery, which introduces a biase against rare population events (Sobol and Kaster, 2023). As in order to achieve 85 % of the samples or beads that contain cells to contain only one, it is necessary to have 90 % no-cell events, hence, only 8.5 % of all samples or beads contain cells at all. This means that to analyse 8500 single cells, it is necessary to screen and process 100,000 beads or samples. Novel, microfluidics based, cell sorters, with cell-size dependent imaging equipment and machine learning supported image analysis can help to solve this issue. This can be done by performing sorting of only single cell events in an image profile driven manner (Kuhn et al., 2024; Nitta et al., 2018; Riba et al., 2016; Wiegand et al., 2021). However, even in this case, several restrictions remain. For example, filamentous microorganisms (e.g. cyanobacteria, streptomycetes and fungi) do not fit in pico/nano-liter volume hydrogel beads (Hosokawa and Nishikawa, 2024). In such cases filtering and similar alternative strategies can mitigate the shortcomings of these assays to widen their application.

Another major challenge in molecular microbial ecology in general, but of utmost importance in single cell analysis, is that of cell lysis (Gaisser et al., 2024). The large variety of microbial cell-wall and membrane structures, and the recalcitrant structures of their dormant states found in complex environments, dictates for the elaborate combination of methods for successful and representative nucleic acids extraction (Lee et al., 2024a). Common cell lysis methods include the use of surfactants (ionic surfactants like sodium dodecyl sulphate and cetrylmethylammonium bromide attacking membranes and charged cell-wall constituents; non-ionic surfactants like Triton X 100 attacking membranes), alkaline solutions ($OH^-$ breaking fatty acid-glycerol ester bonds in phospholipids bilayer), enzymes (attacking the cell walls like e.g. lysozyme and protein structures like proteinase K), and physical methods like heating (e.g. causing denaturation of weak bonds) and bead milling (use of collision force to break cell walls and membranes). Currently, in single-cell genomics only a few chemical and enzymatic cell lysis methods can be used. As a result, genome recovery for difficult to lyse taxa is often low (Hosokawa and Nishikawa, 2024). Nevertheless, state of the art methods used in environmental monitoring might be able to facilitate single cell analysis as well (Lee et al., 2024a). For instance, novel chemical (e.g. ozone-based) and physical/physical-electric (e.g. piezoelectric or sonication derived molecular vibrations, non-ohmic electric field and ionic electric applications, photothermal and photolytic methods) methods have been used at the whole sample scale, however, downscaling to the micro/nano/pico volume scales has not been explored yet.

The current state of the art method to prepare sufficient single cell genomic material, MDA using φ29 polymerases using random hexamer primers (Sobol and Kaster, 2023), adds additional pitfalls as it is biased towards high-GC content, uneven genome coverage due to exponential amplification rates and chimeras produced during MDA. Although the high-GC content bias is reproducible, the chimera production is mostly random, which makes it difficult to bioinformatically resolve their occurrence. However, chimeras can be dealt with, to some extent, by post amplification digestion to smaller fragments. Improved thermostable polymerases (e.g. WGA-X™ of BioSkyrb Genomics, Inc., Durham, NC, USA) and alternative primary template directed amplification (reduction of secondary template amplification) have been demonstrated to partially remedy the issues of φ29 polymerase (Sobol and Kaster, 2023). Finally, methods like primary template-directed amplification (PTA-MDA; using exonuclease terminators for generating smaller amplicons with limited subsequent amplification (Sobol and Kaster, 2023), or the linear amplification via transposon insertion (LIANTI) method (through an elaborate LIANTI transposome DNA fragmentation and T7 promoter tagging, T7 RNA polymerase in vitro transcription mediated linear amplification, and cDNA generation), that result in linear DNA amplification (Chen et al., 2017), are possible solutions to issues associated with the exponential MDA.

Contaminations comprise another common problem impacting single cell genomics, and involves most commonly (Blainey, 2013; Hosokawa and Nishikawa, 2024; Zhang et al., 2006): (i) the contamination with nucleic acids (in the order of a few femtograms); (ii) the presence of nucleic acids in microbially produced reagents; (iii) primer-primer interactions. Next to proper technique and highly purified reagents, suitable control reactions using primers that lack the capacity to cross-hybridize can assist in streamlining the employed MDA protocols and minimizing primer-primer cross-hybridization, while controlling or flagging any other types of contamination. Contamination analysis and control can also be performed bioinformatically, by checking the single copy gene marker redundancies, in a taxon-specific manner with algorithms like CheckM (Parks et al., 2014) or MiGA (Rodriguez-R et al., 2018), with varying levels of confidence per algorithm depending on the existence of affiliate genome sequences in classification databases (e.g. at NCBI or the genome taxonomy database – GTDB) (Parks et al., 2021).

Finally, the high cost of single cell genomic experiments is often prohibitive to perform experiments. Despite the potential accuracy and the reduction of the per genome cost, the use of expensive and exotic equipment during cell separation and processing leads to costs that are usually incompatible with low/mid budget laboratories. However, at the horizon, single cell transcriptomics of eukaryotic organisms and prokaryotes has paved the way for low cost library prep methods employing combinatorial/split-pool barcoding of cDNA, leading to a theoretically cell-specific cDNA labelling via a combination of introduced tags (Gaisser et al., 2024; Kuijpers et al., 2024; Rosenberg et al., 2018). Split-pool barcoding starts with fixation and permeabilization of cells, followed by the in situ polyadenylation, where necessary (in the case of prokaryotes), of mRNA. Afterwards, the polyadenylated mRNA is reverse transcribed to complementary DNA (cDNA) using barcoded oligo(dT) primers, allowing for microtiter well-wise barcode labelling of the cDNA. Then the samples are pooled, washed, and randomly redistributed in a fresh 96-well plate. There, a ligation-based well-wise tagging is performed, which is repeated two or more times. This equipment-wise low-cost solution was demonstrated to be successful for transcriptomics, yet, no similar protocol is available for single cell genomics that could e.g. employ in situ genome amplification, followed by e.g. tagmentation-facilitated combinatorial labelling. Although, e.g. LIANTI employs a similar protocol for in situ tagmentation and reverse transcription of fragmented DNA (Chen et al., 2017), allowing the theoretical implementation of poly-A based tagging as in the split-pool barcoding described above. Establishing methods as they are proposed here would probably dramatically lower the cost of single-cell genomics and their benchtop preparation.

## 4. Concluding remarks

Thus far, sequencing costs drop and smarter heuristics in implemented data analysis algorithms have rendered shotgun metagenomics a yardstick in environmental microbiome analysis. Despite its highly significant contribution, it tends to reach a plateau regarding the quantity and quality of information about relatively rare, yet, highly important microorganisms, and the entirety of the microbial genome. To this end, emerging methodologies described in this review have attempted to fill these gaps, with each answering a different question.

DNA enrichment has great potential at retrieving targeted and, relatively quick, but somewhat restricted, information from environmental DNA. SHS/probe-capture provides a special gene/genome-centric aspect of the metagenome, with its main aim being to answer the question about if that gene/genome we have in a database exists in an environmental sample, and which are its variants. DNA-SIP on the other hand, aims at uncovering the most interesting part of the microbiome with respect to an isotope that can be incorporated to microbial DNA under the tested conditions. Time, space, and microbial identity are

important parameters in complex environments and can, in theory, be manipulated to reveal, from individual actors to near complete trophic networks. EpicPCR has the unique capacity to provide in-depth taxonomic resolution of microbial functions that are shared by multiple members, validating intuitive answers about functional redundancies found in complex microbiomes, and exploring microbial genomic plasticity and dispersal of resistance traits to last line of defence antibiotics that fortified human civilization over the 80 or so years. Hi-C attempts to achieve a similar goal, largely in a database independent manner. Its protocol is more straightforward than that of epicPCR, yet, its payoffs highly depend on a carefully designed experimental setup.

Cell enrichment approaches on the other hand, being more thorough, offer answers to questions associated with the complete functional content of a genome. Their focus ranges from the single-cell level to the population level. Their main advantage over the rest methods (including shotgun metagenomics) is their capacity, in an ideal world, to obtain the complete genomic content of a cell or a population, capturing this way population natural variation among cells. Single-cell analysis is the ideal approach for retrieving clear-cut answers on natural variation, but its interrogation capacity is limited and, in most cases, costly. IMS and its variants requires prior knowledge derived from isolation (for proper antibody designing), yet it is a good and complementary approach in cases where enrichments of such populations are required. FISH-FACS and associated methods have been around for quite some time and have been optimised in several ways given the experience, yet, they require elaborate equipment, often incompatible with low/mid-budget laboratories. Nevertheless, they probably comprise the best solution to date for both single-cell and population level analysis when it comes to cell sorting.

The methods-list described in this document is probably not exhaustive, but covers most of the validated and promising approaches for the targeted analysis of metagenomes. All aforementioned methodologies have the capacity to fill contemporary Microbial Ecology knowledge voids. This, either according to thus far developed protocols, or through out-of-the-box amendments and improvements. This is also somewhat intrinsic to these approaches, since several of them comprise combinations of others. We therefore believe that this review is going to fuel brainstorming for experimental designs that will facilitate the unlocking of the rare and interesting secrets of less or more complex environmental microbiomes.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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