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When FLOW-FISH met FACS: Combining multiparametric, dynamic approaches for microbial single-cell research in the total environment

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Abstract:	In environmental microbiology, the ability to assess, in a high-throughput way, single-cells within microbial communities is key to understand the heterogeneity of the cell. Fluorescence in situ hybridization (FISH) uses fluorescently labeled oligonucleotide probes to detect, identify, and quantify single cells of specific taxonomic groups. The combination of Flow Cytometry (FLOW) with FISH (FLOW-FISH) enables high-throughput quantification of complex whole cell populations, which when associated with fluorescence-activated cell sorting (FACS) enables sorting of target microorganisms. These sorted cells may be investigated in many ways, for instance opening new avenues for cytomics at a single-cell scale. In this review, an overview of FISH and FLOW methodologies is provided, addressing conventional methods, signal amplification approaches, common fluorophores for cell physiology parameters evaluation, and model variation techniques as well. The coupling of FLOW-FISH-FACS is explored in the context of different downstream applications of sorted cells. Current and emerging applications in environmental microbiology to outline the interactions and processes of complex microbial communities within soil, water, animal microbiota, polymicrobial biofilms, and food samples, are described.
Response to Reviewers:	Co Editor-in-Chief Science of the Total Environment Lisboa, 20th of September 2021 Re: When FLOW-FISH met FACS: Combining multiparametric, dynamic approaches for microbial single-cell research in the total environment (Manuscript #: STOTEN-D-21-19205). Dear Editor, We are grateful for the constructive comments made by peers on this work and for the editorial opportunity to revise the manuscript. We carefully addressed reviewer 3 suggestions and made improvements to our original submission by including the comments/suggestions in the revised version of the manuscript. Below we provide a detailed outline of how this manuscript has been revised in light of referee comments. We do believe the current draft is complete and we hope you will now judge it acceptable for publication in Science of the Total Environment. Kind regards, Mónica V. Cunha (PhD), on behalf of all authors Responses to Reviewer comments: Reviewer #1: General comments:

	“The paper is an interesting review paper on new updates and upgrades of FISH, Flow cytometry and fluorescence-activated cell sorting for different environmental microbiological application. The paper is well organized and written. It should be accepted as it is.” Author’s response: We thank the reviewer for the extremely positive comments. Reviewer #3: General comments: “Authors present results of their review work on combining multiparametric, dynamic approaches for microbial single-cell research in the total environment. The manuscript is interesting but looks more like didactics materials than detailed critical review presenting also future developments in the area of topic. I believe Analytica Chimica Acta or Talanta would better fit aims and scope of this work.” Author’s response: We thank the reviewer for the constructive comments and critics. We have tried to build a manuscript that would be adequate for a wide readership, from beginners to advanced users of FISH, Flow cytometry and fluorescence-activated cell sorting in an environmental research area context. Following the referee comments, we developed in the revised version the last section regarding future perspectives, trying to emphasize the promising future applications of FLOW-FISH-FACS in the field of environmental microbiology and microbial ecology. Specific comments: “In GA I miss some relation to data treatment after detecting with flow cyt and FACS. Highlights are informative but too long.” Author’s response: The graphical abstract was changed with the addition of data treatment-related information and the Highlights were rephrased and shorten. “Line 18: to learn heterogeneity of cell? Or heterogeneity of microbial community?” Author’s response: The sentence was rephrased to clarify the meaning of heterogeneity. “Line 90: what is magnitude of being "extremely promising"? what about its cons?” Author’s response: The sentence was rephrased to clarify the disadvantages of FACS technology (line 90). “Line 99: use rRNA from this place in next parts of manuscript as it was already explained.” Author’s response: Change was made. “Line 116: it is one of measures providing specificity.” Author’s response: Change was made. “Line 144: what do You mean by higher costs? Cost of what?” Author’s response: The sentence was rephrased to clarify that PNA probes are more expensive than conventional ones. “Line 147: what parameters govern these obstacles? Please elaborate on that.” Author’s response: Quantum dots are bulky and have lower mobility than free nucleic acids, this leads to decreased efficiency when entering the cell, requiring improved cell permeabilization and longer incubation periods. This information was added to the manuscript to elaborate on this point (line 147). “Line 160: change bad to unsatisfactory” Author’s response: Change was made. “Lines 163-164: remove this question” Author’s response: The question was removed. “2.3 - remove this question as subtitle” Author’s response: The question was removed and the subtitle was rephrased. “Line 179: rephrase to avoid repeating "can"” Author’s response: The paragraph was rephrased.

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Dear Editor of STOTEN,

please find herewith the review article "**When FLOW-FISH met FACS: Combining multiparametric, dynamic approaches for microbial single-cell research in the total environment**".

The importance of single-cell analysis in microbial ecology and environmental microbiology studies and in the context of total environment is greatly increasing. Several methodologies have been used to study complex environmental matrices, with flow cytometry (FLOW) and fluorescence *in situ* hybridization (FISH) allowing the high-throughput assessment of microbial physiological and functional heterogeneity at a single-cell scale. In this manuscript, these unique methods are reviewed and explored in an application-based solution strategy. Moreover, the conjugation of FLOW and FISH methods is explored with the fluorescent-activated cell sorting (FACS) technology to highlight their added value on several downstream analyses, such as multi-omics, again on a single cell approach and opening new avenues in cytomics. We combine and cite work from more than 180 high-impact references that contribute to highlight the prodigious versatility and efficacy hallmark of FLOW and FISH methodologies applied to a wide diversity of environmental matrices, from extreme oligotrophic environments to microniches in polymicrobial biofilms. We begin by reviewing the fundamentals and methodological data on both FLOW and FISH methods, from conventional applications, passing by alternative technical improvements and technical coupling of both methodologies, and finishing up with the supporting consensus of their useful coupling with FACS for multi-omics downstream analysis. We then highlight the complex interplay between techniques, matrices, and ecological niche traits that contribute to the need for technical adaptation to the conventional methodologies that improve single-cell detection, identification, quantification, and sorting across a gradient of multiple spheres in the total environment. Finally, we identify the cornerstones of knowledge involving single microbial cell heterogeneity but also research gaps that need to be addressed, highlighting the overwhelming potential of FLOW-FISH-FACS combination in solving those gaps.

This work adds an unexplored perspective of accumulated knowledge on microbial single cell heterogeneity, approaching microbial ecology and environmental microbiology from several angles and reinforcing the complexity of these multiple environmental matrices.

We hereby declare that the manuscript content has not been published or submitted for publication elsewhere.

All enlisted authors have contributed significantly to this study and they all are in agreement with the content of the manuscript.

We believe that this original review meets high-quality research standards, deserving wide dissemination to the community that will be warranted by publication in STOTEN.

Yours sincerely,

Mónica V. Cunha (on behalf of all authors)

When FLOW-FISH met FACS: Combining multiparametric, dynamic approaches for microbial single-cell research in the total environment

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Co Editor-in-Chief

Science of the Total Environment

Lisboa, 20th of September 2021

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Abstract

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1. Introduction

Environmental microbiology is devoted to the fundamental understanding of complex microbial communities, from taxonomic composition to microbe-microbe interactions, to microbial processes in the environment, e.g. in the hydrosphere, lithosphere, or at the interconnection of multiple spheres. The understanding of the dynamic adaptation of microbial populations to environmental perturbations is also one of the key drivers of environmental microbiology. In these so-called environmental communities, most microorganisms are unculturable or fastidious growers (Stewart, 2012). While in the past bacterial populations from a given taxonomic group within these communities were viewed as clonal and metabolically homogeneous, the development of new methodologies brought insights into the mechanisms leading to dynamic cellular adaptation, metabolic switches, and introduced the notion of phenotypic and genotypic heterogeneity (Delvigne et al., 2018; Lemoine et al., 2017). The development of single-cell techniques has thus enabled the focus on microbial individuality (i.e. individual cell physiology), while enabling consequence inferences at the population level, with widespread application to different contexts (Delvigne et al., 2018; Lemoine et al., 2017). The notion of viability based on the ability to culture microbial cells also became an archaic concept (Hammes et al., 2011), since cultivability not only depends on the physiological state of a microbial cell but also the growth conditions (Hammes et al., 2011). The accurate assessment of microbial viability is important due to the ecological relevance of viable microorganisms, which actively change their environment (Davis, 2014).

As such, the application of methodologies that are capable to capture heterogeneity and, more accurately, assess the physiological status of individual cells within a population are crucial to better understand the taxonomic, metabolic, and functional compositions of microbial communities, their development processes, and their ecological roles (Koch et al., 2014a). Methodologies that capture the hallmark of microbial populations on a cellular level, by means

of a single-cell approach, provide the basis for reconstructing the complexity of microbial communities in different niches, from the industrial to the host-associated, to the most extreme environments (Koch et al., 2014a).

Most studies focusing on environmental communities rely on culture-independent techniques, such as PCR, microarrays, amplicon-based and shotgun sequencing, fluorescence *in situ* hybridization (FISH), and flow cytometry (FLOW). The amplification-based approach has been extensively used to study microorganisms and their genes in complex matrices (Liu et al., 2011). These approaches have several drawbacks since the detection of nucleic acids in the sample cannot be associated with cell viability and do not allow tracing back to the original cell for further analysis (Ju et al., 2016; Liu et al., 2011). Also, the information given by these methods is mostly qualitative based on the presence/absence of taxonomical markers and/or relative abundances of operational taxonomic units.

FISH was introduced 30 years ago as a very valuable molecular diagnostic tool to detect specific DNA or RNA sequences within intact cells through the use of complementary DNA- or RNA-probes labeled with fluorescent dyes (DeLong et al., 1989). FISH has since then developed as a robust technique for the detection, identification, and quantification of single cells of target microorganisms within complex matrices, with widespread applications, namely in environmental microbiology. FISH quantification accuracy is superior to several other approaches, such as the most probable number (MPN), immunological-based methods, and DNA amplification-based methods (Baptista et al., 2014). Besides, FISH is cost-efficient and easy to perform. However, it is time-consuming, with low throughput, and requires the use of expensive microscopes to avoid problems of background fluorescence and resolution (Baptista et al., 2014).

Flow cytometry can overcome several FISH drawbacks, being highly accurate and possessing high-speed single-cell processing capacity (Emerson et al., 2017). FLOW analysis is based on the

detection of light emitted by a laser. Different detectors collect the signal at specific wavelengths after light incidence over individual cells that travel in a fluid, converting the analog signal into a digital one – for an extensive review see (Givan, 2011; Shapiro, 2000). Additionally, FLOW coupled with fluorescence-activated cell sorting (FACS) enables cell sorting of a subpopulation of interest or single-cell sorting (Emerson et al., 2017). The combination of FISH, FLOW and FACS is very powerful and ~~extremely~~ promising to study complex communities from different environmental matrices. However, among FACS ~~possesses has~~ major disadvantages is the need for at least 10^5 initial cells, failure~~ing~~ to isolate single-cells from a poorly~~low quantity~~represented community (in numbers), and the ~~possible~~potential damage ~~of~~to ~~some~~ more fragile cells during ~~the~~ sorting due to high-speed technology (Emerson et al., 2017). Below, we provide an overview of FISH, FLOW, and FACS methodologies, addressing conventional protocols, model variation techniques, signal amplification approaches, and fluorophore choices for cell physiology evaluation, always trying to direct the reader for an application-based solution strategy.

2. Fluorescence *in situ* hybridization (FISH)

2.1. Conventional FISH – principles of an established technique

Bacterial identification by FISH usually explores the use of oligonucleotide/polynucleotide probes targeting ribosomal RNA (rRNA) because of the natural production of rRNA molecules in all cells (Almeida et al., 2013). The r~~Ribosomal~~ rRNA genes have been used as standard phylogenetic markers in microbial taxonomic studies since they are ubiquitously distributed across all archaea and bacteria have an evolutionarily conserved nature and a wide range of variable, discriminatory regions (Almeida et al., 2013; Moter and Gobel, 2000).

A standard FISH protocol targeting the typical rRNA region involves four different steps: fixation/permeabilization, hybridization, washing, and visualization/detection – for an extensive review on conventional FISH, see (Moter and Gobel, 2000).

Briefly, the first step is crucial since it must preserve rRNA integrity, cell shape, and prevent lysis, but at the same time must permeabilize the cell allowing probe diffusion (Moter and Gobel, 2000). Fixation is normally accomplished using paraformaldehyde or formaldehyde for Gram-negative bacteria and ethanol for Gram-positive bacteria (Moter and Gobel, 2000). Permeabilization is achieved with lysozyme, proteases, solvents, detergents, and/or organic acids, depending on the microorganism, leading to physical damage of the cell envelope due to the formation of pores wherein the probe can penetrate the cell (Rocha et al., 2018). The hybridization step can be influenced by the pH, ionic strength, and formamide concentration of the hybridization solution, or the hybridization time and temperature (Nettmann et al., 2013). The washing step ensures that all loosely bound or unbound labeled probes are removed from the sample, hence it is one of the measures providing specificity to the detection process (Nettmann et al., 2013). FISH is commonly performed in two formats: with physical cell support, on a microscope slide or filter membrane, followed by visualization using conventional epifluorescence or confocal microscopy; or with cells in suspension, followed by FLOW detection and, eventually, FACS (Amann and Fuchs, 2008).

2.2. How to bait your FISH

A FISH probe catalog of previously designed and used probes is openly available in probeBase (Greuter et al., 2016). For *de novo* probe design, a variety of approaches can be used through different software (Wright et al., 2014), with mathFISH being one of the most used (Yilmaz et al., 2011). Each *de novo* synthesized probe needs to be extensively optimized through an experimental approach using mocked mixed communities to ensure specificity and sensibility of taxonomical identification within complex microbial communities. Complementary, Clone-FISH was developed to investigate the accessibility of the selected target site and evaluate optimal hybridization conditions for probes when no pure cultures are available (Schramm et al., 2002).

The application of this technique potentiates a higher fluorescence signal-to-noise ratio (Kubota et al., 2006).

There are different types of FISH probes (Fig. 1), ranging from a small number of nucleotides, with < 50 nt (oligonucleotides), to enormous probes with almost 1 kb (polynucleotides); from conventionally made probes derived from PCR protocols, such as DNA probes for gene detection, or RNA probes for gene expression analysis, to DNA mimics such as locked nucleic acids (LNA) and peptide nucleic acids (PNA). Polynucleotide probes have higher signal intensities than oligonucleotide probes, being useful for detecting microbes with low ribosome content or aimed at single genes (Trebesius et al., 1994). However, these probes show a reduced hybridization efficiency due to difficulties in penetrating the cell wall (Trebesius et al., 1994). LNA probes have increased affinity towards the target, but high levels of self-annealing, since they possess high thermal stability (Cerqueira et al., 2008). PNA probes are hydrophobic, allowing easy penetration into the cell cytosol and an improved diffusion through bacterial colonies or biofilm structures (Almeida et al., 2013; Lopes et al., 2018)~~.~~, H~~h~~owever, ~~they~~ synthesis of PNA probes ~~are~~is more expensive compared with traditional ones~~have higher costs~~. A recent protocol using quantum dots (QD) as FISH probes was also developed (Liu et al., 2018a). QD allow the resolution of a high number of working wavelengths since they possess narrow emission bands with minimal spectral overlap (Liu et al., 2018a). However, QD are bulky and have lower mobility than free nucleic acids, bringing some obstacles to FISH-based methodologies (Liu et al., 2018a); ~~, this is,~~ QD have decreased efficiency in entering the cell, need~~ing increase~~ improved cell permeabilization and longer incubation periods.

In conventional FISH approaches, probes are directly labeled (Fig. 1a,b) in one of the extremities (5'-end or 3'-end) with a variety of fluorophores with different excitation and emission wavelengths, which can be selected based on the matrix and/or cell autofluorescence signals

and also circumvent technical limitations of the equipment used to visualize the fluorescence signal (Bottari et al., 2006).

Standard FISH suffers from several limitations that may prevent the successful detection of the target microorganisms (Moter and Gobel, 2000). Among probe design and evaluation, problems can occur related to the limited accessibility of probe to the target site, polymicrobial cultures, and difficulties to reach optimal hybridization conditions; regarding detection, the complexity of environmental samples can result in low concentration of target cells or these might not be detected due to low ribosome content, low copy gene, or lack of permeabilization, with additional ~~bad~~ unsatisfactory signal-to-noise ratio; cells within biofilms and cell aggregates can be difficult to count and always require manual counting; finally, the analysis of general metabolic activities and specific functions can be hampered due to the lack of correlation between ribosome content and cells' metabolic activity and physiology (Moter and Gobel, 2000). ~~So how can we overcome these limitations?~~ In the next sections, several improvements to the conventional FISH methodology are proposed to transcend specific limitations ranging from low signal detection, multiple targets, analysis of specific ecological niches or biotic relations, multiparametric assessment of taxonomical and metabolic profiles, and sub-cellular resolution (Table 1).

2.3. ~~Low signal detection?~~ Amplification approaches ~~as solutions~~ to circumvent low signal detection

Conventional FISH has a major limitation: the low signal detection of the cell due to low target accessibility or low target content. Accessibility of probes to target sites can be improved by different methods, however, all of them have specific limitations. For example, the use of unlabeled helper probes (Fuchs et al., 2000) ~~can~~ may improve accessibility but their design is often impossible for probes with broader specificities; the extension of the hybridization time usually resolves the problem, still, this may lead to unspecific probe binding or unspecific dye-

182 binding in complex samples (Yilmaz et al., 2006); the elongation of the probes is also a valid
183 solution, yet it is usually coupled with a specificity decrease (Yilmaz et al., 2006); PNA probes
184 can easily enter cells but are very expensive and are not directly ~~cannot be simply~~ converted
185 from published oligonucleotide probes without a change in specificity (Cerqueira et al., 2008).

186 Indirect probe labeling can help in signal amplification and can be accomplished using: (i) a
187 reporter molecule like digoxygenin (DIG), biotin, or dinitrophenol, that is then detected by a
188 higher intensity fluorescent antibody (Fig. 1c); (ii) horseradish peroxidase (HRP) that uses
189 fluorophore–tyramide as a substrate for enzymatic signal amplification, in a process named
190 tyramide signal amplification (TSA) (Fig. 1d); (iii) the combination of TSA systems with
191 polynucleotide probes internally labeled with a reporter molecule (Bottari et al., 2006) (Fig. 1e).

192 Catalyzed Reporter Deposition (CARD)-FISH is an example of an enzymatic signal amplification
193 technique that has been introduced as an *in situ* signal amplification method based on HRP-
194 labeled oligonucleotide probes and TSA (Hoshino et al., 2008) (Fig. 2a). The resulting
195 fluorescence signal is 26 to 41 times higher than with mono-labeled FISH, making the
196 visualization of hard-to-detect cells possible (Hoshino et al., 2008). Thus, this method has been
197 established for detecting microorganisms in oligotrophic habitats, such as seawater (Kleindienst
198 et al., 2015; McNichol et al., 2018), marine sediments (Hoshino et al., 2017; Pohlner et al., 2017),
199 freshwater (Alfreider et al., 2018; Crognale et al., 2017), permafrost (Winkel et al., 2018), and
200 soil (Cai et al., 2016; Probandt et al., 2017; Schmidt and Eickhorst, 2014). Also, this methodology
201 can be used in microbiota-host interaction studies (Golyshina et al., 2017; Schmidt et al., 2018).
202 However, CARD-FISH is rather expensive, requires enzymatic pretreatment to allow the large
203 HRP-labeled probes to penetrate the target cells, requires inactivation of endogenous
204 peroxidases, and causes a dramatic alteration in the melting behavior of the probes (Pernthaler
205 and Pernthaler, 2007). Furthermore, the use of CARD-FISH to multicolor imaging, targeting

different groups in a single sample, is very time-consuming, because sequential hybridizations for each target need to be made (Pernthaler and Pernthaler, 2007).

The two-pass CARD-FISH protocol is an improvement of the conventional CARD-FISH protocol. This newly improved protocol relies on a sequential catalyzed reporter deposition based on the binding of HRP-conjugated anti-fluorescein antibodies to the fluorophores deposited during the initial CARD-FISH step, followed by a second catalyzed reporter deposition signal amplification with fluorescein-labeled tyramides (Kubota et al., 2006; van de Corput et al., 1998) (Fig. 2b). This protocol was first developed to study eukaryotic cells (van de Corput et al., 1998) and adapted to prokaryotic cells by Kubota and colleagues (2006) (Kubota et al., 2006). Since then, this protocol has been applied to study prokaryotic cells in single-gene detection approaches in anaerobic sludge samples to detect methanogens and sulfate-reducing bacteria (Kawakami et al., 2012) and in freshwater samples to detect ultramicrobacteria (Neuenschwander et al., 2015). Two-pass CARD-FISH increased the detection efficiency up to 98% via the increasing of the length of the probe (up to 820 nt) and adding a second CARD step (Kawakami et al., 2012). The main drawback of the two-way CARD-FISH protocol is its higher cost and more time-consuming protocol when compared with CARD-FISH; also the probe length extension may hamper the efficient probe penetration into the cell.

An additional enzymatic-based method makes use of a nucleotide probe that consists of single-stranded RNA generated via *in vitro* transcription (Zwirglmaier et al., 2005; Zwirglmaier et al., 2004). During transcription, the probe is labeled with a reporter molecule that has a labeling density of about one labeled nucleotide every 10–20 nt (Pratscher et al., 2009). The probe then anchors other probes, which in turn form a network around the cell periphery, resulting in a halo-shaped fluorescent signal (Pratscher et al., 2009). This phenomenon occurs due to the folding of the single strand of RNA probe into secondary structures, forming a complex network in the cell during hybridization (Pratscher et al., 2009) (Fig. 2c). This network concept allows the

number of probe molecules contributing to the signal to greatly exceed the number of target molecules and, thus, to amplify the signal intensity (Zwirglmaier et al., 2004). This method is named Recognition of Individual Genes (RING)-FISH and it increases signal amplification between 10- and 50-fold when compared to conventional FISH (Pratscher et al., 2009; Zwirglmaier et al., 2005; Zwirglmaier et al., 2004). RING-FISH is typically used to study the phylogenetic affiliation of members of denitrifier communities in enriched activated sludge (Pratscher et al., 2009), the composition of archaeal and bacterial communities in marine plankton samples (Pernthaler et al., 2002), and the distribution of secretion systems in *Vibrio parahaemolyticus* from water, oyster, and sediment samples (Noriea et al., 2010). However, this technique has some limitations, such as: probe penetration due to the preferential use of polynucleotide probes; increased cost, due to the high amounts of probe used; and low specificity, since this method generates a halo-shaped signal (Zwirglmaier et al., 2005).

Besides probe systems modifications, target amplification can be performed by PCR-based technologies or by isothermal amplification-based technologies. Among these technologies, a recent approach was developed to overcome the identification of microorganisms in environmental samples that contain low rRNA content, when targeting low copy genes, or probes that have low permeability (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). This method relies on *in situ* DNA-hybridization chain reaction (HCR) (Fig. 2d). HCR is based on two different fluorescently labeled hairpins probes and an initiator nucleotide comprising the complementary sequence of the target gene and the complementary sequence to one of the hairpin probes (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). These hairpins probes are stable in the absence of the initiator. In its presence, the initiator hybridizes with the portion of the complementary hairpin probe, leaving the remaining single-strand portion of the first hairpin probe to hybridize with a portion of the second hairpin probe (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). This

hybridization leads to the formation of a single strand part of the second hairpin probe similar to the initiator, promoting the hybridization of the first hairpin probe (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). This cascade of polymerization reactions of the fluorescently labeled hairpin probes linearly increases the fluorescent signal intensity (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). This method showed an up to 8-fold higher sensitivity than conventional FISH, being a suitable alternative to CARD-FISH, particularly for cases when strong cell permeabilization or CARD reactions should be avoided. This methodology was used in gene expression studies involving symbiotic partners (Nikolakakis et al., 2015). More recently, HCR-FISH was optimized to be used in the detection of anaerobic methanotrophic archaea in marine sediments (Jia et al., 2021).

2.4. Multiple probes for multiple targets

FISH offers the possibility to detect/identify more than one target in one single experiment. This possesses great interest in multiple taxonomic identifications, concomitant taxonomical and functional identification, or intracellular relationships between guest and host cells.

Double labeling of oligonucleotide probes (DOPE)-FISH (Pernthaler et al., 2002; Stoecker et al., 2010) offers a doubled signal intensity. This technique offers more options for the design of specific probes: probes may be labeled at the 5' and 3' ends with the same fluorophores or two different ones (Fig. 2e). These options not only enable signal amplification when the same fluorophore is used but also enable multicolor probe combinations that can target up to six organisms in a single FISH experiment (Behnam et al., 2012; Schimak et al., 2016). Recently, DOPE-FISH has been applied in different contexts, such as the evaluation of bacterial endophytes composition (Glassner et al., 2015), assessment of changes in plant microbiomes (Mitter et al., 2017), the discovery of iron-oxidizing biofilms in stromatolitic iron-rich structures (Heim et al., 2017), examination of active microbial biofilms composition in subsurface rocks (Escudero et al., 2018), assistance in the isolation process of "*Candidatus*" species (Beam et al., 2015; Lehtovirta-

Morley et al., 2016), detection of novel extracellular gut symbionts (Kroer et al., 2016), identification of primary fermenters in anaerobic digesters of sludge (McIlroy et al., 2017), and investigation of the structure and abundance of methanogenic archaea and methane-oxidizing bacteria in peat bog lakes (Lew and Glińska-Lewczuk, 2018). A drawback of DOPE-FISH is the limited number of taxa that can be labeled simultaneously in one sample, due to the highly overlapping excitation and emission spectra of fluorophores (Valm et al., 2012; Valm et al., 2011).

To increase the number of microorganisms that can be concomitantly targeted in polymicrobial samples, Combinatorial LAbeling and Spectral Imaging FISH (CLASI-FISH) can be employed (Valm et al., 2012; Valm et al., 2011). This approach is based on the premise that a unique spectral tag is allocated to each target group of microorganisms by conjugation of mono-labeled probes carrying one fluorophore each to the same binding site (Fig. 2f). The combination of emitted wavelengths is revealed by linear unmixing and spectral imaging with modern confocal laser scanning microscopy (CLSM). However, the expected 50:50 ratio of probes can be switched to one side due to the difference of affinity between probes, further complicating the identification of target organisms (Behnam et al., 2012; Schimak et al., 2016). Behnam and colleagues (2012) proposed the combination of DOPE-FISH with spectral unmixing (such as in CLASI-FISH), enabling the use of oligonucleotide probes labeled with different binary combinations of dyes to increase the potential of multicolor FISH for the analyses of samples with elevated levels of background fluorescence (Behnam et al., 2012).

Welch and colleagues (2016) studied the human oral microbiota employing high-throughput sequencing data to identify the major bacterial taxa in the supragingival plaque, followed by CLASI-FISH for the direct visualization of their spatial structure, which allowed the description of a complex, spatially organized, multi-genera consortium (Mark Welch et al., 2016). This workflow by Welch and colleagues (2016) might be considered as a case study in microbial

biogeography at the micron scale (Mark Welch et al., 2016). Following this study, the same approach was used to study the spatial organization in gnotobiotic mice's gut of a defined 15-member taxa community present in the human gut (Mark Welch et al., 2017).

Schimak and colleagues (2016) demonstrated the applicability of multi-labeled oligonucleotide probes synthesized by a "click" reaction on complex microbial populations (Schimak et al., 2016). This procedure named MiL-FISH allows the simultaneous targeting of up to seven microbial groups using a multi-labeling oligonucleotide probe strategy that can improve amplification signal, when conjugated with acrylic resin for precise localization of individual microbial cells, or sort unfixed environmental microorganisms (Schimak et al., 2016). This methodology was used to study the small bacterial community of gutless oligochaete *Olavius algarvensis* (Schimak et al., 2016). This new type of probe can also be used in CLASI-FISH with a single site targeted by a four-times-labeled probe, instead of using four mono-labeled probes targeting four sites on the 16S rRNA, thus resulting in increased specificity and sensitivity (Schimak et al., 2016). To improve signal amplification in low rRNA content targets, four-times-labeled probes carrying the same fluorophore combination can be applied to several sites on the 16S rRNA molecule (Schimak et al., 2016).

2.5. Taxonomy, function, and metabolism – a multi-parametric analysis of microbial communities

Marking multiple cells in the same sample can be accomplished as previously stated, however, targeting multiple genes in the same cell is also possible. One of the first methods applying this principle was GeneFISH. It consists of the detection of two different targets: first, the gene of interest (normally, a functional gene) and then the rRNA of the microorganism under study (Moraru et al., 2010). The first step uses double-stranded DNA probes labeled with DIG molecules, in a CARD-FISH methodology. The rRNA detection for taxonomical identification is accomplished by CARD-FISH using HRP-labelled oligonucleotide probes. GeneFISH was

successfully used in microbial identification in a diversity of environments, including marine sediment enrichments (Lenk et al., 2012), marine seep sediments (Stagars et al., 2016), marine bacterial - eukaryotic symbiotic systems (Bernhard et al., 2012; Petersen et al., 2011), upwelling seawater samples for the study of planktonic microbial communities (Moraru et al., 2010), and groundwater samples (Matturro and Rossetti, 2015).

Targeting two genes in the same cell is not the only way to assess both taxonomical and functional information about environmental microorganisms. Isotopic-based techniques can also be used, namely microautoradiography (MAR)-FISH. This method is a microscopic technique that allows simultaneous visualization of the target microorganism and specific activity at the single-cell level, due to the uptake of radioactively-labeled substrates (for a review see (Wagner et al., 2006)). However, several limitations are reported (Wagner, 2009): the impossibility to detect several labelling isotopes simultaneously and low single-cell resolution in dense microbial aggregates; the risk of radiation exposure; inexistence of isotopes with adequate half-life time for some important elements (e.g. nitrogen and oxygen), impairing the application of this technique to study nitrogen fixation, for example; the quantification of incorporated isotope requires an internal standard of bacteria with known isotopic composition; time-consuming process; and impaired downstream analysis due to cell fixation. Besides all these limitations, this technique is still used nowadays for water samples (McIlroy et al., 2016; Teira et al., 2017). Additionally, MAR-FISH can be coupled with a signal amplification methodology, CARD-FISH, applied to mRNA or single genes as the target or in the context of oligotrophic environments. MAR-CARD-FISH was used to calculate a specialization index regarding bacterioplankton ability to use phytoplankton-derived dissolved organic carbon from different phytoplankton species and at different concentrations (Sarmento et al., 2016); to identify which microbial groups are involved in dark dissolved inorganic carbon uptake in the Atlantic Ocean (Guerrero-Feijóo et al., 2018); to quantify the biomass production of specific taxonomical bacterial groups present in ocean samples between subtropical and Antarctica locations (Bakenhus et al., 2018); and to

358 characterize the incorporation ability of bicarbonate carbon sources by ammonia-oxidizing
359 archaea "*Candidatus* Nitrosocosmicus exaquare" recovered from municipality wastewater
360 treatment plants (Sauder et al., 2017).

361 Besides MAR-FISH, stable-isotope probing (SIP) also relies on isotopically labeled cells. SIP
362 method is based on the incorporation of an isotope with low natural abundance into
363 components of the biomass of microbial cells using an isotopically enriched substrate
364 (Radajewski et al., 2000). When associated with FISH, this method allows linking the diversity,
365 abundance, and function of a given single-microbial cell. SIP-FISH can be used in the study of
366 complex biogeochemical cycles. This technique was used to identify active methanol-utilizing
367 microorganisms from soil samples (linked to carbon cycle) (Radajewski et al., 2000); to identify
368 active denitrifiers in full-scale nutrient removal wastewater treatment plants (nitrogen cycle)
369 (McIlroy et al., 2016); and to identify active sulfate-reducing bacteria at marine seeps (sulfur
370 cycle) (Kleindienst et al., 2014); as well as in the identification and characterization of propane-
371 and butane-degrading communities from marine hydrocarbon cold seeps (Jaekel et al., 2012).

372 A non-isotopic dependent technique can also be applied as an alternative to study microbial
373 ecophysiology, relying instead on the active cell-labeling with chemical modification of
374 biomolecule analogs (Hatzenpichler et al., 2014). This synthetic metabolites-based approach
375 named biorthogonal noncanonical amino acid tagging (BONCAT) has been coupled with FISH
376 methods to study archaea and bacteria within marine seawater and sediments (Hatzenpichler
377 et al., 2016; Hatzenpichler et al., 2014; Leizeaga et al., 2017). After the uptake of the
378 biorthogonal synthetic amino acid, the aminoacyl tRNA synthetase incorporates this amino acid
379 into *de novo* peptides (Hatzenpichler et al., 2016) (Fig. 2g). The protein-active cells can be
380 visualized using a reaction that conjugates a modified fluorescence dye to a chemical reporter
381 group of the amino acid (Hatzenpichler et al., 2016).

382 **2.6. Parallel detection of virus and host cells**

The study of specific ecological niches requires specific assessment techniques, specially designed or adapted to those scenarios. One of those ecological niches is guest-host interactions, both pathogenic, parasitic, or symbiotic. The unique interplay between virus and their hosts is one example of such interaction. Allers and colleagues (2013) introduced a method based on GeneFISH for targeting bacteriophages (phageFISH), in the host-context (Allers et al., 2013; Dang et al., 2015). This new methodology enables the detection of phage genes in free virus particles, with a sensitivity of about 100%; in addition, it enables the reliable simultaneous visualization of phage genes and host rRNA inside infected cells, as well as the relative quantification of the intracellular phage DNA signal (Allers et al., 2013). The phageFISH technique holds great potential for the development of viral ecology in the context of environmental samples with complex matrices. However, the necessity of prior knowledge of phage sequence variation for probe design is a disadvantage. More recently, Castillo et al. (2020) developed a similar method, named VirusFISH that allows the visualization and quantification of virus-infected cells during an infection cycle under experimental conditions (Castillo et al., 2020). Besides the application *in vitro*, the potential transposition of this approach to polymicrobial communities is enormous, for instance in the context of research focusing on pathogenic and symbiotic relationships naturally occurring in environmental settings, not only in virus-host interactions but also in the study of predatory and symbiotic relationships between bacteria and protozoa.

2.7. Plant leaves matrices and their autofluorescence – how to overcome this limitation?

Some matrices can be particularly hard to work with, especially due to high autofluorescence. It is the case of leaves and epiphytic microbial communities. This problem can be overcome using TAPE-FISH, a FISH protocol that relies on the transference of surface microorganisms to an adhesive tape (Bisha and Brehm-Stecher, 2009; Remus-Emsermann et al., 2014). After the transfer, the recovered microorganisms are dehydrated and hybridized directly on adhesive

tape, allowing the analysis of spatial arrangement patterns of both adaxial and abaxial phylloplanes (Bisha and Brehm-Stecher, 2009; Remus-Emsermann et al., 2014). A recent study explored this method (Leaf-FISH), developing a robust and easily transferable protocol, based on ethanol-mediated pigment removal and CLASI-FISH, to create a tridimensional image of the leaf enabling the visualization of multispecies microbiota that can directly be associated with the leaf microstructure (Peredo and Simmons, 2018). This methodology has the potential to disclose microbial preferences for microhabitats, colonization strategies, microbial interactions, and plant/host interactions with particular emphasis to leaf microbiome (Peredo and Simmons, 2018), but also to any spatial microbiome study in a variety of biological and substrate surfaces.

2.8. How to study cell-cell and cell-substrate interactions in biofilms and cell aggregates?

One of the major limitations of FISH technologies is the difficulty associated with studying intact biofilms and cell aggregates since the discrimination of fluorescence signals from overlapping cells through more conventional image acquisition systems is hampered. Atomic force microscopy (AFM) enables the quantification of the interactions between microorganisms and surfaces. It is used, in particular, in studies focusing on surface colonization and biofilm formation. AFM can be combined with FISH, providing a detailed analysis of interactions between microorganisms and different surfaces, such as sludge digesters matrices (Hao et al., 2018), and the formation and development processes of microbial biofilms (Bao et al., 2018).

Besides microscopic imaging improvements, other methodologies can be applied to better understand natural polymicrobial biofilms. Pernthaler team (2008) developed a new FISH method named magneto-FISH to enrich and characterize microbial community associations in environmental samples (Pernthaler et al., 2008). Magneto-FISH combines an in-solution CARD-FISH approach using 16S rRNA oligonucleotide probes with the immunomagnetic sediment matrix separation mechanism that captures hybridized cells. Paramagnetic beads coated with an antibody targeting the fluorophore applied in the CARD-FISH procedure are used for matrix

433 separation (Pernthaler et al., 2008). This method enables the capture and assessment of whole
434 microorganisms and cell aggregates from complex environments, without the loss of existent
435 interspecies associations and corresponding metabolic properties (Pernthaler et al., 2008).

436 In a study by Escudero *et al.* (2018), several FISH signal enhancement methods, such as CARD-
437 FISH, DOPE-FISH, and MiL-FISH, were used to improve signal detection and increase the number
438 of different taxa that could be detected simultaneously (Escudero et al., 2018). Additionally, a
439 Gene-FISH hybridization buffer was used to decrease background noise due to the presence of
440 salmon sperm DNA or yeast RNA that act as extra blocking reagents (Escudero et al., 2018).
441 Moreover, a fluorescence lectin-binding assay (FLBA) was also performed based on fluorophore-
442 labeled lectins in combination with other specific stains for DNA, proteins, and lipids (Escudero
443 et al., 2018). This polyphasic approach was used to study biofilm formation, cell aggregates, and
444 cell attachment to the substrate in deep poor porous continental subsurface rocks (Escudero et
445 al., 2018).

446 **2.9. More than a single cell – a higher resolution at a sub-cellular level**

447 In the last decade, several improvements were made towards increased resolution, not only
448 concerning single-cell visualization but also the sub-cellular visualization of important microbial
449 sub-compartments. These efforts are driven by the increased interest in microbial metabolites
450 in ecological microbiology, and the sub-cellular precision needed to evaluate their localization
451 and quantification *in situ* (Kaltenpoth et al., 2015). To accomplish this, both mass-spectrometric
452 imaging (MSI) and super-resolution microscopy (SRM) techniques were shown to be excellent
453 tools. Matrix-assisted laser desorption ionization time-of-flight MSI (MALDI-TOF/MSI) was used
454 coupled with FISH to concomitantly monitor antibiotic production and taxonomic identification
455 in the defensive symbiosis between beewolf wasps and *"Candidatus* Streptomyces philanthi"
456 (Kaltenpoth et al., 2015). The combination of both methodologies was used to identify active
457 microbes in the mouse fecal microbiota (Berry et al., 2015). In this study, the incorporation of

heavy water (D_2O) was analyzed by Raman spectroscopy (an MSI technique) to detect incorporation into microbial biomass, together with nanoscale-resolution secondary ion mass spectrometry (NanoSIMS; SRM technique) to obtain the labeling pattern of D_2O incorporation (Berry et al., 2015). The detection of D_2O incorporation can additionally be coupled with FISH to identify metabolically active microbes (Berry et al., 2015). Moreover, the combination of epifluorescence microscopy with scanning electron microscopy (SEM) was developed to allow the evaluation of CARD-FISH on a cellular level, followed by a subcellular level evaluation of probe-target location, significantly increasing resolution (Schmidt et al., 2012). This new method named Gold-FISH is enabled by the high-affinity binding of streptavidin-Alexa Fluor 488 fluorophore/nanogold conjugates to biotin, in the last CARD-FISH step, followed by autometallographic enhancement of nanogold particles, before scanning electronic microscopy (SEM) detection (Schmidt et al., 2012). The applicability of this technique was optimized in rice roots and marine sediment samples (Schmidt et al., 2012).

3. Flow Cytometry (FLOW)

3.1. Principles and workflow of a rising technique

In the environment, bacteria tend to grow embedded in microcolonies or biofilms, adopting a community lifestyle. For single-cell analyses using flow cytometry, sessile cells must be put in suspension, requiring the use of efficient detachment methodologies for cell-substrate and cell-cell dissociation (Müller and Nebe-von-Caron, 2010). Cell detachment can be accomplished using chemical and/or physical processes. Different classes of chemical reagents can be used for cell detachment: non-ionic surfactants (e.g. Tween®) (Amalfitano and Fazi, 2008; Lavergne et al., 2014; Vignola et al., 2018); salts (e.g. trisodium citrate) (Hickey et al., 2018; Vignola et al., 2018); redox agents (e.g. dithiothreitol) (Ben-Amor et al., 2005); or ionic dispersants (e.g. sodium pyrophosphate) (Amalfitano and Fazi, 2008; Nettmann et al., 2013). These chemical reagents usually work by weakening intermolecular forces that attach the cell to the substrate (Müller

and Nebe-von-Caron, 2010). Physical detaching can be more often accomplished by vortex (Bressan et al., 2015), sonication (Amalfitano and Fazi, 2008), or ultrasounds (Frossard et al., 2016). These physical methods release bacteria that are entrapped in micropores or channels (Müller and Nebe-von-Caron, 2010).

After detachment, cells and environmental matrices coexist in solution and need to be separated. To do this, density centrifugation (Amalfitano and Fazi, 2008; Frossard et al., 2016), or simply low-speed centrifugation (150 x *g*) (Bressan et al., 2015), can help deposit large and/or dense particles. To remove smaller particles, filtration with different pore size filters can be applied (Albright and Martiny, 2017; Plominsky et al., 2018).

In many studies, FLOW analysis is performed with fixed cells. The fixation is important to maintain cell stability and morphology characteristics (Müller and Nebe-von-Caron, 2010). Several types of fixating agents can be used, namely formaldehyde (van Gelder et al., 2018), paraformaldehyde (Takahashi et al., 2015), and glutaraldehyde (Vignola et al., 2018). However, fixating agents can cause cell agglutination and increased autofluorescence, obscuring FLOW analysis (Müller and Nebe-von-Caron, 2010). Agglutination is due to cells' production of exopolysaccharides in response to stress led by the fixation agent (Müller and Nebe-von-Caron, 2010). Nevertheless, fixation allows cell conservation at -20˚C for several months. When the final goal of the FLOW analysis is cell sorting and further cell utilization, fixation of cells is highly unrecommended due to loss of cultivability and difficulty in DNA sequencing arising from covalent cross-links between DNA molecules (Müller and Nebe-von-Caron, 2010).

In flow cytometry, microbial cells can be directly analysed and/or stained with fluorophores (Fig. 3). The direct analysis allows the evaluation of two main cell characteristics: cell size and cell complexity. Cell size can be assessed through the fraction of scattered light collected in the same direction as the incident light (named Forward Scatter, FSC), which is as intense as the cell size (Léonard et al., 2016). Cell complexity can be assessed through the fraction scattered of light

508 collected orthogonally to the incident light (named Side Scatter, SSC), which is related to
509 morphological characteristics, such as cell surface roughness, cell membrane, nucleus, internal
510 granular material, and organelles (Léonard et al., 2016). Besides cell size and complexity, direct
511 analysis can also be performed on autofluorescence cells, such as chlorophyll-containing ones.

512 The use of fluorophores enables multiparametric cell morpho-physiology assessment
513 approaches, including membrane integrity, intake activity, membrane potential, pH gradient,
514 metabolic activity, lipid content, and Gram character (Table 2; Fig. 4).

515 Staining bacteria is a complex process due to a variety of parameters that can influence this
516 process, namely dye chemistry, target organism, and staining conditions (mostly time and
517 temperature) (Buysschaert et al., 2016). To minimize the difficulty of the process, several steps
518 need to be optimized and standardised, namely dye concentration, the buffer used to resuspend
519 cells, time and temperature of dye incubation, and utilization (or not) of different types of
520 fixation and permeabilization methodologies (Buysschaert et al., 2016).

521 The population's physiological status using a single fluorophore is sometimes difficult to assess,
522 due to complex relations between physiological parameters, for example, membrane integrity,
523 metabolic activity, membrane potential, and stress response. The absence of membrane
524 potential could be related to the loss of membrane integrity or instead be related to the lack of
525 metabolic ability to maintain that potential. For this reason, a multi-parameter flow cytometric
526 analysis using different fluorophores simultaneously is normally performed. This approach
527 overcomes interpretation problems associated with the complexity between viability
528 parameters, but also the characterization of intermediate states, such as dormancy (Léonard et
529 al., 2016). To combine different fluorophores, it is important to choose those who possess the
530 right spectral proprieties, to determine their incubation time, incubation conditions, and
531 concentration for each one separately, and then assess possible interferences (Léonard et al.,
532 2016). There are three main interferences in staining procedures: overspill, fluorescence

resonance electron transfer (FRET), and/or matrix quenching (Buysschaert et al., 2016). The first can be solved by applying a compensation matrix during data analysis, the last two lead to decreased fluorescence of one of the fluorophores by quenching and should be solved by selecting different fluorophore combinations (Buysschaert et al., 2016). If two fluorophores can be used simultaneously, one of two things can happen (Léonard et al., 2016): either both fluorophores have discriminatory power and four quadrants can be observed corresponding to four different physiological states; or only one fluorophore has discriminatory power and the other can enter all cells, resulting in two possible physiological states.

3.2. Community profiling

Environmental microbial communities can be profiled using different parameters, being the most used: cell size (FSC); cell complexity (SSC); autofluorescence; and/or permeant fluorophores that label cells indiscriminately.

One common application of autofluorescence analysis is the phytoplankton community profiling which possesses both autotrophic picoeukaryotes (chlorophyll-containing cells) and autotrophic prokaryotes (Bernard et al., 2019). Chlorophyll (green pigment) detection can be accomplished by its emission of red fluorescent light (Bernard et al., 2019). Another example focuses on the analysis of phycoerythrin by its orange fluorescence emission light to discriminate photosynthetic and heterotrophic cells from cave biofilm samples (Borderie et al., 2016).

The analysis of scattering plots of morphological cell parameters (FSC or SSC) *vs* fluorescence emitted by the permeant fluorophore allows the definition of a community cytometric fingerprint that represents the microbial community structure by the number and position of clusters and the number of cells within each cluster.

There are a set of frequently used permeant fluorophores to perform community profiling: SYTO™ dyes, SYBR™ Green dyes, and 4',6-diamidino-2-phenylindole (DAPI). The first group is extensively used due to their advantageous characteristics, namely high signal to background

fluorescence, high molar absorptivity, permeant to nearly all cell membranes, and high quantum yields when bound to nucleic acids (Hammes et al., 2011; Léonard et al., 2016). SYBR™ Green dyes, such as SYBR™ Green I and II, are green-permeant fluorophores (Grégori et al., 2001). SYBR™ Green II shows higher sensitivity and higher quantum yield while keeping a strong affinity for double-stranded DNA, about half of SYBR™ Green I (Grégori et al., 2001). Other fluorescent dyes can be used, namely 4',6-diamidino-2-phenylindole (DAPI) that can be used to stain all cells due to its high cell permeability and high affinity to DNA, being excited using UV light and emitting in the blue wavelength (460 nm) (Hammes et al., 2011; Léonard et al., 2016). Moreover, some fluorophores can selectively bind to RNA molecules, namely pyronin-Y, allowing the discrimination between high and low RNA content cells, dead cells, and debris (Peris-Bondia et al., 2011). In environmental samples, DNA-permeant stained cells are normally grouped in two clusters: high (HNA) and low (LHA) nucleic acid content cells. HNA cells have been shown to have an increased cell division and high metabolic activity comparing to LNA cells (Hammes and Egli, 2010; Krause et al., 2020; Liu and Müller, 2020; Santos et al., 2019; Wang et al., 2010).

Four methods can be used to evaluate cytometric fingerprints: Dalmatian Plot, Cytometric Histogram Image Comparison (CHIC), Cytometric Barcoding (CyBar), and FlowFP (for a full review and a systematic comparison, see (Koch et al., 2014b)). Furthermore, a more recently developed tool, *FlowEMM*. This is a fast tool that determines the number of gates automatically, separating the cell clusters from background clusters containing irrelevant information and calculates the real number of data points for each cell cluster (Ludwig et al., 2019).

3.3. Membrane integrity as the ultimate measure of cell viability

For membrane integrity evaluation, dye exclusion methods are normally preferable (Hammes et al., 2011; Léonard et al., 2016). These dyes bind to nucleic acids emitting fluorescence upon binding. Only membrane-compromised cells are permeable to the dyes. Propidium Iodide (PI) is one of the most frequently used to detect dead cells, showing exclusion proprieties due to its

two positive charges (Hammes et al., 2011; Léonard et al., 2016). PI emits red fluorescence, being normally used in association with SYTO™ 9, an SYTO™ dye that produces green fluorescence (Hammes et al., 2011; Léonard et al., 2016). This methodology is very frequently used in pure culture analysis, however, it is rare in polymicrobial culture due to species-specific staining heterogeneity. Nevertheless, it has been previously used to assess membrane integrity in freshwater samples treated with UV light (Berney et al., 2007). Contrary, the conjugation of SYBR™ Green I and PI results in more homogeneous and reproducible staining profiles. These fluorophores were recently used to assess bacterial viability present in groundwater samples recovered after temperature and pH disturbances (Song et al., 2019) and in freshwater samples at a river catchment scale (Liu et al., 2019).

A recent integrative study assessed the effect of arsenic particles resultant from biotransformation of soluble arsenic by rhizosphere fungi on soil-dwelling bacteria physiology using several fluorophores to analyze membrane integrity and permeability. Propidium iodide (PI) was used to assess membrane cell integrity as a proxy of cell viability, 1-N-phenylnaphthylamine (NPN) to assess outer membrane permeability, and o-nitrophenyl-b-D-galactopyranoside (ONPG) to assess inner membrane permeability (Mohd et al., 2019).

3.4. Cell intake activity – measuring efflux pump activity and glucose intake

Efflux pump activity is usually evaluated by the measurement of ethidium bromide (EB)-associated fluorescence (Thomas et al., 1997). This dye possesses a positive charge with a monovalent capacity towards DNA. When the membrane efflux pumps of the cell, namely non-specific proton antiport transport system, are actively working, EB is pumped out of the cell, but when the cell membrane is damaged and/or the efflux pumps are malfunctioning, EB is accumulated intracellularly, binding to DNA and emitting yellow fluorescence (Thomas et al., 1997). To our knowledge, there are no reports describing the use of this fluorophore to assess pump activity in environmental polymicrobial communities.

Glucose intake can be investigated as a measure of cell viability. For this purpose, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) can be used as substrate. This fluorophore is an analog of glucose, being specifically taken inside the cell by the phosphoenolpyruvate phosphotransferase system (Strauber and Muller, 2010). A recent study takes advantage of this approach to identify uncultured rumen bacteria that can intake glucose, as a proof-of-concept to the use of NBD as a labeling fluorophore for substrate utilization by uncultured bacteria (Tao et al., 2019). The use of NDB can be applied in the characterization of bacterial niches by their intake capacity of different substrates.

3.5. Assessing membrane potential and pH gradient – two sides of the same coin

The membrane potential is an important cell physiological parameter since only living cells can maintain their membrane potential. When the membrane potential reaches zero, it means that membrane structure is compromised, and ions can freely cross the membrane. To evaluate membrane potential, different dyes can be used, but all of them have common proprieties: they are lipophilic molecules that can cross the membrane and be accumulated inside or outside the cell, depending on their charge (Buysschaert et al., 2016). The fluorescent signal is directly related to cell energy levels, however, due to their outer membrane, Gram-negative bacteria can be resistant to staining (Buysschaert et al., 2016). This problem can be overcome by washing cells with Tris-EDTA or citric acid (Buysschaert et al., 2016). The different dyes can be divided into two groups: cationic dyes that are accumulated inside polarized cells, and anionic dyes that are only accumulated inside cells with an altered polarization (Maurice et al., 2013; Pepè Sciarria et al., 2019). Among cationic dyes, carbocyanine ($DiOC_n(3)$) is the most common (Pepè Sciarria et al., 2019). In a recent study by Pepè Sciarria and colleagues (2019), microbial fuel cells were studied, namely both the anode biofilm and the planktonic broth of an acetate fed-batch (Pepè Sciarria et al., 2019). Both SYBR™ Green I and $DiOC_6(3)$ were used, the first to enumerate total microbial populations and the second to enumerate metabolically active microbial populations

(Pepè Sciarria et al., 2019). Anionic dyes, when inside the cell, become associated with non-specific intracellular proteins, increasing dye concentration, and consequently fluorescence intensity, being the most common bis-(1,3-dibutyl barbituric acid)-trimethine oxonol ($DiBAC_4(3)$) (Maurice et al., 2013). This fluorophore was used in a study where the action of xenobiotics over the physiology of the human gut microbiome was assessed (Maurice et al., 2013). In this study, $DiBAC_4(3)$ proved useful as it was accumulated inside depolarized cells, indicating increased damage after antibiotic exposure, with seasonal physiological variations to this exposure (Maurice et al., 2013).

The ability of cells to maintain a pH gradient is also a measurable viability parameter, strongly related to membrane potential. The intracellular pH can be assessed using the pH-dependent fluorescent probe carboxyfluorescein succinimidyl ester (cFSE) (Breeuwer et al., 1996). cFSE can conjugate with aliphatic amines and be retained within the cell, with green fluorescence (Breeuwer et al., 1996). A methodology using this fluorophore to detect viable *Clavibacter michiganensis* subsp. *michiganensis*, the causative agent of bacterial canker of tomato, has been previously ~~was recently~~ developed (Chitarra et al., 2000).

3.6. Metabolic activity – respiration, enzymatic activity, and oxidative stress

Metabolic activity is positively correlated with viability only if the population under study does not show any signal of membrane damage, dormancy, or starvation (Creach et al., 2003; Hoefel et al., 2003). This parameter is analyzed using a non-fluorescent substrate that upon entrance into cells is enzymatically transformed into a fluorescent molecule accumulating inside the cell (Creach et al., 2003; Hoefel et al., 2003). The usually measured activities are respiration and enzymatic activity. The first is usually assessed using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) that competes with oxygen as an electron donor and is reduced by the active electron transport system of respiring cells in an insoluble fluorescent molecule named formazan with red fluorescence, that is retained inside the cell (Creach et al., 2003). The most metabolically

active cells can be positively detected, but cells with low respiratory activity may not be detected due to the inner accumulation of CTC being highly cytotoxic (Creach et al., 2003). This fluorophore was used, in a recent study, to evaluate microbial activity in Antarctica permafrost (La Ferla et al., 2017). The second is assessed using fluorescein diacetate (FDA) or derivates, such as carboxyfluorescein diacetate (cFDA), a lipophilic non-fluorescent precursor that enters the cell, is hydrolyzed by unspecific esterases into carboxyfluorescein (cF) that, due to its extra negative charges, is retained within the cell (Hoefel et al., 2003). However, this fluorophore has several disadvantages, namely dead cells that only recently died can possess residual enzymatic activity; many bacteria cannot uptake cFDA; the fluorescence signal tends to be weak; and fluorescence intensity may change with pH (Hoefel et al., 2003). Nevertheless, this fluorophore has been successfully used to assess bacterial viability in soil samples (Espina, 2020). Carboxyfluorescein diacetate succinimidyl ester (cFDA/SE) is similar to cFDA but differs in the presence of a succinimidyl ester (SE) group with the ability to bind to free amines (Hoefel et al., 2003). Its hydrolyzation by nonspecific esterases results in the formation of cF/SE that is a highly fluorescent amine-reactive fluorophore (Hoefel et al., 2003). Thus, cFDA/SE can more easily be retained inside bacterial cells, resulting in higher fluorescent intensity. However, the use of this fluorophore to evaluate river, reservoir, and marine water samples, showed a low differentiation capacity between active and inactive bacterial cells, with lower fluorescence signal intensity, when compared with cFDA (Hoefel et al., 2003).

Oxidative stress can also be evaluated using hydroethidine (HE). This fluorophore suffers oxidation by reactive oxygen species (ROS) and reactive nitrogen species (RNS), forming ethidium that intercalates DNA (Buysschaert et al., 2016). This fluorophore has been recently used to assess oxidative stress caused by salt on thermophilic starter cultures applied to cheese manufacture (Hickey et al., 2018). Moreover, CellROX™ Green is a fluorophore used to measure ROS in live cells, being a non-fluorescent permeant dye that upon oxidation binds to DNA and becomes fluorescent (Hickey et al., 2018). This fluorophore was used, in conjugation with PI, to

assess free total ROS and membrane integrity of probiotic bacteria after several drying techniques (Fallico et al., 2020).

3.7. Mining lipid content

Lipid content can be evaluated to study cell physiology, but not as a proxy of cell viability. Many cells can storage lipids in vesicles. These lipids can be detected using Nile Red or boron-dipyrromethene (BODIPY™), a benzophenoxazone dye that detects non-polar lipid droplets inside cells, as well as the presence of storage lipids (PHA/PHB) (Buysschaert et al., 2016). To our knowledge, these fluorophores were not yet applied to environmental microbiology studies, however, they have the potential to selectively label intensive microbial producers and accumulators of lipids, which can thus be sorted and applied into biotechnological processes, such as biodiesel production.

3.8. Gram character – a modernized perspective on a historical character

The Gram stain is one of the most important and widely used differential stains for the taxonomic differentiation of bacteria. Gram character can be evaluated using flow cytometry: a combination of SYTO™ dyes that label both Gram-negative and Gram-positive bacteria, resulting in green fluorescent cells; and the nucleic acid stain, hexidium iodide (HI), that selectively stains almost all Gram-positive bacteria, resulting in red fluorescent cells, since it is blocked by the lipopolysaccharide layer of Gram-negative bacteria (Mason et al., 1998). However, this methodology only allows the selective staining of live bacteria, since dead bacteria may stain variably (Mason et al., 1998). Thus, this fluorophore combination was never used to assess the Gram character of environmental bacterial communities.

Additionally, Gram staining can also be evaluated using wheat germ agglutinin that can bind to N-acetylglucosamine in the peptidoglycan layer of the cell wall (Holm and Jespersen, 2003). However, due to the presence of the lipopolysaccharide in Gram-negative, these bacteria do not stain, but Gram-positive bacteria do so. The wheat germ agglutinin does not possess any

fluorescence, so it must be conjugated with a fluorophore to be detectable through FLOW (Holm and Jespersen, 2003). This technique has been previously applied to analyze milk samples (Holm and Jespersen, 2003) and pH influence on microbiota intestinal samples (Duquenoy et al., 2020).

4. Fluorescence *in situ* hybridization coupled with Flow Cytometry (FLOW-FISH)

FLOW-FISH allows a high-throughput characterization of the individual microbial cells based on their fluorescence resulting from *in situ* hybridization alone or together with physiology evaluating fluorophores, enabling the combination of both taxonomic identification, physiological characterization, and rapid and precise quantification of microbial communities (Müller and Nebe-von-Caron, 2010).

Several examples of FLOW-FISH applications to environmental microbial studies can be found in the literature. Friedrich and Lenke (2006) used this approach with fixation-free in-solution FISH to detect and quantify lactic acid bacteria, in particular, *Leuconostoc* and *Lactococcus* genera, from dairy starter cultures together with cell viability evaluation of the labeled bacteria with PI (Friedrich and Lenke, 2006). This project showed the advantage of FLOW-FISH to quantify the starter bacteria as an alternative to the conventional plate counting method (Friedrich and Lenke, 2006). Jen et al. (2007) labeled the clostridial hydrogenase gene using in-solution FISH with fixed cells of anaerobic hydrogen-producing systems from brewery yeast waste (Jen et al., 2007). In 2011, three very interesting articles were published using this methodology. The first one, by Manti and team (2011), described a very elegant protocol for in-solution and on-filter CARD-FISH applied to seawater samples to quantify the overall bacteria burden using FLOW (Manti et al., 2011). The utilization of on-filter CARD-FISH improved the recovery rate from seawater samples, even though the detachment of bacterial cells from the filter was imperative for the subsequent FLOW analysis (Manti et al., 2011). Secondly, a miniaturization of the FLOW-FISH method was obtained by microfluidic application, allowing the detection of *Desulfovibrio* spp. and *Pseudomonas* spp. from water samples (Liu et al., 2011). The last one used LNA probes

734 to perform CARD-FISH to detect bacterial noncoding RNA (Robertson and Vora, 2012). Besides
735 the very low amount of noncoding RNA present in bacterial cells, good signal intensity was
736 obtained, due to the use of LNA together with CARD-FISH as a signal amplification approach
737 (Robertson and Vora, 2012). Nettmanni et al. (2013) studied the bacterial community of an
738 anaerobically fermented liquor by developing a new FISH-FLOW protocol (Nettmann et al.,
739 2013). This protocol is based on the hybridization of the bacterial cells with fluorophore-labeled
740 universal probes targeting bacteria and archaea members, followed by FLOW to quantify the
741 labeled community and performance of a dehydrogenase activity essay using CTC to evaluate
742 cell's metabolic activity (Nettmann et al., 2013). Takahashi team (2015) applied a FISH-FLOW
743 protocol to monitor the beer brewing process, targeting universal regions of bacteria and
744 eukaryotic cells (Takahashi et al., 2015). FLOW-FISH was also applied to identify different
745 taxonomical groups of bacteria present in samples of arsenic-rich waters of geothermal origin
746 using CARD-FISH (Crognale et al., 2017) and in rat feces to study the effect of cocoa compounds
747 using conventional FISH (Martin-Pelaez et al., 2017). Furthermore, Branco team (2019)
748 developed a FLOW-FISH protocol based on RNA-FISH to detect *Dekkera bruxellensis* in wine
749 samples using a species-specific probe targeting the 26S rRNA gene labeled with a red
750 fluorophore to avoid background noise from wine autofluorescence compounds (Branco et al.,
751 2019). Recently, the same team developed another FLOW-FISH protocol with a different probe,
752 this time a DNA-FISH probe to avoid the use of formamide due to its toxicity. The RNA target
753 was the same and was labeled with the same fluorophore to be applied equally in the wine
754 environment (Branco et al., 2020).

755 **5. FLOW-FISH coupled with fluorescence-activated cell sorting (FACS)**

756 **5.1. FACS general principles**

757 Cells can be singly sorted after FLOW analysis based on their autofluorescence, size, complexity,
758 viability, taxonomy, functional group, among others (Chen et al., 2017; Czechowska et al., 2008;

759 Koch et al., 2014a). This can be performed using different methodologies, being one of the most
760 used the droplet sorting principle. This technique is based on the formation of charged droplets
761 containing a cell of interest when passing by the area downstream of the light collection point
762 (Müller and Nebe-von-Caron, 2010). Droplets of interest are charged and then sorted using an
763 electrostatic field that is generated by deflection plates, while uncharged droplets that contain
764 other cells pass into the waste (Müller and Nebe-von-Caron, 2010). Additionally, the flow
765 diversion sort principle can also be applied. This approach relies on a pressure wave
766 (piezoelectricity) that directs the cells of interest into a different channel, while the remaining
767 cells pass into the waste (Müller and Nebe-von-Caron, 2010). This approach is advantageous
768 when compared to the previous one, since it is performed enclosed in the tubing system,
769 reducing the risk of aerosols of hazardous materials and the contamination of the sorted cells
770 (Müller and Nebe-von-Caron, 2010). FLOW-FISH-FACS allow the sorted cells to be used in
771 different applications, ranging from metagenomics, cultivation of functional communities of
772 microorganisms, characterization of microorganisms using single-cell omics, and depiction of
773 the viable community or spore community, allowing the understanding of their role on an
774 ecological niche (Chen et al., 2017; Czechowska et al., 2008; Koch et al., 2014a) (Fig. 5).

775 **5.2. FLOW-FISH & FACS for gene target amplification and sequencing**

776 One of the first works focusing on the downstream application of FLOW-FISH-FACS on
777 environmental samples was performed by Wallner and colleagues (1997). This team evaluated
778 activated sludge, lake water, and lake sediment using 16S rRNA oligonucleotide fluorescent
779 probes targeting *Acinetobacter* spp. and *Leptothrix* spp. in fixed cells, followed by detection and
780 cell sorting by FACS to subsequently perform 16S rRNA gene PCR amplification and sequencing
781 (Wallner et al., 1997). Additionally, on-filter CARD-FISH using fixed cell followed by FACS was
782 performed in marine water samples to detect bacterioplankton using conjugation of different
783 16S rRNA targeting probes to different bacterial taxonomical groups (namely α-, β-, and γ-

784 proteobacteria); they then performed 16S rRNA sequencing, allowing a diversity study of these
785 different groups (Sekar et al., 2004). FLOW-FISH was also used to study
786 "*Candidatus* Accumulibacter phosphatis" in activated sludge with different clade-specific
787 probes, with the main goal of evaluating the polyphosphate kinase-1 gene homologs of the
788 sorted cells by PCR and sequencing (Kim et al., 2010). Furthermore, fecal human samples were
789 previously analyzed to investigate the difference between total bacterial population and active
790 bacterial population using pyronin-Y, to specifically stain RNA, to detect active cells, followed by
791 FACS of the active community and microbial profiling, targeting the 16S rRNA gene (Peris-Bondia
792 et al., 2011). Gougoulias and Shaw (2012) used FLOW-FISH to study the *Lolium perenne*
793 rhizosphere soil, making use of 16S rRNA-targeting probes for detection and identification of
794 *Pseudomonas* spp., followed by FACS and sequencing of 16S rRNA sequence, registering a
795 remarkably high specificity and sensibility of the design probe to the genus but they were unable
796 to recover new *Pseudomonas* species (Gougoulias and Shaw, 2012). Also, CARD-FISH performed
797 on fixed and immobilized cells of activated sludges to the detection of denitrifying bacteria, using
798 as probe-target *nirS* codifying mRNA, was performed (Mota et al., 2012). This work sorted
799 hybridized cells using FACS and completed with denaturing gradient gel electrophoresis (DGGE)
800 of 16S rRNA amplified genes, followed by purification and sequencing of selected gel bands
801 (Mota et al., 2012). The effect evaluation of xenobiotics in the human gut microbiome
802 physiology was performed based on bacterial staining with PI, $DiBAC_4(3)$, and SYBR™ Green I,
803 allowing observation of membrane integrity and membrane potential evaluation and
804 differentiation between LNA and HNA cell populations, respectively (Maurice et al., 2013). FACS
805 was applied to each population, followed by 16S rRNA gene amplification and sequencing, to
806 taxonomically identify changes in the gut microbiome (Maurice et al., 2013). Flow cytometry
807 profiles obtained by cell staining with DAPI, together with FSC evaluation were used to define
808 subcommunities through gate attribution in bacterial cells recovered from wastewater
809 treatment plants (Liu et al., 2018b). Changes in cell abundance in each gate were registered and

stability proprieties were analyzed (Liu et al., 2018b). This analysis is based on the ability of the microbial community to react to environmental disturbances (Liu et al., 2018b). FACS was also applied to the previously defined gates and taxonomical identification of gated cells was completed by 16S rRNA gene sequencing (Liu et al., 2018b). Very recently, FACS was also applied to photosynthetic picoeukaryotes, enabling 18S rRNA gene amplification and sequencing, aiming at the taxonomical identification of these communities from eutrophic shallow lake samples (Shi et al., 2020). This sorting strategy is based on the autofluorescence of chlorophyll-*a* by biplot FSC *vs* far-red fluorescence evaluation (Shi et al., 2020).

FLOW-FISH-FACS for whole-genome amplification (WGA) and sequencing (WGS)

Recently, FACS technology has been used to sort cells and perform WGS for several purposes (Chen et al., 2017; Czechowska et al., 2008; Koch et al., 2014a). However, presently, there subsist yield limitations of targeted cell enrichment by FACS (between 10^5–10^6 cells), demanding WGA before WGS. However, current WGA methods are not compatible with formaldehyde fixation due to alterations of DNA molecules, so alternative fixation protocols, such as ethanol fixation, are necessary to amplify genomic material from FACS (Chen et al., 2017; Czechowska et al., 2008; Koch et al., 2014a).

Several examples of this application were described in the last decade. Yilmaz et al. (2010) performed an in-solution fixation-free FISH methodology followed by FACS and subsequent WGA and 16S rRNA gene sequencing to study bioreactor sludges and termite hindgut samples to detect both "*Candidatus* Accumulibacter phosphatis" and methanotrophic bacteria (Yilmaz et al., 2010). This approach was one of the first steps to in-solution fixation-free FISH protocol, facilitating WGA and sequencing of sorted cells since DNA has no crosslink from paraformaldehyde or similar fixating agents (Yilmaz et al., 2010). Lee and team (2015) performed FISH, using *Dehaloccoccoides mccartyi* specific 16S rRNA targeting probe, followed by FACS and WGA of sorted cells on activated sludge and contaminated groundwater samples (Lee et al.,

2015). The amplified DNA was used for microarrays analysis, and functional and comparative genomics to improve the knowledge of this dechlorinating species (Lee et al., 2015). BONCAT-FISH and BONCAT-CARD-FISH were coupled with FACS to study the anaerobic oxidation of methane consortia in methane seep sediments (Hatzenpichler et al., 2016). This approach allowed the detection of active cells and concomitant functional identification of consortia, followed by FACS and WGA to 16S rRNA gene sequencing to evaluate microbial interactions occurring in these consortia (Hatzenpichler et al., 2016). De Corte team (2019) studied the viral community of the Global Deep Ocean Conveyor Belt system by FACS followed by WGA and WGS (De Corte et al., 2019). To accomplish this study, viral particles present in water samples were fixated with a low concentration of glutaraldehyde, to increase downstream application efficiency, and stained with SYBR™ Green I (De Corte et al., 2019). Very recently, forest soil samples were evaluated by SYBR™ Green I staining and flow cytometric profile analysis (as described above) (Alteio et al., 2020). The obtained gates were sorted, followed by WGA and WGS of those mini-metagenomes (Alteio et al., 2020). This analysis was coupled with metabolic predictions to better reconstruct the microbial diversity of those soil samples (Alteio et al., 2020).

5.3. Examples of FLOW-FISH-FACS for other downstream applications

Bacteria recovered from lake water were labelled with nitrogen and carbon-heavy isotopes, followed by FACS according to the autofluorescence of the target species *Chlorobium phaeobacteroides* (Zimmermann et al., 2015). The sorted bacteria were labelled by CARD-FISH targeting 16S rRNA and analysed by NanoSIMS, to measure the incorporated heavy isotopes in this specific species (Zimmermann et al., 2015).

Moreover, Batani team (2019) developed a FISH protocol that allows the labeling of cells keeping them alive through the omission of fixation, optimization of centrifugation steps and buffers, in parallel with chemical transformation, resulting in specific hybridization of DNA probes (Batani

et al., 2019). Following FISH of Baltic surface seawater, labeled cells were sorted by FACS and cultivated, with successful growth (Batani et al., 2019). Espina (2020) also developed an optimized FLOW protocol, followed by FACS, to increase the success rate of cultivation of soil bacteria using cFDA and PI to sort reproductively viable bacteria (Espina, 2020). These new methodologies hold great promise to improve the isolation and cultivation of new microorganisms from environmental matrices.

Besides the typical FACS based on direct/natural morphophysiological characteristics of cells and fluorophore staining of cells, genetic engineering can also be coupled with this technology. In a recent study by Liu and colleagues (2019), the evaluation of heavy metals facilitating the transfer of antibiotic resistance genes between bacteria in activated sludge was assessed by FLOW using an *E. coli* strain transformed with a plasmid-encoding antibiotic resistance gene together with the *Lac* system (Lin et al., 2019). So, donor cells possess chromosomal red fluorescence protein-producing gene under the influence of $LacI^q$ and a plasmid with a green fluorescence protein-producing gene with a $LacI^q$ repressor (Lin et al., 2019). Donor cells are fluorescence red, while plasmid recipient cells acquire green fluorescence (Lin et al., 2019). Cells that are unable to acquire the plasmid-encoding antibiotic resistance gene have no fluorescence (Lin et al., 2019). After recipient cells were sorted by FACS, 16S rRNA gene amplification and sequencing were performed, together with microbial metabolic function prediction using PICRUSt (Lin et al., 2019). This study allowed the identification of the *Pseudomonas* genus as the dominant plasmid recipient in the presence of arsenic and mercury, and *Aeromonas* and *Enterobacter* genera as the main recipients in the presence of lead (Lin et al., 2019). ATP-binding cassette transporters were related to heavy metal transport in the microbial metabolic function prediction of recipient bacteria (Lin et al., 2019).

6. Perspectives

Flow cytometry is an amazing tool to study microbial communities within environmental samples, enabling quantification and viability evaluation in a single-cell and high-throughput approach that can overcome culture-dependent methods. Besides, it enables the evaluation of population heterogeneity, thus favouring microbial-driven ecological studies in natural environments. Additionally, this technology can be coupled with FISH to recover taxonomical and functional information. So, FLOW-FISH can be used, not only for signal intensity measurements but also combined with FACS, followed by several downstream analyses of sorted cells, such as omics to complete genome-wide analysis that provides a deeper understanding of cell biology. This combined workflow is recent and its potential is still unexplored, but we believe that in the coming years several research works will take advantage of this marriage. Cytomics will thus arise as a key strategy in environmental microbiology and microbial ecology, combining ~~structural and functional information from single-cell characterization with individual cell contribution to the community state.~~ multiparametric and dynamic approaches (both structural, functional, and metabolic information) for microbial single-cell characterization and research (Müller and Nebe-von-Caron, 2010). This will allow the multitudinous operations of microorganisms in the total environment to be followed, improving our understanding of microbial cell behaviour in complex natural matrices and their contribution to the community state. This information can be further used to develop predictive models, e.g. metabolic models, in systems biology approaches through the use of machine learning and artificial intelligence. The use of new ~~biological~~molecular, mathematical, and bioinformatic~~al~~ technologies and methodologies will be fundamental in downstream steps to help us handle the enormous amount of data that ~~can~~may be generated ~~on the downstream analysis through~~by omics-related methods. ~~Ultimately, a~~An in-depth understanding of metabolic pathways regulation and coexistence processes of microbial cells within populations or complex consortia will be achieved. Ultimately, this brave new world will contribute to outstanding improvements in nature-based biotechnological and medical microbial applications.

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Table 1 – Overview of signal amplification approaches: advantages, disadvantages, and applications to environmental microbiology.

Methodologies	Technique	Description	Advantages	Disadvantages	Applications	References (e.g.)
Multi-labeled probes or multiple probes	DOPE-FISH and MiL-FISH	Probes labeled at the 5' and 3' ends (same or different fluorophores)	As simple as conventional FISH; Up to seven target groups in a single experience	N/A	Aquatic environments; Isolation of *"Candidatus"* species; Plant and animal microbiota; Biofilms	(Beam et al., 2015; Escudero et al., 2018; Glassner et al., 2015; Kroer et al., 2016; Lehtovirta-Morley et al., 2016; Mitter et al., 2017)
	CLASI-FISH (TAPE-FISH and Leaf-FISH)	Unique spectral tag to each target group by conjugation of mono-labeled probes	TAPE-FISH and Leaf-FISH allows the visualization of the spatial structure	Expected 50:50 ratio of probes can be switch	Plant and animal microbiota	(Bisha and Brehm-Stecher, 2009; Mark Welch et al., 2017; Peredo and Simmons, 2018)
Enzymatic-based	CARD-FISH	HRP-labeled oligonucleotide probes and fluorophore-labeled tyramides	26 to 41 times higher fluorescent signal intensity than conventional FISH	Expensive; Enzymatic pretreatment of cells; Inactivation of endogenous peroxidases; Dramatic alteration in melting behavior of the probes; Multi-targeting groups are time-consuming	Animal microbiota; Aquatic environments; Soil and sediments	(Alfreider et al., 2018; Cai et al., 2016; Crognale et al., 2017; Golyshina et al., 2017; Hoshino et al., 2017; McNichol et al., 2018; Pohlner et al., 2017; Probandt et al., 2017; Schmidt and Eickhorst, 2014; Schmidt et al., 2018; Winkel et al., 2018)
	2-pass CARD-FISH	Sequential CARD with HRP-conjugated anti-fluorophore antibodies to the hybridized fluorophore, followed by a second CARD signal amplification with fluorophore-labeled tyramides	Increased detection efficiency of low ribosomal content cells	Same problems as CARD-FISH, with increased time-consumption	Aquatic environments	(Kawakami et al., 2012; Neuenschwander et al., 2015)
	GeneFISH (and PhageFISH)	Detection of the gene of interest and the rRNA of the microorganism,	Simultaneous evaluation of taxonomy and functionality	Same as CARD-FISH	Aquatic environments; Archaeal viruses;	(Allers et al., 2013; Bernhard et al., 2012; Dang et al., 2015; Lenk et al., 2012; Matturro

		both using CARD-FISH methodology			Phage/host interactions; Sediments; Symbiosis relations	and Rossetti, 2015; Moraru et al., 2010; Petersen et al., 2011; Stagars et al., 2016)
	RING-FISH	Use of a probe labeled with a reporter molecule that anchors other probes, that in turn form a network around the cell periphery resulting in a halo-shaped fluorescent signal	10- and 50-fold signal amplification when compared to conventional FISH	Low probe penetration; High cost; Low specificity; Lack of sub-cellular definition	Aquatic environments; Sediments	(Noriea et al., 2010; Pratscher et al., 2009)
Amplification-based	HCR-FISH	An initial probe partially hybridizes to the target sequence and the remaining part becomes a trigger for the polymerization of the two fluorescently labeled amplifier probes	Up to 8-fold higher sensitivity than conventional FISH; Substitute of CARD-FISH with less aggressive permeabilization	N/A	Aquatic environments; Sediments; Symbiosis relations	(Nikolakakis et al., 2015; Yamaguchi et al., 2015a)
Synthetic metabolites-based	BONCAT-FISH	A biorthogonal synthetic amino acid is taken by the cell and acts as a substrate of aminoacyl tRNA synthetase to be incorporated into *de novo* peptides, reacting to a modified fluorescence dye	A nondestructive technique that is an alternative to study microbial ecophysiology independently of isotopes	Expensive; Hard to perform	Animal microbiota; Aquatic environments; Sediments; Symbiosis relations	(Hatzenpichler et al., 2016; Hatzenpichler et al., 2014)

FISH – Fluorescence *in situ* hybridization; DOPE – Double labeling of oligonucleotide probes; MiL – multi-labeled oligonucleotide probes; CLASI – combinatorial labeling and spectral imaging; CARD – Catalyzed reported deposition; HRP – horseradish peroxidase; RING – recognition of individual genes; HCR – *in situ* DNA hybridization chain reaction; BONCAT – biorthogonal noncanonical amino acid tagging; N/A – not applied.

Table 2 – Overview of morpho-physiological parameters assessed using flow cytometry in environmental microbiology: fluorophores and applications.

Parameter	Fluorophores	Description	Environmental applications	References*
Community cytometric profile	Nucleic acids staining dyes: DAPI Hoechst 33342 SYBR™ Green I and II SYTO™ dyes	In environmental samples, stained cells are normally grouped in two clusters: high (HNA) and low (LNA) nucleic acid content cells. HNA cells have increased cell division and high metabolic activity	Animal microbiota; Aquatic environments; Biofilms; Microbial fuel cells; Virus-bacteria differentiation; Soil; Wastewater treatment plants	(Bartelme et al., 2020; Berney et al., 2007; Borderie et al., 2016; Gözdereliler et al., 2013; Khalili et al., 2019; Krause et al., 2020; La Ferla et al., 2017; Liu et al., 2019; Liu and Müller, 2020; Müller et al., 2012; Plominsky et al., 2018; Song et al., 2019; Storesund et al., 2015; van Gelder et al., 2018)
Membrane integrity	Exclusion dyes: Propidium Iodide (PI) TOTO-1	Double staining with an exclusion dye and an all-staining dye that binds to DNA and/or RNA	Animal microbiota; Aquatic environments; Biofilms; Dairy products; Microbial fuel cells; Soil; Wastewater treatment plants	(Abzazou et al., 2015; Ben-Amor et al., 2005; Foladori et al., 2015; Gözdereliler et al., 2013; Grégori et al., 2001; La Ferla et al., 2017; Liu et al., 2019; Matos and Lopes da Silva, 2013; Maurice et al., 2013; Mohd et al., 2019; Peris-Bondia et al., 2011; Song et al., 2019; Wang et al., 2019)
Pump activity	Ethidium bromide (EB)	Cells with malfunctioning efflux pumps accumulate intracellularly the fluorophore	NA	NA
Glucose intake	2-NBDG	This analog of glucose is specifically uptake by the phosphoenolpyruvate phosphotransferase system	Animal microbiota	(Tao et al., 2019)
Membrane potential	Cationic dyes: Carbocyanine ($DiOC_2(3)$) Rhodamine 123 (Rh123) Anionic dyes: $DiBAC_4(3)$	Cationic dyes: accumulate inside viable cells Anionic dyes: accumulate inside dead cells	Animal microbiota; Microbial fuel cells; Soil	(Maurice et al., 2013; Mohd et al., 2019; Pepè Sciarria et al., 2019)
pH gradient	cFSE	A pH-dependent fluorescent probe	Plant microbiota	(Chitarra et al., 2000)
Metabolic activity (e.g. respiratory and enzymatic activities)	Respiratory activity: CTC Enzymatic activity: Fluorescein diacetate (FDA) or derivates	A non-fluorescent substrate that upon cell entrance is enzymatically transformed into a fluorescent substance that is accumulated	Aquatic environments; Dairy products; Soil; Wastewater treatment plants	(Bunthof and Abee, 2002; Espina, 2020; Hoefel et al., 2003; La Ferla et al., 2017; Lentendu et al., 2013; Ziglio et al., 2002)
Oxidative stress	CellROX™ Green Hydroethidine (HE)	The fluorophore suffers oxidation by reactive oxygen and/or nitrogen	Dairy products	(Fallico et al., 2020; Hickey et al., 2018)

		species, forming ethidium that intercalates with DNA		
Lipid content	BODIPY™ Nile Red	Binds to non-polar lipid droplets and stored lipids (polyhydroxyalkanoates) inside cells	NA	NA
Gram character	SYTO™ dye + Hexidium iodide Wheat germ agglutinin conjugated with a fluorophore	Hexidium iodide only stains Gram-positive bacteria Wheat germ agglutinin only binds to Gram-positive bacteria	Animal microbiota; Dairy products	(Duquenoy et al., 2020; Holm and Jespersen, 2003)

DAPI – 4′,6-diamidino-2-phenylindole; $DiBAC_4(3)$ – bis-(1,3-dibutyl barbituric acid)-trimethine oxol; CTC – 5-cyano-2,3-ditolyl tetrazolium chloride; cFSE – 5(6)-carboxy-fluorescein succinimidyl ester; 2-NBDG – 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; BODIPY™ – boron-dipyrromethene. * – some referenced examples are not developed in the manuscript.

Figure captions

Fig. 1 – **Type of fluorescence *in situ* hybridization (FISH) probes and labelling methods.** Four different type probes are represented, namely peptide nucleic acid (PNA), locked nucleic acid (LNA), DNA, and RNA probes. Five probe labelling options are denoted: direct [(a) and (b)] and indirect [(c) to (f)] labelled probes. Direct probes can be labelled in a mono (a) or multiple (b) way. Indirectly labelled probes can be made using a reporter molecule, like digoxygenin, biotin, or dinitrophenol (c); a horseradish peroxidase (HRP) that uses fluorophore–tyramide (TSA) as a substrate (d); and a combination of TSA system and a probe with a reported molecule (e).

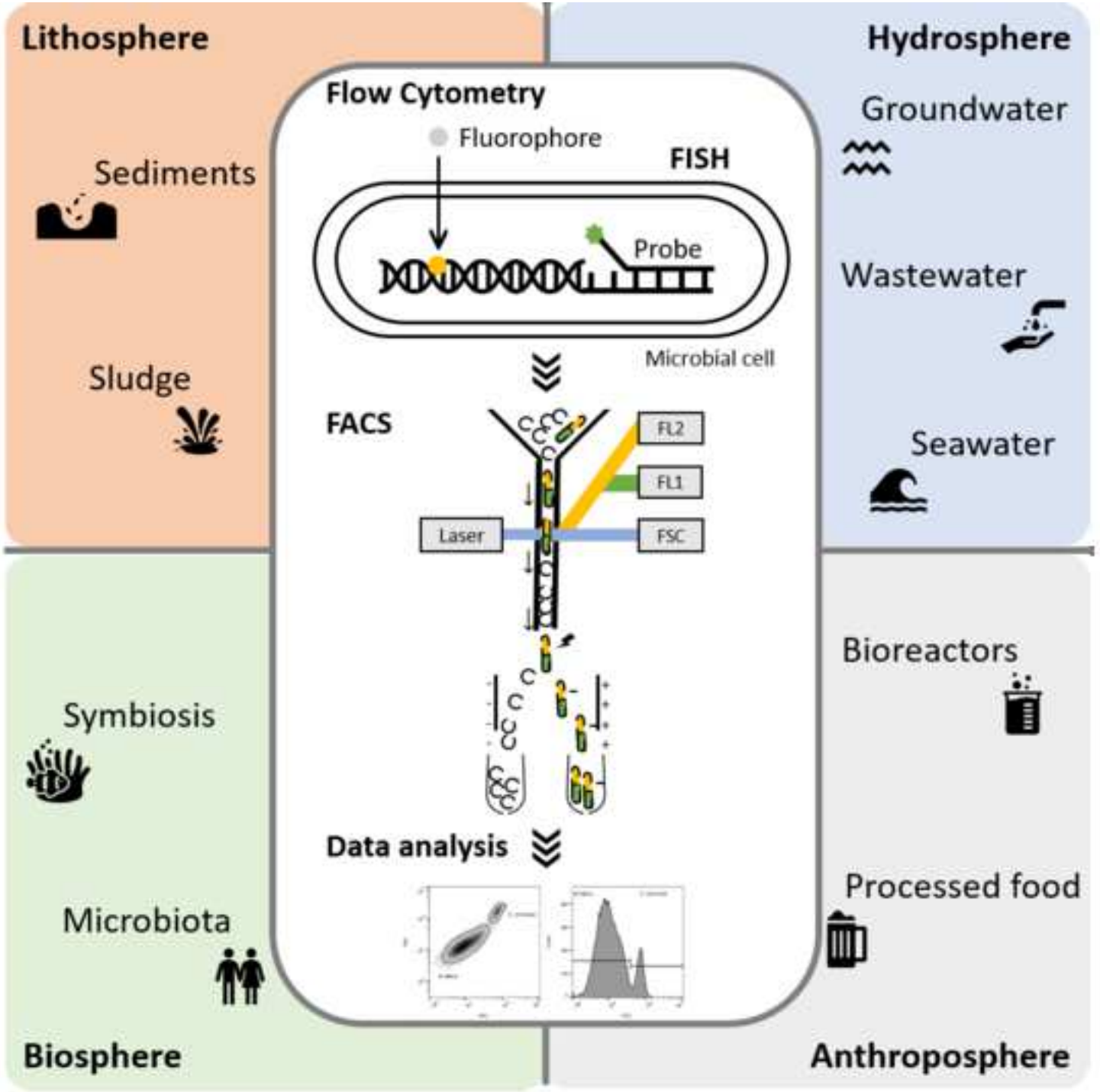
Fig. 2 – **Signal amplification systems used in fluorescence *in situ* hybridization (FISH)**. a) CARD-FISH; b) Two-CARD-FISH; c) RING-FISH; d) HCR-FISH; e) DOPE-FISH; f) CLASI-FISH; g) BONCAT-FISH. HRP – horseradish peroxidase; AHA – L-azidohomoalanine; ALP – azide-labelled protein; AMD – alkyne-modified dye.

Fig. 3 – **Graphical outputs of flow cytometry analysis.** A) *Micrococcus luteus* and *Saccharomyces cerevisiae* were directly analysed (without the use of fluorophores), allowing the differentiation between microbial populations according to cell size (FSC) and cell complexity (SSC), using both a biplot of SSC *vs* FSC (left) and a histogram of FSC counts (right). B) Two populations of live (grey) and dead (black) cells of *Pseudomonas denitrificans* (left) and *Pseudomonas stutzeri* (right) were stained with different fluorophores: (i) SYTO™ 9 and PI; (ii) cFDA-AM; and (iii) $DiBAC_3(4)$. Despite the two species belong to the same genus, different responses can be perceived to be species- and fluorophore-specific. (i) *P. denitrificans* showing increase red fluorescence in damaged cells, while *P. stutzeri* showed decreased green fluorescence, but both showed differentiation capacity between live and dead cells. None of the tested species had their metabolic activity correctly differentiated using cFDA-AM (ii). However, $DiBAC_3(4)$ showed to be useful to better differentiate polarized and depolarized cells of *P. denitrificans versus* cells of *P. stutzeri*.

Fig. 4 – Fluorophores and their applications to measure physiological cell parameters. Different physiological parameters are denoted, namely pH gradient, membrane potential, metabolic activity, glucose intake, permeability, membrane integrity, oxidative stress, pump activity, and lipid content. FDA – Fluorescein diacetate; CTC – 5-cyano-2,3-ditolyl tetrazolium chloride; 2-NBDG – 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; FFS – phosphoenolpyruvate phosphotransferase system; NPN – 1-N-phenylnaphthylamine; ONPG – o-nitrophenyl-b-D-galactopyranoside; ONP – o-nitrophenyl; PI – Propidium iodide; HE – Hydroethidine; ROS/RNS – Reactive oxygen species/reactive nitrogen species; EB – Ethidium bromide; cFSE – 5(6)-carboxy-fluorescein succinimidyl ester.

Fig. 5 – General workflow of fluorescence *in situ* hybridization (FISH) coupled with flow cytometry (FLOW) and fluorescence-activated cell sorting (FACS). Initially, an environmental sample is collected and the microbial community is separated from the matrix by physical and/or chemical methods. Next, FISH is performed: microbial cells are then fixated (e.g. paraformaldehyde) and/or dehydrated (e.g. ethanol), permeabilized (e.g. lysozyme), hybridized with a specific probe (e.g. taxonomical or functional gene identification), and washed to avoid unspecific hybridization. Detection of hybridized cells can be accomplished by epifluorescence microscopy or by FLOW, the last one allowing further analysis by FACS and downstream applications (e.g. omics or metabolic studies).

Graphical Abstract



Highlights

1. Community profiling and single-cell research are key in environmental microbiology.
2. The principles and advances of FLOW and FISH applied to microbial communities are reviewed.
3. Examples of FLOW-FISH-FACS for high resolution downstream analyses of single-cells are illustrated.
4. Overviews of morpho-physiological and metabolic parameters, signal amplification strategies, and troubleshooting are provided.
5. Multi-parametric approaches to co-assess taxonomy, function, and metabolism of microbial communities are highlighted.

When FLOW-FISH met FACS: Combining multiparametric, dynamic approaches for microbial single-cell research in the total environment

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Abstract

In environmental microbiology, the ability to assess, in a high-throughput way, single-cells within microbial communities is key to understand their heterogeneity. Fluorescence *in situ* hybridization (FISH) uses fluorescently labeled oligonucleotide probes to detect, identify, and quantify single cells of specific taxonomic groups. The combination of Flow Cytometry (FLOW) with FISH (FLOW-FISH) enables high-throughput quantification of complex whole cell populations, which when associated with fluorescence-activated cell sorting (FACS) enables sorting of target microorganisms. These sorted cells may be investigated in many ways, for instance opening new avenues for cytomics at a single-cell scale. In this review, an overview of FISH and FLOW methodologies is provided, addressing conventional methods, signal amplification approaches, common fluorophores for cell physiology parameters evaluation, and model variation techniques as well. The coupling of FLOW-FISH-FACS is explored in the context of different downstream applications of sorted cells. Current and emerging applications in environmental microbiology to outline the interactions and processes of complex microbial communities within soil, water, animal microbiota, polymicrobial biofilms, and food samples, are described.

Keywords: FISH, flow cytometry, FACS, single-cell, environmental microbiology

1. Introduction

Environmental microbiology is devoted to the fundamental understanding of complex microbial communities, from taxonomic composition to microbe-microbe interactions, to microbial processes in the environment, e.g. in the hydrosphere, lithosphere, or at the interconnection of multiple spheres. The understanding of the dynamic adaptation of microbial populations to environmental perturbations is also one of the key drivers of environmental microbiology. In these so-called environmental communities, most microorganisms are unculturable or fastidious growers (Stewart, 2012). While in the past bacterial populations from a given taxonomic group within these communities were viewed as clonal and metabolically homogeneous, the development of new methodologies brought insights into the mechanisms leading to dynamic cellular adaptation, metabolic switches, and introduced the notion of phenotypic and genotypic heterogeneity (Delvigne et al., 2018; Lemoine et al., 2017). The development of single-cell techniques has thus enabled the focus on microbial individuality (i.e. individual cell physiology), while enabling consequence inferences at the population level, with widespread application to different contexts (Delvigne et al., 2018; Lemoine et al., 2017). The notion of viability based on the ability to culture microbial cells also became an archaic concept (Hammes et al., 2011), since cultivability not only depends on the physiological state of a microbial cell but also the growth conditions (Hammes et al., 2011). The accurate assessment of microbial viability is important due to the ecological relevance of viable microorganisms, which actively change their environment (Davis, 2014).

As such, the application of methodologies that are capable to capture heterogeneity and, more accurately, assess the physiological status of individual cells within a population are crucial to better understand the taxonomic, metabolic, and functional compositions of microbial communities, their development processes, and their ecological roles (Koch et al., 2014a). Methodologies that capture the hallmark of microbial populations on a cellular level, by means

of a single-cell approach, provide the basis for reconstructing the complexity of microbial communities in different niches, from the industrial to the host-associated, to the most extreme environments (Koch et al., 2014a).

Most studies focusing on environmental communities rely on culture-independent techniques, such as PCR, microarrays, amplicon-based and shotgun sequencing, fluorescence *in situ* hybridization (FISH), and flow cytometry (FLOW). The amplification-based approach has been extensively used to study microorganisms and their genes in complex matrices (Liu et al., 2011). These approaches have several drawbacks since the detection of nucleic acids in the sample cannot be associated with cell viability and do not allow tracing back to the original cell for further analysis (Ju et al., 2016; Liu et al., 2011). Also, the information given by these methods is mostly qualitative based on the presence/absence of taxonomical markers and/or relative abundances of operational taxonomic units.

FISH was introduced 30 years ago as a very valuable molecular diagnostic tool to detect specific DNA or RNA sequences within intact cells through the use of complementary DNA- or RNA-probes labeled with fluorescent dyes (DeLong et al., 1989). FISH has since then developed as a robust technique for the detection, identification, and quantification of single cells of target microorganisms within complex matrices, with widespread applications, namely in environmental microbiology. FISH quantification accuracy is superior to several other approaches, such as the most probable number (MPN), immunological-based methods, and DNA amplification-based methods (Baptista et al., 2014). Besides, FISH is cost-efficient and easy to perform. However, it is time-consuming, with low throughput, and requires the use of expensive microscopes to avoid problems of background fluorescence and resolution (Baptista et al., 2014).

Flow cytometry can overcome several FISH drawbacks, being highly accurate and possessing high-speed single-cell processing capacity (Emerson et al., 2017). FLOW analysis is based on the

detection of light emitted by a laser. Different detectors collect the signal at specific wavelengths after light incidence over individual cells that travel in a fluid, converting the analog signal into a digital one – for an extensive review see (Givan, 2011; Shapiro, 2000). Additionally, FLOW coupled with fluorescence-activated cell sorting (FACS) enables cell sorting of a subpopulation of interest or single-cell sorting (Emerson et al., 2017). The combination of FISH, FLOW and FACS is very powerful and promising to study complex communities from different environmental matrices. However, among FACS major disadvantages is the need for at least 10^5 initial cells, failure to isolate single-cells from a poorlyrepresented community (in numbers), and the potential damage to more fragile cells during sorting due to high-speed technology (Emerson et al., 2017). Below, we provide an overview of FISH, FLOW, and FACS methodologies, addressing conventional protocols, model variation techniques, signal amplification approaches, and fluorophore choices for cell physiology evaluation, always trying to direct the reader for an application-based solution strategy.

2. Fluorescence *in situ* hybridization (FISH)

2.1. Conventional FISH – principles of an established technique

Bacterial identification by FISH usually explores the use of oligonucleotide/polynucleotide probes targeting ribosomal RNA (rRNA) because of the natural production of rRNA molecules in all cells (Almeida et al., 2013). The rRNA genes have been used as standard phylogenetic markers in microbial taxonomic studies since they are ubiquitously distributed across all archaea and bacteria have an evolutionarily conserved nature and a wide range of variable, discriminatory regions (Almeida et al., 2013; Moter and Gobel, 2000).

A standard FISH protocol targeting the typical rRNA region involves four different steps: fixation/permeabilization, hybridization, washing, and visualization/detection – for an extensive review on conventional FISH, see (Moter and Gobel, 2000).

Briefly, the first step is crucial since it must preserve rRNA integrity, cell shape, and prevent lysis, but at the same time must permeabilize the cell allowing probe diffusion (Moter and Gobel, 2000). Fixation is normally accomplished using paraformaldehyde or formaldehyde for Gram-negative bacteria and ethanol for Gram-positive bacteria (Moter and Gobel, 2000). Permeabilization is achieved with lysozyme, proteases, solvents, detergents, and/or organic acids, depending on the microorganism, leading to physical damage of the cell envelope due to the formation of pores wherein the probe can penetrate the cell (Rocha et al., 2018). The hybridization step can be influenced by the pH, ionic strength, and formamide concentration of the hybridization solution, or the hybridization time and temperature (Nettmann et al., 2013). The washing step ensures that all loosely bound or unbound labeled probes are removed from the sample, hence it is one of the measures providing specificity to the detection process (Nettmann et al., 2013). FISH is commonly performed in two formats: with physical cell support, on a microscope slide or filter membrane, followed by visualization using conventional epifluorescence or confocal microscopy; or with cells in suspension, followed by FLOW detection and, eventually, FACS (Amann and Fuchs, 2008).

2.2. How to bait your FISH

A FISH probe catalog of previously designed and used probes is openly available in probeBase (Greuter et al., 2016). For *de novo* probe design, a variety of approaches can be used through different software (Wright et al., 2014), with mathFISH being one of the most used (Yilmaz et al., 2011). Each *de novo* synthesized probe needs to be extensively optimized through an experimental approach using mocked mixed communities to ensure specificity and sensibility of taxonomical identification within complex microbial communities. Complementary, Clone-FISH was developed to investigate the accessibility of the selected target site and evaluate optimal hybridization conditions for probes when no pure cultures are available (Schramm et al., 2002).

133 The application of this technique potentiates a higher fluorescence signal-to-noise ratio (Kubota
134 et al., 2006).

135 There are different types of FISH probes (Fig. 1), ranging from a small number of nucleotides,
136 with < 50 nt (oligonucleotides), to enormous probes with almost 1 kb (polynucleotides); from
137 conventionally made probes derived from PCR protocols, such as DNA probes for gene
138 detection, or RNA probes for gene expression analysis, to DNA mimics such as locked nucleic
139 acids (LNA) and peptide nucleic acids (PNA). Polynucleotide probes have higher signal intensities
140 than oligonucleotide probes, being useful for detecting microbes with low ribosome content or
141 aimed at single genes (Trebesius et al., 1994). However, these probes show a reduced
142 hybridization efficiency due to difficulties in penetrating the cell wall (Trebesius et al., 1994).
143 LNA probes have increased affinity towards the target, but high levels of self-annealing, since
144 they possess high thermal stability (Cerqueira et al., 2008). PNA probes are hydrophobic,
145 allowing easy penetration into the cell cytosol and an improved diffusion through bacterial
146 colonies or biofilm structures (Almeida et al., 2013; Lopes et al., 2018). However, synthesis of
147 PNA probes is more expensive compared with traditional ones. A recent protocol using quantum
148 dots (QD) as FISH probes was also developed (Liu et al., 2018a). QD allow the resolution of a high
149 number of working wavelengths since they possess narrow emission bands with minimal
150 spectral overlap (Liu et al., 2018a). However, QD are bulky and have lower mobility than free
151 nucleic acids, bringing some obstacles to FISH-based methodologies (Liu et al., 2018a); QD have
152 decreased efficiency in entering the cell, need improved cell permeabilization and longer
153 incubation periods.

154 In conventional FISH approaches, probes are directly labeled (Fig. 1a,b) in one of the extremities
155 (5'-end or 3'-end) with a variety of fluorophores with different excitation and emission
156 wavelengths, which can be selected based on the matrix and/or cell autofluorescence signals

and also circumvent technical limitations of the equipment used to visualize the fluorescence signal (Bottari et al., 2006).

Standard FISH suffers from several limitations that may prevent the successful detection of the target microorganisms (Moter and Gobel, 2000). Among probe design and evaluation, problems can occur related to the limited accessibility of probe to the target site, polymicrobial cultures, and difficulties to reach optimal hybridization conditions; regarding detection, the complexity of environmental samples can result in low concentration of target cells or these might not be detected due to low ribosome content, low copy gene, or lack of permeabilization, with additional unsatisfactory signal-to-noise ratio; cells within biofilms and cell aggregates can be difficult to count and always require manual counting; finally, the analysis of general metabolic activities and specific functions can be hampered due to the lack of correlation between ribosome content and cells' metabolic activity and physiology (Moter and Gobel, 2000). In the next sections, several improvements to the conventional FISH methodology are proposed to transcend specific limitations ranging from low signal detection, multiple targets, analysis of specific ecological niches or biotic relations, multiparametric assessment of taxonomical and metabolic profiles, and sub-cellular resolution (Table 1).

2.3. Amplification approaches to circumvent low signal detection

Conventional FISH has a major limitation: the low signal detection of the cell due to low target accessibility or low target content. Accessibility of probes to target sites can be improved by different methods, however, all of them have specific limitations. For example, the use of unlabeled helper probes (Fuchs et al., 2000) may improve accessibility but their design is often impossible for probes with broader specificities; the extension of the hybridization time usually resolves the problem, still, this may lead to unspecific probe binding or unspecific dye-binding in complex samples (Yilmaz et al., 2006); the elongation of the probes is also a valid solution, yet it is usually coupled with a specificity decrease (Yilmaz et al., 2006); PNA probes can easily enter

cells but are very expensive and are not directly converted from published oligonucleotide probes without a change in specificity (Cerqueira et al., 2008).

Indirect probe labeling can help in signal amplification and can be accomplished using: (i) a reporter molecule like digoxygenin (DIG), biotin, or dinitrophenol, that is then detected by a higher intensity fluorescent antibody (Fig. 1c); (ii) horseradish peroxidase (HRP) that uses fluorophore–tyramide as a substrate for enzymatic signal amplification, in a process named tyramide signal amplification (TSA) (Fig. 1d); (iii) the combination of TSA systems with polynucleotide probes internally labeled with a reporter molecule (Bottari et al., 2006) (Fig. 1e).

Catalyzed Reporter Deposition (CARD)-FISH is an example of an enzymatic signal amplification technique that has been introduced as an *in situ* signal amplification method based on HRP-labeled oligonucleotide probes and TSA (Hoshino et al., 2008) (Fig. 2a). The resulting fluorescence signal is 26 to 41 times higher than with mono-labeled FISH, making the visualization of hard-to-detect cells possible (Hoshino et al., 2008). Thus, this method has been established for detecting microorganisms in oligotrophic habitats, such as seawater (Kleindienst et al., 2015; McNichol et al., 2018), marine sediments (Hoshino et al., 2017; Pohlner et al., 2017), freshwater (Alfreider et al., 2018; Crognale et al., 2017), permafrost (Winkel et al., 2018), and soil (Cai et al., 2016; Probandt et al., 2017; Schmidt and Eickhorst, 2014). Also, this methodology can be used in microbiota-host interaction studies (Golyshina et al., 2017; Schmidt et al., 2018). However, CARD-FISH is rather expensive, requires enzymatic pretreatment to allow the large HRP-labeled probes to penetrate the target cells, requires inactivation of endogenous peroxidases, and causes a dramatic alteration in the melting behavior of the probes (Pernthaler and Pernthaler, 2007). Furthermore, the use of CARD-FISH to multicolor imaging, targeting different groups in a single sample, is very time-consuming, because sequential hybridizations for each target need to be made (Pernthaler and Pernthaler, 2007).

206 The two-pass CARD-FISH protocol is an improvement of the conventional CARD-FISH protocol.
207 This newly improved protocol relies on a sequential catalyzed reporter deposition based on the
208 binding of HRP-conjugated anti-fluorescein antibodies to the fluorophores deposited during the
209 initial CARD-FISH step, followed by a second catalyzed reporter deposition signal amplification
210 with fluorescein-labeled tyramides (Kubota et al., 2006; van de Corput et al., 1998) (Fig. 2b). This
211 protocol was first developed to study eukaryotic cells (van de Corput et al., 1998) and adapted
212 to prokaryotic cells by Kubota and colleagues (2006) (Kubota et al., 2006). Since then, this
213 protocol has been applied to study prokaryotic cells in single-gene detection approaches in
214 anaerobic sludge samples to detect methanogens and sulfate-reducing bacteria (Kawakami et
215 al., 2012) and in freshwater samples to detect ultramicrobacteria (Neuenschwander et al.,
216 2015). Two-pass CARD-FISH increased the detection efficiency up to 98% via the increasing of
217 the length of the probe (up to 820 nt) and adding a second CARD step (Kawakami et al., 2012).
218 The main drawback of the two-way CARD-FISH protocol is its higher cost and more time-
219 consuming protocol when compared with CARD-FISH; also the probe length extension may
220 hamper the efficient probe penetration into the cell.

221 An additional enzymatic-based method makes use of a nucleotide probe that consists of single-
222 stranded RNA generated via *in vitro* transcription (Zwirglmaier et al., 2005; Zwirglmaier et al.,
223 2004). During transcription, the probe is labeled with a reporter molecule that has a labeling
224 density of about one labeled nucleotide every 10–20 nt (Pratscher et al., 2009). The probe then
225 anchors other probes, which in turn form a network around the cell periphery, resulting in a
226 halo-shaped fluorescent signal (Pratscher et al., 2009). This phenomenon occurs due to the
227 folding of the single strand of RNA probe into secondary structures, forming a complex network
228 in the cell during hybridization (Pratscher et al., 2009) (Fig. 2c). This network concept allows the
229 number of probe molecules contributing to the signal to greatly exceed the number of target
230 molecules and, thus, to amplify the signal intensity (Zwirglmaier et al., 2004). This method is
231 named Recognition of Individual Genes (RING)-FISH and it increases signal amplification

between 10- and 50-fold when compared to conventional FISH (Pratscher et al., 2009; Zwirglmaier et al., 2005; Zwirglmaier et al., 2004). RING-FISH is typically used to study the phylogenetic affiliation of members of denitrifier communities in enriched activated sludge (Pratscher et al., 2009), the composition of archaeal and bacterial communities in marine plankton samples (Pernthaler et al., 2002), and the distribution of secretion systems in *Vibrio parahaemolyticus* from water, oyster, and sediment samples (Noriea et al., 2010). However, this technique has some limitations, such as: probe penetration due to the preferential use of polynucleotide probes; increased cost, due to the high amounts of probe used; and low specificity, since this method generates a halo-shaped signal (Zwirglmaier et al., 2005).

Besides probe systems modifications, target amplification can be performed by PCR-based technologies or by isothermal amplification-based technologies. Among these technologies, a recent approach was developed to overcome the identification of microorganisms in environmental samples that contain low rRNA content, when targeting low copy genes, or probes that have low permeability (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). This method relies on *in situ* DNA-hybridization chain reaction (HCR) (Fig. 2d). HCR is based on two different fluorescently labeled hairpins probes and an initiator nucleotide comprising the complementary sequence of the target gene and the complementary sequence to one of the hairpin probes (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). These hairpins probes are stable in the absence of the initiator. In its presence, the initiator hybridizes with the portion of the complementary hairpin probe, leaving the remaining single-strand portion of the first hairpin probe to hybridize with a portion of the second hairpin probe (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). This hybridization leads to the formation of a single strand part of the second hairpin probe similar to the initiator, promoting the hybridization of the first hairpin probe (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). This cascade of polymerization reactions of

the fluorescently labeled hairpin probes linearly increases the fluorescent signal intensity (Nikolakakis et al., 2015; Yamaguchi et al., 2015a; Yamaguchi et al., 2015b). This method showed an up to 8-fold higher sensitivity than conventional FISH, being a suitable alternative to CARD-FISH, particularly for cases when strong cell permeabilization or CARD reactions should be avoided. This methodology was used in gene expression studies involving symbiotic partners (Nikolakakis et al., 2015). More recently, HCR-FISH was optimized to be used in the detection of anaerobic methanotrophic archaea in marine sediments (Jia et al., 2021).

2.4. Multiple probes for multiple targets

FISH offers the possibility to detect/identify more than one target in one single experiment. This possesses great interest in multiple taxonomic identifications, concomitant taxonomical and functional identification, or intracellular relationships between guest and host cells.

Double labeling of oligonucleotide probes (DOPE)-FISH (Pernthaler et al., 2002; Stoecker et al., 2010) offers a doubled signal intensity. This technique offers more options for the design of specific probes: probes may be labeled at the 5' and 3' ends with the same fluorophores or two different ones (Fig. 2e). These options not only enable signal amplification when the same fluorophore is used but also enable multicolor probe combinations that can target up to six organisms in a single FISH experiment (Behnam et al., 2012; Schimak et al., 2016). Recently, DOPE-FISH has been applied in different contexts, such as the evaluation of bacterial endophytes composition (Glassner et al., 2015), assessment of changes in plant microbiomes (Mitter et al., 2017), the discovery of iron-oxidizing biofilms in stromatolitic iron-rich structures (Heim et al., 2017), examination of active microbial biofilms composition in subsurface rocks (Escudero et al., 2018), assistance in the isolation process of "*Candidatus*" species (Beam et al., 2015; Lehtovirta-Morley et al., 2016), detection of novel extracellular gut symbionts (Kroer et al., 2016), identification of primary fermenters in anaerobic digesters of sludge (McIlroy et al., 2017), and investigation of the structure and abundance of methanogenic archaea and methane-oxidizing

bacteria in peat bog lakes (Lew and Glińska-Lewczuk, 2018). A drawback of DOPE-FISH is the limited number of taxa that can be labeled simultaneously in one sample, due to the highly overlapping excitation and emission spectra of fluorophores (Valm et al., 2012; Valm et al., 2011).

To increase the number of microorganisms that can be concomitantly targeted in polymicrobial samples, Combinatorial LAbeling and Spectral Imaging FISH (CLASI-FISH) can be employed (Valm et al., 2012; Valm et al., 2011). This approach is based on the premise that a unique spectral tag is allocated to each target group of microorganisms by conjugation of mono-labeled probes carrying one fluorophore each to the same binding site (Fig. 2f). The combination of emitted wavelengths is revealed by linear unmixing and spectral imaging with modern confocal laser scanning microscopy (CLSM). However, the expected 50:50 ratio of probes can be switched to one side due to the difference of affinity between probes, further complicating the identification of target organisms (Behnam et al., 2012; Schimak et al., 2016). Behnam and colleagues (2012) proposed the combination of DOPE-FISH with spectral unmixing (such as in CLASI-FISH), enabling the use of oligonucleotide probes labeled with different binary combinations of dyes to increase the potential of multicolor FISH for the analyses of samples with elevated levels of background fluorescence (Behnam et al., 2012).

Welch and colleagues (2016) studied the human oral microbiota employing high-throughput sequencing data to identify the major bacterial taxa in the supragingival plaque, followed by CLASI-FISH for the direct visualization of their spatial structure, which allowed the description of a complex, spatially organized, multi-genera consortium (Mark Welch et al., 2016). This workflow by Welch and colleagues (2016) might be considered as a case study in microbial biogeography at the micron scale (Mark Welch et al., 2016). Following this study, the same approach was used to study the spatial organization in gnotobiotic mice's gut of a defined 15-member taxa community present in the human gut (Mark Welch et al., 2017).

Schimak and colleagues (2016) demonstrated the applicability of multi-labeled oligonucleotide probes synthesized by a “click” reaction on complex microbial populations (Schimak et al., 2016). This procedure named MiL-FISH allows the simultaneous targeting of up to seven microbial groups using a multi-labeling oligonucleotide probe strategy that can improve amplification signal, when conjugated with acrylic resin for precise localization of individual microbial cells, or sort unfixed environmental microorganisms (Schimak et al., 2016). This methodology was used to study the small bacterial community of gutless oligochaete *Olavius algarvensis* (Schimak et al., 2016). This new type of probe can also be used in CLASI-FISH with a single site targeted by a four-times-labeled probe, instead of using four mono-labeled probes targeting four sites on the 16S rRNA, thus resulting in increased specificity and sensitivity (Schimak et al., 2016). To improve signal amplification in low rRNA content targets, four-times-labeled probes carrying the same fluorophore combination can be applied to several sites on the 16S rRNA molecule (Schimak et al., 2016).

2.5. Taxonomy, function, and metabolism – a multi-parametric analysis of microbial communities

Marking multiple cells in the same sample can be accomplished as previously stated, however, targeting multiple genes in the same cell is also possible. One of the first methods applying this principle was GeneFISH. It consists of the detection of two different targets: first, the gene of interest (normally, a functional gene) and then the rRNA of the microorganism under study (Moraru et al., 2010). The first step uses double-stranded DNA probes labeled with DIG molecules, in a CARD-FISH methodology. The rRNA detection for taxonomical identification is accomplished by CARD-FISH using HRP-labelled oligonucleotide probes. GeneFISH was successfully used in microbial identification in a diversity of environments, including marine sediment enrichments (Lenk et al., 2012), marine seep sediments (Stagars et al., 2016), marine bacterial - eukaryotic symbiotic systems (Bernhard et al., 2012; Petersen et al., 2011), upwelling

seawater samples for the study of planktonic microbial communities (Moraru et al., 2010), and groundwater samples (Matturro and Rossetti, 2015).

Targeting two genes in the same cell is not the only way to assess both taxonomical and functional information about environmental microorganisms. Isotopic-based techniques can also be used, namely microautoradiography (MAR)-FISH. This method is a microscopic technique that allows simultaneous visualization of the target microorganism and specific activity at the single-cell level, due to the uptake of radioactively-labeled substrates (for a review see (Wagner et al., 2006)). However, several limitations are reported (Wagner, 2009): the impossibility to detect several labelling isotopes simultaneously and low single-cell resolution in dense microbial aggregates; the risk of radiation exposure; inexistence of isotopes with adequate half-life time for some important elements (e.g. nitrogen and oxygen), impairing the application of this technique to study nitrogen fixation, for example; the quantification of incorporated isotope requires an internal standard of bacteria with known isotopic composition; time-consuming process; and impaired downstream analysis due to cell fixation. Besides all these limitations, this technique is still used nowadays for water samples (McIlroy et al., 2016; Teira et al., 2017). Additionally, MAR-FISH can be coupled with a signal amplification methodology, CARD-FISH, applied to mRNA or single genes as the target or in the context of oligotrophic environments. MAR-CARD-FISH was used to calculate a specialization index regarding bacterioplankton ability to use phytoplankton-derived dissolved organic carbon from different phytoplankton species and at different concentrations (Sarmento et al., 2016); to identify which microbial groups are involved in dark dissolved inorganic carbon uptake in the Atlantic Ocean (Guerrero-Feijóo et al., 2018); to quantify the biomass production of specific taxonomical bacterial groups present in ocean samples between subtropical and Antarctica locations (Bakenhus et al., 2018); and to characterize the incorporation ability of bicarbonate carbon sources by ammonia-oxidizing archaea "*Candidatus* Nitrosocosmicus exaquare" recovered from municipality wastewater treatment plants (Sauder et al., 2017).

359 Besides MAR-FISH, stable-isotope probing (SIP) also relies on isotopically labeled cells. SIP
360 method is based on the incorporation of an isotope with low natural abundance into
361 components of the biomass of microbial cells using an isotopically enriched substrate
362 (Radajewski et al., 2000). When associated with FISH, this method allows linking the diversity,
363 abundance, and function of a given single-microbial cell. SIP-FISH can be used in the study of
364 complex biogeochemical cycles. This technique was used to identify active methanol-utilizing
365 microorganisms from soil samples (linked to carbon cycle) (Radajewski et al., 2000); to identify
366 active denitrifiers in full-scale nutrient removal wastewater treatment plants (nitrogen cycle)
367 (McIlroy et al., 2016); and to identify active sulfate-reducing bacteria at marine seeps (sulfur
368 cycle) (Kleindienst et al., 2014); as well as in the identification and characterization of propane-
369 and butane-degrading communities from marine hydrocarbon cold seeps (Jaekel et al., 2012).

370 A non-isotopic dependent technique can also be applied as an alternative to study microbial
371 ecophysiology, relying instead on the active cell-labeling with chemical modification of
372 biomolecule analogs (Hatzenpichler et al., 2014). This synthetic metabolites-based approach
373 named biorthogonal noncanonical amino acid tagging (BONCAT) has been coupled with FISH
374 methods to study archaea and bacteria within marine seawater and sediments (Hatzenpichler
375 et al., 2016; Hatzenpichler et al., 2014; Leizeaga et al., 2017). After the uptake of the
376 biorthogonal synthetic amino acid, the aminoacyl tRNA synthetase incorporates this amino acid
377 into *de novo* peptides (Hatzenpichler et al., 2016) (Fig. 2g). The protein-active cells can be
378 visualized using a reaction that conjugates a modified fluorescence dye to a chemical reporter
379 group of the amino acid (Hatzenpichler et al., 2016).

380 **2.6. Parallel detection of virus and host cells**

381 The study of specific ecological niches requires specific assessment techniques, specially
382 designed or adapted to those scenarios. One of those ecological niches is guest-host
383 interactions, both pathogenic, parasitic, or symbiotic. The unique interplay between virus and

their hosts is one example of such interaction. Allers and colleagues (2013) introduced a method based on GeneFISH for targeting bacteriophages (phageFISH), in the host-context (Allers et al., 2013; Dang et al., 2015). This new methodology enables the detection of phage genes in free virus particles, with a sensitivity of about 100%; in addition, it enables the reliable simultaneous visualization of phage genes and host rRNA inside infected cells, as well as the relative quantification of the intracellular phage DNA signal (Allers et al., 2013). The phageFISH technique holds great potential for the development of viral ecology in the context of environmental samples with complex matrices. However, the necessity of prior knowledge of phage sequence variation for probe design is a disadvantage. More recently, Castillo et al. (2020) developed a similar method, named VirusFISH that allows the visualization and quantification of virus-infected cells during an infection cycle under experimental conditions (Castillo et al., 2020). Besides the application *in vitro*, the potential transposition of this approach to polymicrobial communities is enormous, for instance in the context of research focusing on pathogenic and symbiotic relationships naturally occurring in environmental settings, not only in virus-host interactions but also in the study of predatory and symbiotic relationships between bacteria and protozoa.

2.7. Plant leaves matrices and their autofluorescence – how to overcome this limitation?

Some matrices can be particularly hard to work with, especially due to high autofluorescence. It is the case of leaves and epiphytic microbial communities. This problem can be overcome using TAPE-FISH, a FISH protocol that relies on the transference of surface microorganisms to an adhesive tape (Bisha and Brehm-Stecher, 2009; Remus-Emsermann et al., 2014). After the transfer, the recovered microorganisms are dehydrated and hybridized directly on adhesive tape, allowing the analysis of spatial arrangement patterns of both adaxial and abaxial phylloplanes (Bisha and Brehm-Stecher, 2009; Remus-Emsermann et al., 2014). A recent study explored this method (Leaf-FISH), developing a robust and easily transferable protocol, based

409 on ethanol-mediated pigment removal and CLASI-FISH, to create a tridimensional image of the
410 leaf enabling the visualization of multispecies microbiota that can directly be associated with
411 the leaf microstructure (Peredo and Simmons, 2018). This methodology has the potential to
412 disclose microbial preferences for microhabitats, colonization strategies, microbial interactions,
413 and plant/host interactions with particular emphasis to leaf microbiome (Peredo and Simmons,
414 2018), but also to any spatial microbiome study in a variety of biological and substrate surfaces.

415 **2.8. How to study cell-cell and cell-substrate interactions in biofilms and cell aggregates?**

416 One of the major limitations of FISH technologies is the difficulty associated with studying intact
417 biofilms and cell aggregates since the discrimination of fluorescence signals from overlapping
418 cells through more conventional image acquisition systems is hampered. Atomic force
419 microscopy (AFM) enables the quantification of the interactions between microorganisms and
420 surfaces. It is used, in particular, in studies focusing on surface colonization and biofilm
421 formation. AFM can be combined with FISH, providing a detailed analysis of interactions
422 between microorganisms and different surfaces, such as sludge digesters matrices (Hao et al.,
423 2018), and the formation and development processes of microbial biofilms (Bao et al., 2018).

424 Besides microscopic imaging improvements, other methodologies can be applied to better
425 understand natural polymicrobial biofilms. Pernthaler team (2008) developed a new FISH
426 method named magneto-FISH to enrich and characterize microbial community associations in
427 environmental samples (Pernthaler et al., 2008). Magneto-FISH combines an in-solution CARD-
428 FISH approach using 16S rRNA oligonucleotide probes with the immunomagnetic sediment
429 matrix separation mechanism that captures hybridized cells. Paramagnetic beads coated with
430 an antibody targeting the fluorophore applied in the CARD-FISH procedure are used for matrix
431 separation (Pernthaler et al., 2008). This method enables the capture and assessment of whole
432 microorganisms and cell aggregates from complex environments, without the loss of existent
433 interspecies associations and corresponding metabolic properties (Pernthaler et al., 2008).

In a study by Escudero *et al.* (2018), several FISH signal enhancement methods, such as CARD-FISH, DOPE-FISH, and MiL-FISH, were used to improve signal detection and increase the number of different taxa that could be detected simultaneously (Escudero et al., 2018). Additionally, a Gene-FISH hybridization buffer was used to decrease background noise due to the presence of salmon sperm DNA or yeast RNA that act as extra blocking reagents (Escudero et al., 2018). Moreover, a fluorescence lectin-binding assay (FLBA) was also performed based on fluorophore-labeled lectins in combination with other specific stains for DNA, proteins, and lipids (Escudero et al., 2018). This polyphasic approach was used to study biofilm formation, cell aggregates, and cell attachment to the substrate in deep poor porous continental subsurface rocks (Escudero et al., 2018).

2.9. More than a single cell – a higher resolution at a sub-cellular level

In the last decade, several improvements were made towards increased resolution, not only concerning single-cell visualization but also the sub-cellular visualization of important microbial sub-compartments. These efforts are driven by the increased interest in microbial metabolites in ecological microbiology, and the sub-cellular precision needed to evaluate their localization and quantification *in situ* (Kaltenpoth et al., 2015). To accomplish this, both mass-spectrometric imaging (MSI) and super-resolution microscopy (SRM) techniques were shown to be excellent tools. Matrix-assisted laser desorption ionization time-of-flight MSI (MALDI-TOF/MSI) was used coupled with FISH to concomitantly monitor antibiotic production and taxonomic identification in the defensive symbiosis between beewolf wasps and *"Candidatus* Streptomyces philanthi" (Kaltenpoth et al., 2015). The combination of both methodologies was used to identify active microbes in the mouse fecal microbiota (Berry et al., 2015). In this study, the incorporation of heavy water (D_2O) was analyzed by Raman spectroscopy (an MSI technique) to detect incorporation into microbial biomass, together with nanoscale-resolution secondary ion mass spectrometry (NanoSIMS; SRM technique) to obtain the labeling pattern of D_2O incorporation

(Berry et al., 2015). The detection of D_2O incorporation can additionally be coupled with FISH to identify metabolically active microbes (Berry et al., 2015). Moreover, the combination of epifluorescence microscopy with scanning electron microscopy (SEM) was developed to allow the evaluation of CARD-FISH on a cellular level, followed by a subcellular level evaluation of probe-target location, significantly increasing resolution (Schmidt et al., 2012). This new method named Gold-FISH is enabled by the high-affinity binding of streptavidin-Alexa Fluor 488 fluorophore/nanogold conjugates to biotin, in the last CARD-FISH step, followed by autometallographic enhancement of nanogold particles, before scanning electronic microscopy (SEM) detection (Schmidt et al., 2012). The applicability of this technique was optimized in rice roots and marine sediment samples (Schmidt et al., 2012).

3. Flow Cytometry (FLOW)

3.1. Principles and workflow of a rising technique

In the environment, bacteria tend to grow embedded in microcolonies or biofilms, adopting a community lifestyle. For single-cell analyses using flow cytometry, sessile cells must be put in suspension, requiring the use of efficient detachment methodologies for cell-substrate and cell-cell dissociation (Müller and Nebe-von-Caron, 2010). Cell detachment can be accomplished using chemical and/or physical processes. Different classes of chemical reagents can be used for cell detachment: non-ionic surfactants (e.g. Tween®) (Amalfitano and Fazi, 2008; Lavergne et al., 2014; Vignola et al., 2018); salts (e.g. trisodium citrate) (Hickey et al., 2018; Vignola et al., 2018); redox agents (e.g. dithiothreitol) (Ben-Amor et al., 2005); or ionic dispersants (e.g. sodium pyrophosphate) (Amalfitano and Fazi, 2008; Nettmann et al., 2013). These chemical reagents usually work by weakening intermolecular forces that attach the cell to the substrate (Müller and Nebe-von-Caron, 2010). Physical detaching can be more often accomplished by vortex (Bressan et al., 2015), sonication (Amalfitano and Fazi, 2008), or ultrasounds (Frossard et al.,

2016). These physical methods release bacteria that are entrapped in micropores or channels (Müller and Nebe-von-Caron, 2010).

After detachment, cells and environmental matrices coexist in solution and need to be separated. To do this, density centrifugation (Amalfitano and Fazi, 2008; Frossard et al., 2016), or simply low-speed centrifugation (150 x *g*) (Bressan et al., 2015), can help deposit large and/or dense particles. To remove smaller particles, filtration with different pore size filters can be applied (Albright and Martiny, 2017; Plominsky et al., 2018).

In many studies, FLOW analysis is performed with fixed cells. The fixation is important to maintain cell stability and morphology characteristics (Müller and Nebe-von-Caron, 2010). Several types of fixating agents can be used, namely formaldehyde (van Gelder et al., 2018), paraformaldehyde (Takahashi et al., 2015), and glutaraldehyde (Vignola et al., 2018). However, fixating agents can cause cell agglutination and increased autofluorescence, obscuring FLOW analysis (Müller and Nebe-von-Caron, 2010). Agglutination is due to cells' production of exopolysaccharides in response to stress led by the fixation agent (Müller and Nebe-von-Caron, 2010). Nevertheless, fixation allows cell conservation at -20˚C for several months. When the final goal of the FLOW analysis is cell sorting and further cell utilization, fixation of cells is highly unrecommended due to loss of cultivability and difficulty in DNA sequencing arising from covalent cross-links between DNA molecules (Müller and Nebe-von-Caron, 2010).

In flow cytometry, microbial cells can be directly analysed and/or stained with fluorophores (Fig. 3). The direct analysis allows the evaluation of two main cell characteristics: cell size and cell complexity. Cell size can be assessed through the fraction of scattered light collected in the same direction as the incident light (named Forward Scatter, FSC), which is as intense as the cell size (Léonard et al., 2016). Cell complexity can be assessed through the fraction scattered of light collected orthogonally to the incident light (named Side Scatter, SSC), which is related to morphological characteristics, such as cell surface roughness, cell membrane, nucleus, internal

granular material, and organelles (Léonard et al., 2016). Besides cell size and complexity, direct analysis can also be performed on autofluorescence cells, such as chlorophyll-containing ones.

The use of fluorophores enables multiparametric cell morpho-physiology assessment approaches, including membrane integrity, intake activity, membrane potential, pH gradient, metabolic activity, lipid content, and Gram character (Table 2; Fig. 4).

Staining bacteria is a complex process due to a variety of parameters that can influence this process, namely dye chemistry, target organism, and staining conditions (mostly time and temperature) (Buysschaert et al., 2016). To minimize the difficulty of the process, several steps need to be optimized and standardised, namely dye concentration, the buffer used to resuspend cells, time and temperature of dye incubation, and utilization (or not) of different types of fixation and permeabilization methodologies (Buysschaert et al., 2016).

The population's physiological status using a single fluorophore is sometimes difficult to assess, due to complex relations between physiological parameters, for example, membrane integrity, metabolic activity, membrane potential, and stress response. The absence of membrane potential could be related to the loss of membrane integrity or instead be related to the lack of metabolic ability to maintain that potential. For this reason, a multi-parameter flow cytometric analysis using different fluorophores simultaneously is normally performed. This approach overcomes interpretation problems associated with the complexity between viability parameters, but also the characterization of intermediate states, such as dormancy (Léonard et al., 2016). To combine different fluorophores, it is important to choose those who possess the right spectral proprieties, to determine their incubation time, incubation conditions, and concentration for each one separately, and then assess possible interferences (Léonard et al., 2016). There are three main interferences in staining procedures: overspill, fluorescence resonance electron transfer (FRET), and/or matrix quenching (Buysschaert et al., 2016). The first can be solved by applying a compensation matrix during data analysis, the last two lead to

decreased fluorescence of one of the fluorophores by quenching and should be solved by selecting different fluorophore combinations (Buysschaert et al., 2016). If two fluorophores can be used simultaneously, one of two things can happen (Léonard et al., 2016): either both fluorophores have discriminatory power and four quadrants can be observed corresponding to four different physiological states; or only one fluorophore has discriminatory power and the other can enter all cells, resulting in two possible physiological states.

3.2. Community profiling

Environmental microbial communities can be profiled using different parameters, being the most used: cell size (FSC); cell complexity (SSC); autofluorescence; and/or permeant fluorophores that label cells indiscriminately.

One common application of autofluorescence analysis is the phytoplankton community profiling which possesses both autotrophic picoeukaryotes (chlorophyll-containing cells) and autotrophic prokaryotes (Bernard et al., 2019). Chlorophyll (green pigment) detection can be accomplished by its emission of red fluorescent light (Bernard et al., 2019). Another example focuses on the analysis of phycoerythrin by its orange fluorescence emission light to discriminate photosynthetic and heterotrophic cells from cave biofilm samples (Borderie et al., 2016).

The analysis of scattering plots of morphological cell parameters (FSC or SSC) *vs* fluorescence emitted by the permeant fluorophore allows the definition of a community cytometric fingerprint that represents the microbial community structure by the number and position of clusters and the number of cells within each cluster.

There are a set of frequently used permeant fluorophores to perform community profiling: SYTO™ dyes, SYBR™ Green dyes, and 4',6-diamidino-2-phenylindole (DAPI). The first group is extensively used due to their advantageous characteristics, namely high signal to background fluorescence, high molar absorptivity, permeant to nearly all cell membranes, and high quantum yields when bound to nucleic acids (Hammes et al., 2011; Léonard et al., 2016). SYBR™ Green

dyes, such as SYBR™ Green I and II, are green-permeant fluorophores (Grégori et al., 2001). SYBR™ Green II shows higher sensitivity and higher quantum yield while keeping a strong affinity for double-stranded DNA, about half of SYBR™ Green I (Grégori et al., 2001). Other fluorescent dyes can be used, namely 4',6-diamidino-2-phenylindole (DAPI) that can be used to stain all cells due to its high cell permeability and high affinity to DNA, being excited using UV light and emitting in the blue wavelength (460 nm) (Hammes et al., 2011; Léonard et al., 2016). Moreover, some fluorophores can selectively bind to RNA molecules, namely pyronin-Y, allowing the discrimination between high and low RNA content cells, dead cells, and debris (Peris-Bondia et al., 2011). In environmental samples, DNA-permeant stained cells are normally grouped in two clusters: high (HNA) and low (LHA) nucleic acid content cells. HNA cells have been shown to have an increased cell division and high metabolic activity comparing to LNA cells (Hammes and Egli, 2010; Krause et al., 2020; Liu and Müller, 2020; Santos et al., 2019; Wang et al., 2010).

Four methods can be used to evaluate cytometric fingerprints: Dalmatian Plot, Cytometric Histogram Image Comparison (CHIC), Cytometric Barcoding (CyBar), and FlowFP (for a full review and a systematic comparison, see (Koch et al., 2014b)). Furthermore, a more recently developed tool, *FlowEMM*. This is a fast tool that determines the number of gates automatically, separating the cell clusters from background clusters containing irrelevant information and calculates the real number of data points for each cell cluster (Ludwig et al., 2019).

3.3. Membrane integrity as the ultimate measure of cell viability

For membrane integrity evaluation, dye exclusion methods are normally preferable (Hammes et al., 2011; Léonard et al., 2016). These dyes bind to nucleic acids emitting fluorescence upon binding. Only membrane-compromised cells are permeable to the dyes. Propidium Iodide (PI) is one of the most frequently used to detect dead cells, showing exclusion proprieties due to its two positive charges (Hammes et al., 2011; Léonard et al., 2016). PI emits red fluorescence, being normally used in association with SYTO™ 9, an SYTO™ dye that produces green

583 fluorescence (Hammes et al., 2011; Léonard et al., 2016). This methodology is very frequently
584 used in pure culture analysis, however, it is rare in polymicrobial culture due to species-specific
585 staining heterogeneity. Nevertheless, it has been previously used to assess membrane integrity
586 in freshwater samples treated with UV light (Berney et al., 2007). Contrary, the conjugation of
587 SYBR™ Green I and PI results in more homogeneous and reproducible staining profiles. These
588 fluorophores were recently used to assess bacterial viability present in groundwater samples
589 recovered after temperature and pH disturbances (Song et al., 2019) and in freshwater samples
590 at a river catchment scale (Liu et al., 2019).

591 A recent integrative study assessed the effect of arsenic particles resultant from
592 biotransformation of soluble arsenic by rhizosphere fungi on soil-dwelling bacteria physiology
593 using several fluorophores to analyze membrane integrity and permeability. Propidium iodide
594 (PI) was used to assess membrane cell integrity as a proxy of cell viability, 1-N-
595 phenylnaphthylamine (NPN) to assess outer membrane permeability, and o-nitrophenyl-b-D-
596 galactopyranoside (ONPG) to assess inner membrane permeability (Mohd et al., 2019).

597 **3.4. Cell intake activity – measuring efflux pump activity and glucose intake**

598 Efflux pump activity is usually evaluated by the measurement of ethidium bromide (EB)-
599 associated fluorescence (Thomas et al., 1997). This dye possesses a positive charge with a
600 monovalent capacity towards DNA. When the membrane efflux pumps of the cell, namely non-
601 specific proton antiport transport system, are actively working, EB is pumped out of the cell, but
602 when the cell membrane is damaged and/or the efflux pumps are malfunctioning, EB is
603 accumulated intracellularly, binding to DNA and emitting yellow fluorescence (Thomas et al.,
604 1997). To our knowledge, there are no reports describing the use of this fluorophore to assess
605 pump activity in environmental polymicrobial communities.

606 Glucose intake can be investigated as a measure of cell viability. For this purpose,
607 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) can be used as

substrate. This fluorophore is an analog of glucose, being specifically taken inside the cell by the phosphoenolpyruvate phosphotransferase system (Strauber and Muller, 2010). A recent study takes advantage of this approach to identify uncultured rumen bacteria that can intake glucose, as a proof-of-concept to the use of NBD as a labeling fluorophore for substrate utilization by uncultured bacteria (Tao et al., 2019). The use of NDB can be applied in the characterization of bacterial niches by their intake capacity of different substrates.

3.5. Assessing membrane potential and pH gradient – two sides of the same coin

The membrane potential is an important cell physiological parameter since only living cells can maintain their membrane potential. When the membrane potential reaches zero, it means that membrane structure is compromised, and ions can freely cross the membrane. To evaluate membrane potential, different dyes can be used, but all of them have common proprieties: they are lipophilic molecules that can cross the membrane and be accumulated inside or outside the cell, depending on their charge (Buysschaert et al., 2016). The fluorescent signal is directly related to cell energy levels, however, due to their outer membrane, Gram-negative bacteria can be resistant to staining (Buysschaert et al., 2016). This problem can be overcome by washing cells with Tris-EDTA or citric acid (Buysschaert et al., 2016). The different dyes can be divided into two groups: cationic dyes that are accumulated inside polarized cells, and anionic dyes that are only accumulated inside cells with an altered polarization (Maurice et al., 2013; Pepè Sciarria et al., 2019). Among cationic dyes, carbocyanine ($DiOC_n(3)$) is the most common (Pepè Sciarria et al., 2019). In a recent study by Pepè Sciarria and colleagues (2019), microbial fuel cells were studied, namely both the anode biofilm and the planktonic broth of an acetate fed-batch (Pepè Sciarria et al., 2019). Both SYBR™ Green I and $DiOC_6(3)$ were used, the first to enumerate total microbial populations and the second to enumerate metabolically active microbial populations (Pepè Sciarria et al., 2019). Anionic dyes, when inside the cell, become associated with non-specific intracellular proteins, increasing dye concentration, and consequently fluorescence

intensity, being the most common bis-(1,3-dibutyl barbituric acid)-trimethine oxonol ($DiBAC_4(3)$) (Maurice et al., 2013). This fluorophore was used in a study where the action of xenobiotics over the physiology of the human gut microbiome was assessed (Maurice et al., 2013). In this study, $DiBAC_4(3)$ proved useful as it was accumulated inside depolarized cells, indicating increased damage after antibiotic exposure, with seasonal physiological variations to this exposure (Maurice et al., 2013).

The ability of cells to maintain a pH gradient is also a measurable viability parameter, strongly related to membrane potential. The intracellular pH can be assessed using the pH-dependent fluorescent probe carboxyfluorescein succinimidyl ester (cFSE) (Breeuwer et al., 1996). cFSE can conjugate with aliphatic amines and be retained within the cell, with green fluorescence (Breeuwer et al., 1996). A methodology using this fluorophore to detect viable *Clavibacter michiganensis* subsp. *michiganensis*, the causative agent of bacterial canker of tomato, has been previously developed (Chitarra et al., 2000).

3.6. Metabolic activity – respiration, enzymatic activity, and oxidative stress

Metabolic activity is positively correlated with viability only if the population under study does not show any signal of membrane damage, dormancy, or starvation (Creach et al., 2003; Hoefel et al., 2003). This parameter is analyzed using a non-fluorescent substrate that upon entrance into cells is enzymatically transformed into a fluorescent molecule accumulating inside the cell (Creach et al., 2003; Hoefel et al., 2003). The usually measured activities are respiration and enzymatic activity. The first is usually assessed using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) that competes with oxygen as an electron donor and is reduced by the active electron transport system of respiring cells in an insoluble fluorescent molecule named formazan with red fluorescence, that is retained inside the cell (Creach et al., 2003). The most metabolically active cells can be positively detected, but cells with low respiratory activity may not be detected due to the inner accumulation of CTC being highly cytotoxic (Creach et al., 2003). This

fluorophore was used, in a recent study, to evaluate microbial activity in Antarctica permafrost (La Ferla et al., 2017). The second is assessed using fluorescein diacetate (FDA) or derivates, such as carboxyfluorescein diacetate (cFDA), a lipophilic non-fluorescent precursor that enters the cell, is hydrolyzed by unspecific esterases into carboxyfluorescein (cF) that, due to its extra negative charges, is retained within the cell (Hoefel et al., 2003). However, this fluorophore has several disadvantages, namely dead cells that only recently died can possess residual enzymatic activity; many bacteria cannot uptake cFDA; the fluorescence signal tends to be weak; and fluorescence intensity may change with pH (Hoefel et al., 2003). Nevertheless, this fluorophore has been successfully used to assess bacterial viability in soil samples (Espina, 2020). Carboxyfluorescein diacetate succinimidyl ester (cFDA/SE) is similar to cFDA but differs in the presence of a succinimidyl ester (SE) group with the ability to bind to free amines (Hoefel et al., 2003). Its hydrolyzation by nonspecific esterases results in the formation of cF/SE that is a highly fluorescent amine-reactive fluorophore (Hoefel et al., 2003). Thus, cFDA/SE can more easily be retained inside bacterial cells, resulting in higher fluorescent intensity. However, the use of this fluorophore to evaluate river, reservoir, and marine water samples, showed a low differentiation capacity between active and inactive bacterial cells, with lower fluorescence signal intensity, when compared with cFDA (Hoefel et al., 2003).

Oxidative stress can also be evaluated using hydroethidine (HE). This fluorophore suffers oxidation by reactive oxygen species (ROS) and reactive nitrogen species (RNS), forming ethidium that intercalates DNA (Buysschaert et al., 2016). This fluorophore has been recently used to assess oxidative stress caused by salt on thermophilic starter cultures applied to cheese manufacture (Hickey et al., 2018). Moreover, CellROX™ Green is a fluorophore used to measure ROS in live cells, being a non-fluorescent permeant dye that upon oxidation binds to DNA and becomes fluorescent (Hickey et al., 2018). This fluorophore was used, in conjugation with PI, to assess free total ROS and membrane integrity of probiotic bacteria after several drying techniques (Fallico et al., 2020).

3.7. Mining lipid content

Lipid content can be evaluated to study cell physiology, but not as a proxy of cell viability. Many cells can storage lipids in vesicles. These lipids can be detected using Nile Red or boron-dipyrromethene (BODIPY™), a benzophenoxazone dye that detects non-polar lipid droplets inside cells, as well as the presence of storage lipids (PHA/PHB) (Buysschaert et al., 2016). To our knowledge, these fluorophores were not yet applied to environmental microbiology studies, however, they have the potential to selectively label intensive microbial producers and accumulators of lipids, which can thus be sorted and applied into biotechnological processes, such as biodiesel production.

3.8. Gram character – a modernized perspective on a historical character

The Gram stain is one of the most important and widely used differential stains for the taxonomic differentiation of bacteria. Gram character can be evaluated using flow cytometry: a combination of SYTO™ dyes that label both Gram-negative and Gram-positive bacteria, resulting in green fluorescent cells; and the nucleic acid stain, hexidium iodide (HI), that selectively stains almost all Gram-positive bacteria, resulting in red fluorescent cells, since it is blocked by the lipopolysaccharide layer of Gram-negative bacteria (Mason et al., 1998). However, this methodology only allows the selective staining of live bacteria, since dead bacteria may stain variably (Mason et al., 1998). Thus, this fluorophore combination was never used to assess the Gram character of environmental bacterial communities.

Additionally, Gram staining can also be evaluated using wheat germ agglutinin that can bind to N-acetylglucosamine in the peptidoglycan layer of the cell wall (Holm and Jespersen, 2003). However, due to the presence of the lipopolysaccharide in Gram-negative, these bacteria do not stain, but Gram-positive bacteria do so. The wheat germ agglutinin does not possess any fluorescence, so it must be conjugated with a fluorophore to be detectable through FLOW (Holm

and Jespersen, 2003). This technique has been previously applied to analyze milk samples (Holm and Jespersen, 2003) and pH influence on microbiota intestinal samples (Duquenoy et al., 2020).

4. Fluorescence *in situ* hybridization coupled with Flow Cytometry (FLOW-FISH)

FLOW-FISH allows a high-throughput characterization of the individual microbial cells based on their fluorescence resulting from *in situ* hybridization alone or together with physiology evaluating fluorophores, enabling the combination of both taxonomic identification, physiological characterization, and rapid and precise quantification of microbial communities (Müller and Nebe-von-Caron, 2010).

Several examples of FLOW-FISH applications to environmental microbial studies can be found in the literature. Friedrich and Lenke (2006) used this approach with fixation-free in-solution FISH to detect and quantify lactic acid bacteria, in particular, *Leuconostoc* and *Lactococcus* genera, from dairy starter cultures together with cell viability evaluation of the labeled bacteria with PI (Friedrich and Lenke, 2006). This project showed the advantage of FLOW-FISH to quantify the starter bacteria as an alternative to the conventional plate counting method (Friedrich and Lenke, 2006). Jen et al. (2007) labeled the clostridial hydrogenase gene using in-solution FISH with fixed cells of anaerobic hydrogen-producing systems from brewery yeast waste (Jen et al., 2007). In 2011, three very interesting articles were published using this methodology. The first one, by Manti and team (2011), described a very elegant protocol for in-solution and on-filter CARD-FISH applied to seawater samples to quantify the overall bacteria burden using FLOW (Manti et al., 2011). The utilization of on-filter CARD-FISH improved the recovery rate from seawater samples, even though the detachment of bacterial cells from the filter was imperative for the subsequent FLOW analysis (Manti et al., 2011). Secondly, a miniaturization of the FLOW-FISH method was obtained by microfluidic application, allowing the detection of *Desulfovibrio* spp. and *Pseudomonas* spp. from water samples (Liu et al., 2011). The last one used LNA probes to perform CARD-FISH to detect bacterial noncoding RNA (Robertson and Vora, 2012). Besides

733 the very low amount of noncoding RNA present in bacterial cells, good signal intensity was
734 obtained, due to the use of LNA together with CARD-FISH as a signal amplification approach
735 (Robertson and Vora, 2012). Nettmanni et al. (2013) studied the bacterial community of an
736 anaerobically fermented liquor by developing a new FISH-FLOW protocol (Nettmann et al.,
737 2013). This protocol is based on the hybridization of the bacterial cells with fluorophore-labeled
738 universal probes targeting bacteria and archaea members, followed by FLOW to quantify the
739 labeled community and performance of a dehydrogenase activity essay using CTC to evaluate
740 cell's metabolic activity (Nettmann et al., 2013). Takahashi team (2015) applied a FISH-FLOW
741 protocol to monitor the beer brewing process, targeting universal regions of bacteria and
742 eukaryotic cells (Takahashi et al., 2015). FLOW-FISH was also applied to identify different
743 taxonomical groups of bacteria present in samples of arsenic-rich waters of geothermal origin
744 using CARD-FISH (Crognale et al., 2017) and in rat feces to study the effect of cocoa compounds
745 using conventional FISH (Martin-Pelaez et al., 2017). Furthermore, Branco team (2019)
746 developed a FLOW-FISH protocol based on RNA-FISH to detect *Dekkera bruxellensis* in wine
747 samples using a species-specific probe targeting the 26S rRNA gene labeled with a red
748 fluorophore to avoid background noise from wine autofluorescence compounds (Branco et al.,
749 2019). Recently, the same team developed another FLOW-FISH protocol with a different probe,
750 this time a DNA-FISH probe to avoid the use of formamide due to its toxicity. The RNA target
751 was the same and was labeled with the same fluorophore to be applied equally in the wine
752 environment (Branco et al., 2020).

753 **5. FLOW-FISH coupled with fluorescence-activated cell sorting (FACS)**

754 **5.1. FACS general principles**

755 Cells can be singly sorted after FLOW analysis based on their autofluorescence, size, complexity,
756 viability, taxonomy, functional group, among others (Chen et al., 2017; Czechowska et al., 2008;
757 Koch et al., 2014a). This can be performed using different methodologies, being one of the most

758 used the droplet sorting principle. This technique is based on the formation of charged droplets
759 containing a cell of interest when passing by the area downstream of the light collection point
760 (Müller and Nebe-von-Caron, 2010). Droplets of interest are charged and then sorted using an
761 electrostatic field that is generated by deflection plates, while uncharged droplets that contain
762 other cells pass into the waste (Müller and Nebe-von-Caron, 2010). Additionally, the flow
763 diversion sort principle can also be applied. This approach relies on a pressure wave
764 (piezoelectricity) that directs the cells of interest into a different channel, while the remaining
765 cells pass into the waste (Müller and Nebe-von-Caron, 2010). This approach is advantageous
766 when compared to the previous one, since it is performed enclosed in the tubing system,
767 reducing the risk of aerosols of hazardous materials and the contamination of the sorted cells
768 (Müller and Nebe-von-Caron, 2010). FLOW-FISH-FACS allow the sorted cells to be used in
769 different applications, ranging from metagenomics, cultivation of functional communities of
770 microorganisms, characterization of microorganisms using single-cell omics, and depiction of
771 the viable community or spore community, allowing the understanding of their role on an
772 ecological niche (Chen et al., 2017; Czechowska et al., 2008; Koch et al., 2014a) (Fig. 5).

773 **5.2. FLOW-FISH & FACS for gene target amplification and sequencing**

774 One of the first works focusing on the downstream application of FLOW-FISH-FACS on
775 environmental samples was performed by Wallner and colleagues (1997). This team evaluated
776 activated sludge, lake water, and lake sediment using 16S rRNA oligonucleotide fluorescent
777 probes targeting *Acinetobacter* spp. and *Leptothrix* spp. in fixed cells, followed by detection and
778 cell sorting by FACS to subsequently perform 16S rRNA gene PCR amplification and sequencing
779 (Wallner et al., 1997). Additionally, on-filter CARD-FISH using fixed cell followed by FACS was
780 performed in marine water samples to detect bacterioplankton using conjugation of different
781 16S rRNA targeting probes to different bacterial taxonomical groups (namely α-, β-, and γ-
782 proteobacteria); they then performed 16S rRNA sequencing, allowing a diversity study of these

783 different groups (Sekar et al., 2004). FLOW-FISH was also used to study
784 "*Candidatus* Accumulibacter phosphatis" in activated sludge with different clade-specific
785 probes, with the main goal of evaluating the polyphosphate kinase-1 gene homologs of the
786 sorted cells by PCR and sequencing (Kim et al., 2010). Furthermore, fecal human samples were
787 previously analyzed to investigate the difference between total bacterial population and active
788 bacterial population using pyronin-Y, to specifically stain RNA, to detect active cells, followed by
789 FACS of the active community and microbial profiling, targeting the 16S rRNA gene (Peris-Bondia
790 et al., 2011). Gougoulias and Shaw (2012) used FLOW-FISH to study the *Lolium perenne*
791 rhizosphere soil, making use of 16S rRNA-targeting probes for detection and identification of
792 *Pseudomonas* spp., followed by FACS and sequencing of 16S rRNA sequence, registering a
793 remarkably high specificity and sensibility of the design probe to the genus but they were unable
794 to recover new *Pseudomonas* species (Gougoulias and Shaw, 2012). Also, CARD-FISH performed
795 on fixed and immobilized cells of activated sludges to the detection of denitrifying bacteria, using
796 as probe-target *nirS* codifying mRNA, was performed (Mota et al., 2012). This work sorted
797 hybridized cells using FACS and completed with denaturing gradient gel electrophoresis (DGGE)
798 of 16S rRNA amplified genes, followed by purification and sequencing of selected gel bands
799 (Mota et al., 2012). The effect evaluation of xenobiotics in the human gut microbiome
800 physiology was performed based on bacterial staining with PI, $DiBAC_4(3)$, and SYBR™ Green I,
801 allowing observation of membrane integrity and membrane potential evaluation and
802 differentiation between LNA and HNA cell populations, respectively (Maurice et al., 2013). FACS
803 was applied to each population, followed by 16S rRNA gene amplification and sequencing, to
804 taxonomically identify changes in the gut microbiome (Maurice et al., 2013). Flow cytometry
805 profiles obtained by cell staining with DAPI, together with FSC evaluation were used to define
806 subcommunities through gate attribution in bacterial cells recovered from wastewater
807 treatment plants (Liu et al., 2018b). Changes in cell abundance in each gate were registered and
808 stability proprieties were analyzed (Liu et al., 2018b). This analysis is based on the ability of the

809 microbial community to react to environmental disturbances (Liu et al., 2018b). FACS was also
810 applied to the previously defined gates and taxonomical identification of gated cells was
811 completed by 16S rRNA gene sequencing (Liu et al., 2018b). Very recently, FACS was also applied
812 to photosynthetic picoeukaryotes, enabling 18S rRNA gene amplification and sequencing,
813 aiming at the taxonomical identification of these communities from eutrophic shallow lake
814 samples (Shi et al., 2020). This sorting strategy is based on the autofluorescence of chlorophyll-
815 *a* by biplot FSC *vs* far-red fluorescence evaluation (Shi et al., 2020).

816 **FLOW-FISH-FACS for whole-genome amplification (WGA) and sequencing (WGS)**

817 Recently, FACS technology has been used to sort cells and perform WGS for several purposes
818 (Chen et al., 2017; Czechowska et al., 2008; Koch et al., 2014a). However, presently, there subsist
819 yield limitations of targeted cell enrichment by FACS (between 10^5–10^6 cells), demanding WGA
820 before WGS. However, current WGA methods are not compatible with formaldehyde fixation
821 due to alterations of DNA molecules, so alternative fixation protocols, such as ethanol fixation,
822 are necessary to amplify genomic material from FACS (Chen et al., 2017; Czechowska et al., 2008;
823 Koch et al., 2014a).

824 Several examples of this application were described in the last decade. Yilmaz et al. (2010)
825 performed an in-solution fixation-free FISH methodology followed by FACS and subsequent
826 WGA and 16S rRNA gene sequencing to study bioreactor sludges and termite hindgut samples
827 to detect both "*Candidatus* Accumulibacter phosphatis" and methanotrophic bacteria (Yilmaz
828 et al., 2010). This approach was one of the first steps to in-solution fixation-free FISH protocol,
829 facilitating WGA and sequencing of sorted cells since DNA has no crosslink from
830 paraformaldehyde or similar fixating agents (Yilmaz et al., 2010). Lee and team (2015) performed
831 FISH, using *Dehaloccoccoides mccartyi* specific 16S rRNA targeting probe, followed by FACS and
832 WGA of sorted cells on activated sludge and contaminated groundwater samples (Lee et al.,
833 2015). The amplified DNA was used for microarrays analysis, and functional and comparative

genomics to improve the knowledge of this dechlorinating species (Lee et al., 2015). BONCAT-FISH and BONCAT-CARD-FISH were coupled with FACS to study the anaerobic oxidation of methane consortia in methane seep sediments (Hatzenpichler et al., 2016). This approach allowed the detection of active cells and concomitant functional identification of consortia, followed by FACS and WGA to 16S rRNA gene sequencing to evaluate microbial interactions occurring in these consortia (Hatzenpichler et al., 2016). De Corte team (2019) studied the viral community of the Global Deep Ocean Conveyor Belt system by FACS followed by WGA and WGS (De Corte et al., 2019). To accomplish this study, viral particles present in water samples were fixated with a low concentration of glutaraldehyde, to increase downstream application efficiency, and stained with SYBR™ Green I (De Corte et al., 2019). Very recently, forest soil samples were evaluated by SYBR™ Green I staining and flow cytometric profile analysis (as described above) (Alteio et al., 2020). The obtained gates were sorted, followed by WGA and WGS of those mini-metagenomes (Alteio et al., 2020). This analysis was coupled with metabolic predictions to better reconstruct the microbial diversity of those soil samples (Alteio et al., 2020).

5.3. Examples of FLOW-FISH-FACS for other downstream applications

Bacteria recovered from lake water were labelled with nitrogen and carbon-heavy isotopes, followed by FACS according to the autofluorescence of the target species *Chlorobium phaeobacteroides* (Zimmermann et al., 2015). The sorted bacteria were labelled by CARD-FISH targeting 16S rRNA and analysed by NanoSIMS, to measure the incorporated heavy isotopes in this specific species (Zimmermann et al., 2015).

Moreover, Batani team (2019) developed a FISH protocol that allows the labeling of cells keeping them alive through the omission of fixation, optimization of centrifugation steps and buffers, in parallel with chemical transformation, resulting in specific hybridization of DNA probes (Batani et al., 2019). Following FISH of Baltic surface seawater, labeled cells were sorted by FACS and

cultivated, with successful growth (Batani et al., 2019). Espina (2020) also developed an optimized FLOW protocol, followed by FACS, to increase the success rate of cultivation of soil bacteria using cFDA and PI to sort reproductively viable bacteria (Espina, 2020). These new methodologies hold great promise to improve the isolation and cultivation of new microorganisms from environmental matrices.

Besides the typical FACS based on direct/natural morphophysiological characteristics of cells and fluorophore staining of cells, genetic engineering can also be coupled with this technology. In a recent study by Liu and colleagues (2019), the evaluation of heavy metals facilitating the transfer of antibiotic resistance genes between bacteria in activated sludge was assessed by FLOW using an *E. coli* strain transformed with a plasmid-encoding antibiotic resistance gene together with the *Lac* system (Lin et al., 2019). So, donor cells possess chromosomal red fluorescence protein-producing gene under the influence of $LacI^q$ and a plasmid with a green fluorescence protein-producing gene with a $LacI^q$ repressor (Lin et al., 2019). Donor cells are fluorescence red, while plasmid recipient cells acquire green fluorescence (Lin et al., 2019). Cells that are unable to acquire the plasmid-encoding antibiotic resistance gene have no fluorescence (Lin et al., 2019). After recipient cells were sorted by FACS, 16S rRNA gene amplification and sequencing were performed, together with microbial metabolic function prediction using PICRUSt (Lin et al., 2019). This study allowed the identification of the *Pseudomonas* genus as the dominant plasmid recipient in the presence of arsenic and mercury, and *Aeromonas* and *Enterobacter* genera as the main recipients in the presence of lead (Lin et al., 2019). ATP-binding cassette transporters were related to heavy metal transport in the microbial metabolic function prediction of recipient bacteria (Lin et al., 2019).

6. Perspectives

Flow cytometry is an amazing tool to study microbial communities within environmental samples, enabling quantification and viability evaluation in a single-cell and high-throughput

approach that can overcome culture-dependent methods. Besides, it enables the evaluation of population heterogeneity, thus favouring microbial-driven ecological studies in natural environments. Additionally, this technology can be coupled with FISH to recover taxonomical and functional information. So, FLOW-FISH can be used, not only for signal intensity measurements but also combined with FACS, followed by several downstream analyses of sorted cells, such as omics to complete genome-wide analysis that provides a deeper understanding of cell biology. This combined workflow is recent and its potential is still unexplored, but we believe that in the coming years several research works will take advantage of this marriage. Cytomics will thus arise as a key strategy in environmental microbiology and microbial ecology, combining multiparametric and dynamic approaches (both structural, functional, and metabolic information) for microbial single-cell characterization and research (Müller and Nebe-von-Caron, 2010). This will allow the multitudinous operations of microorganisms in the total environment to be followed, improving our understanding of microbial cell behaviour in complex natural matrices and their contribution to the community state. This information can be further used to develop predictive models, e.g. metabolic models, in systems biology approaches through the use of machine learning and artificial intelligence. The use of new molecular, mathematical, and bioinformatic technologies and methodologies will be fundamental in downstream steps to help us handle the enormous amount of data that may be generated by omics-related methods. An in-depth understanding of metabolic pathways regulation and coexistence processes of microbial cells within populations or complex consortia will be achieved. Ultimately, this brave new world will contribute to outstanding improvements in nature-based biotechnological and medical microbial applications.

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Figure captions

Fig. 1 – **Type of fluorescence *in situ* hybridization (FISH) probes and labelling methods.** Four different type probes are represented, namely peptide nucleic acid (PNA), locked nucleic acid (LNA), DNA, and RNA probes. Five probe labelling options are denoted: direct [(a) and (b)] and indirect [(c) to (f)] labelled probes. Direct probes can be labelled in a mono (a) or multiple (b) way. Indirectly labelled probes can be made using a reporter molecule, like digoxygenin, biotin, or dinitrophenol (c); a horseradish peroxidase (HRP) that uses fluorophore–tyramide (TSA) as a substrate (d); and a combination of TSA system and a probe with a reported molecule (e).

Fig. 2 – **Signal amplification systems used in fluorescence *in situ* hybridization (FISH)**. a) CARD-FISH; b) Two-CARD-FISH; c) RING-FISH; d) HCR-FISH; e) DOPE-FISH; f) CLASI-FISH; g) BONCAT-FISH. HRP – horseradish peroxidase; AHA – L-azidohomoalanine; ALP – azide-labelled protein; AMD – alkyne-modified dye.

Fig. 3 – **Graphical outputs of flow cytometry analysis.** A) *Micrococcus luteus* and *Saccharomyces cerevisiae* were directly analysed (without the use of fluorophores), allowing the differentiation between microbial populations according to cell size (FSC) and cell complexity (SSC), using both a biplot of SSC *vs* FSC (left) and a histogram of FSC counts (right). B) Two populations of live (grey) and dead (black) cells of *Pseudomonas denitrificans* (left) and *Pseudomonas stutzeri* (right) were stained with different fluorophores: (i) SYTO™ 9 and PI; (ii) cFDA-AM; and (iii) $DiBAC_3(4)$. Despite the two species belong to the same genus, different responses can be perceived to be species- and fluorophore-specific. (i) *P. denitrificans* showing increase red fluorescence in damaged cells, while *P. stutzeri* showed decreased green fluorescence, but both showed differentiation capacity between live and dead cells. None of the tested species had their metabolic activity correctly differentiated using cFDA-AM (ii). However, $DiBAC_3(4)$ showed to be useful to better differentiate polarized and depolarized cells of *P. denitrificans versus* cells of *P. stutzeri*.

Fig. 4 – Fluorophores and their applications to measure physiological cell parameters. Different physiological parameters are denoted, namely pH gradient, membrane potential, metabolic activity, glucose intake, permeability, membrane integrity, oxidative stress, pump activity, and lipid content. FDA – Fluorescein diacetate; CTC – 5-cyano-2,3-ditolyl tetrazolium chloride; 2-NBDG – 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; FFS – phosphoenolpyruvate phosphotransferase system; NPN – 1-N-phenylnaphthylamine; ONPG – o-nitrophenyl-b-D-galactopyranoside; ONP – o-nitrophenyl; PI – Propidium iodide; HE – Hydroethidine; ROS/RNS – Reactive oxygen species/reactive nitrogen species; EB – Ethidium bromide; cFSE – 5(6)-carboxy-fluorescein succinimidyl ester.

Fig. 5 – General workflow of fluorescence *in situ* hybridization (FISH) coupled with flow cytometry (FLOW) and fluorescence-activated cell sorting (FACS). Initially, an environmental sample is collected and the microbial community is separated from the matrix by physical and/or chemical methods. Next, FISH is performed: microbial cells are then fixated (e.g. paraformaldehyde) and/or dehydrated (e.g. ethanol), permeabilized (e.g. lysozyme), hybridized with a specific probe (e.g. taxonomical or functional gene identification), and washed to avoid unspecific hybridization. Detection of hybridized cells can be accomplished by epifluorescence microscopy or by FLOW, the last one allowing further analysis by FACS and downstream applications (e.g. omics or metabolic studies).

Table 1 – Overview of signal amplification approaches: advantages, disadvantages, and applications to environmental microbiology.

Methodologies	Technique	Description	Advantages	Disadvantages	Applications	References (e.g.)
Multi-labeled probes or multiple probes	DOPE-FISH and MiL-FISH	Probes labeled at the 5' and 3' ends (same or different fluorophores)	As simple as conventional FISH; Up to seven target groups in a single experience	N/A	Aquatic environments; Isolation of "*Candidatus*" species; Plant and animal microbiota; Biofilms	(Beam et al., 2015; Escudero et al., 2018; Glassner et al., 2015; Kroer et al., 2016; Lehtovirta-Morley et al., 2016; Mitter et al., 2017)
	CLASI-FISH (TAPE-FISH and Leaf-FISH)	Unique spectral tag to each target group by conjugation of mono-labeled probes	TAPE-FISH and Leaf-FISH allows the visualization of the spatial structure	Expected 50:50 ratio of probes can be switch	Plant and animal microbiota	(Bisha and Brehm-Stecher, 2009; Mark Welch et al., 2017; Peredo and Simmons, 2018)
Enzymatic-based	CARD-FISH	HRP-labeled oligonucleotide probes and fluorophore-labeled tyramides	26 to 41 times higher fluorescent signal intensity than conventional FISH	Expensive; Enzymatic pretreatment of cells; Inactivation of endogenous peroxidases; Dramatic alteration in melting behavior of the probes; Multi-targeting groups are time-consuming	Animal microbiota; Aquatic environments; Soil and sediments	(Alfreider et al., 2018; Cai et al., 2016; Crognale et al., 2017; Golyshina et al., 2017; Hoshino et al., 2017; McNichol et al., 2018; Pohlner et al., 2017; Probandt et al., 2017; Schmidt and Eickhorst, 2014; Schmidt et al., 2018; Winkel et al., 2018)
	2-pass CARD-FISH	Sequential CARD with HRP conjugated anti-fluorophore antibodies to the hybridized fluorophore, followed by a second CARD signal amplification with fluorophore-labeled tyramides	Increased detection efficiency of low ribosomal content cells	Same problems as CARD-FISH, with increased time-consumption	Aquatic environments	(Kawakami et al., 2012; Neuenschwander et al., 2015)
	GeneFISH (and PhageFISH)	Detection of the gene of interest and the rRNA of the microorganism,	Simultaneous evaluation of taxonomy and functionality	Same as CARD-FISH	Aquatic environments; Archaeal viruses;	(Allers et al., 2013; Bernhard et al., 2012; Dang et al., 2015; Lenk et al., 2012; Matturro

		both using CARD-FISH methodology			Phage/host interactions; Sediments; Symbiosis relations	and Rossetti, 2015; Moraru et al., 2010; Petersen et al., 2011; Stagars et al., 2016)
	RING-FISH	Use of a probe labeled with a reporter molecule that anchors other probes, that in turn form a network around the cell periphery resulting in a halo-shaped fluorescent signal	10- and 50-fold signal amplification when compared to conventional FISH	Low probe penetration; High cost; Low specificity; Lack of sub-cellular definition	Aquatic environments; Sediments	(Noriea et al., 2010; Pratscher et al., 2009)
Amplification-based	HCR-FISH	An initial probe partially hybridizes to the target sequence and the remaining part becomes a trigger for the polymerization of the two fluorescently labeled amplifier probes	Up to 8-fold higher sensitivity than conventional FISH; Substitute of CARD-FISH with less aggressive permeabilization	N/A	Aquatic environments; Sediments; Symbiosis relations	(Nikolakakis et al., 2015; Yamaguchi et al., 2015a)
Synthetic metabolites-based	BONCAT-FISH	A biorthogonal synthetic amino acid is taken by the cell and acts as a substrate of aminoacyl tRNA synthetase to be incorporated into *de novo* peptides, reacting to a modified fluorescence dye	A nondestructive technique that is an alternative to study microbial ecophysiology independently of isotopes	Expensive; Hard to perform	Animal microbiota; Aquatic environments; Sediments; Symbiosis relations	(Hatzenpichler et al., 2016; Hatzenpichler et al., 2014)

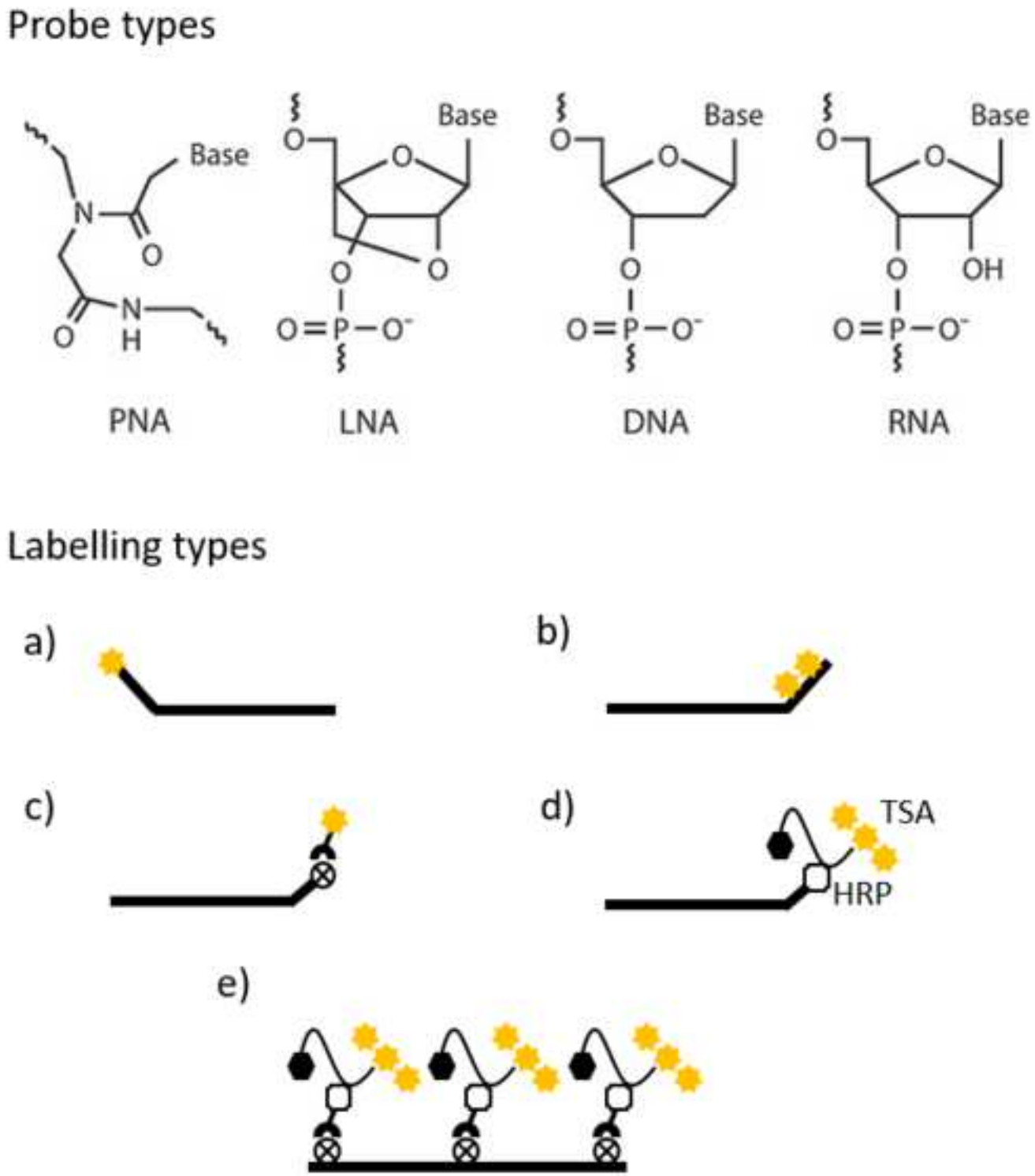
FISH – Fluorescence *in situ* hybridization; DOPE – Double labeling of oligonucleotide probes; MiL – multi-labeled oligonucleotide probes; CLASI – combinatorial labeling and spectral imaging; CARD – Catalyzed reported deposition; HRP – horseradish peroxidase; RING – recognition of individual genes; HCR – *in situ* DNA-hybridization chain reaction; BONCAT – biorthogonal noncanonical amino acid tagging; N/A – not applied.

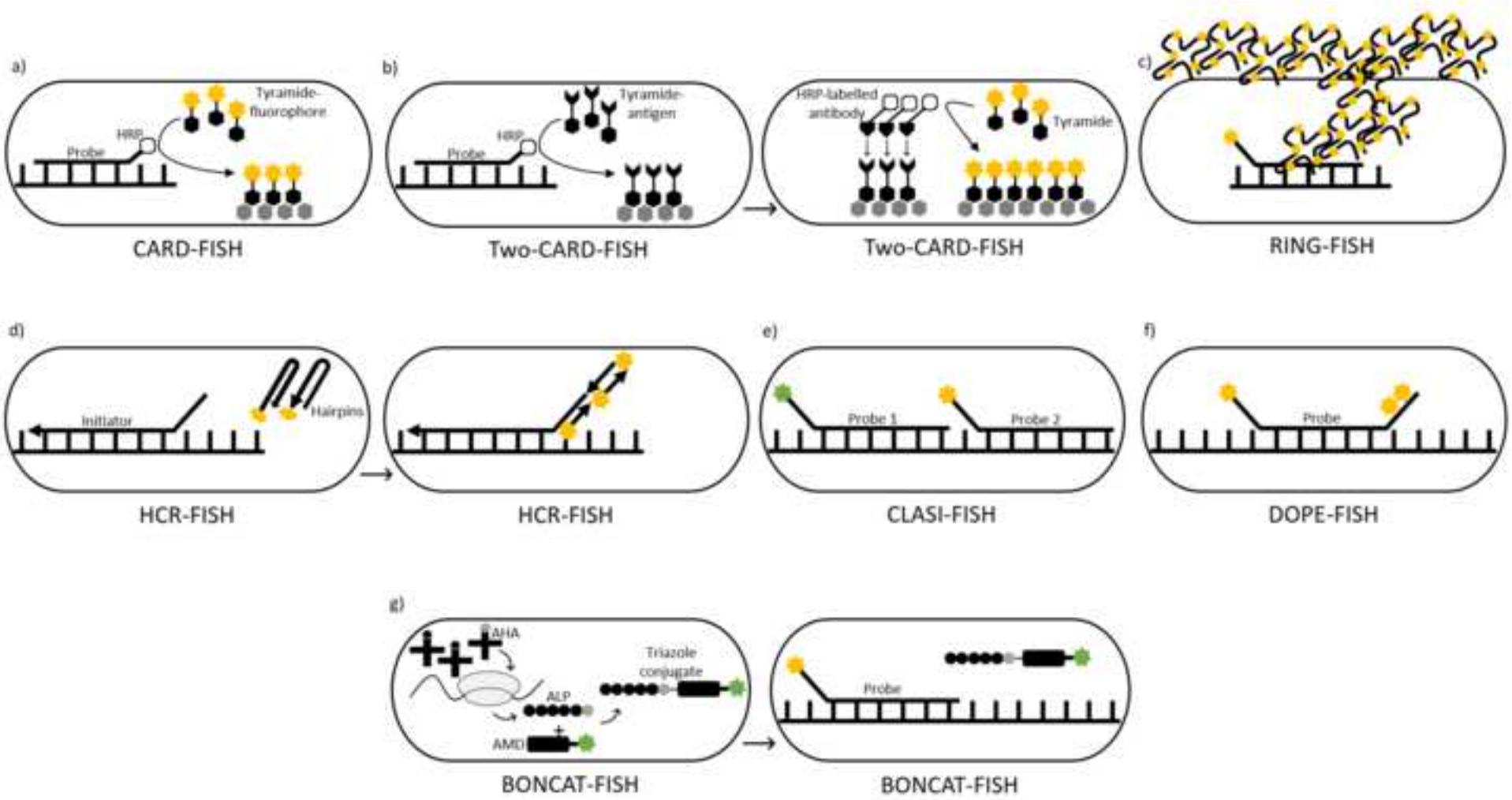
Table 2 – Overview of morpho-physiological parameters that may be assessed using flow cytometry in environmental microbiology and their relation with fluorophores and applications.

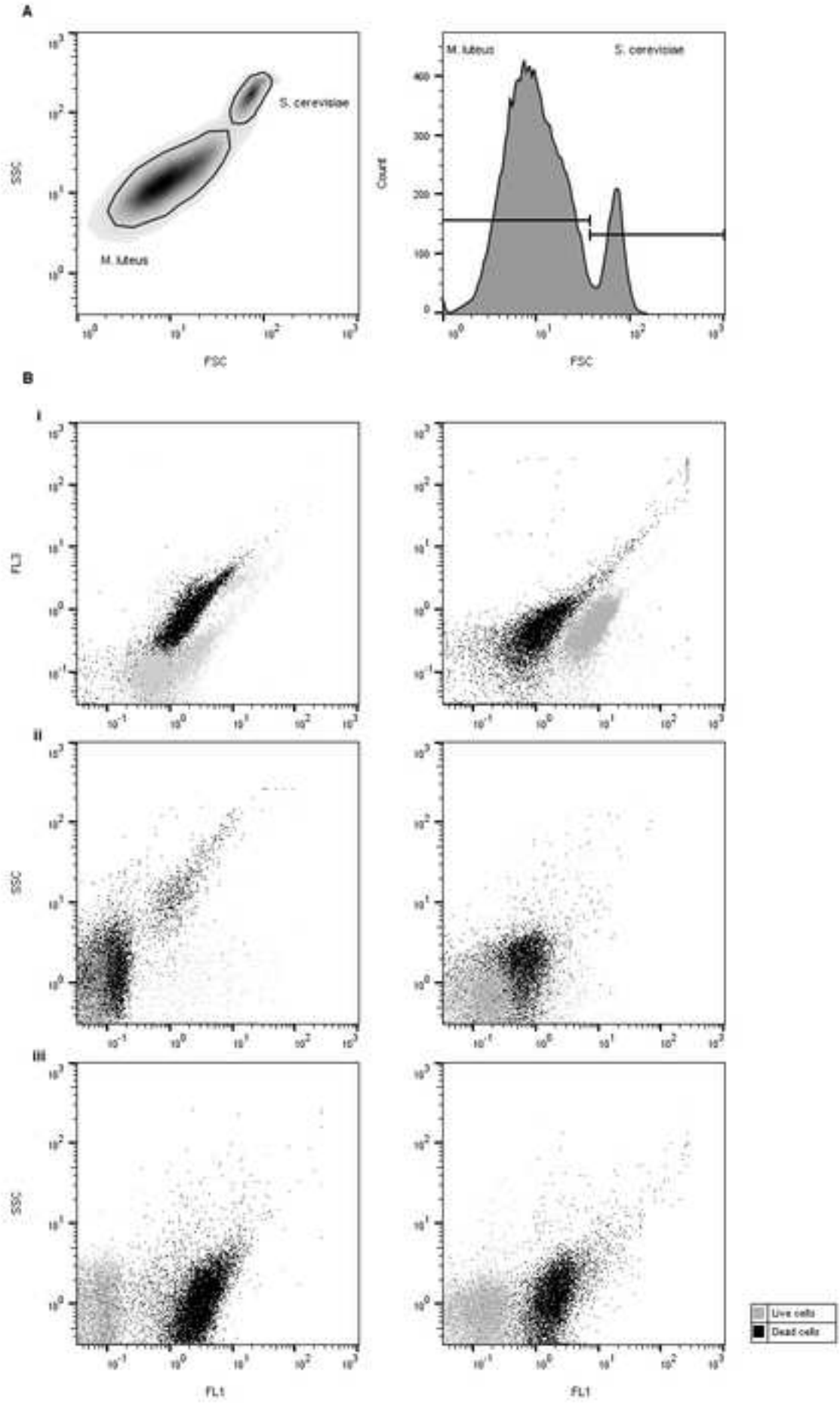
Parameter	Fluorophores	Description	Environmental applications	References*
Community cytometric profile	Nucleic acids staining dyes: DAPI Hoechst 33342 SYBR™ Green I and II SYTO™ dyes	In environmental samples, stained cells are normally grouped in two clusters: high (HNA) and low (LHA) nucleic acid content cells. HNA cells have increased cell division and high metabolic activity	Animal microbiota; Aquatic environments; Biofilms; Microbial fuel cells; Virus-bacteria differentiation; Soil; Wastewater treatment plants	(Bartelme et al., 2020; Berney et al., 2007; Borderie et al., 2016; Gözdereliler et al., 2013; Khalili et al., 2019; Krause et al., 2020; La Ferla et al., 2017; Liu et al., 2019; Liu and Müller, 2020; Müller et al., 2012; Plominsky et al., 2018; Song et al., 2019; Storesund et al., 2015; van Gelder et al., 2018)
Membrane integrity	Exclusion dyes: Propidium Iodide (PI) TOTO-1	Double staining with an exclusion dye and an all-staining dye that binds to DNA and/or RNA	Animal microbiota; Aquatic environments; Biofilms; Dairy products; Microbial fuel cells; Soil; Wastewater treatment plants	(Abzazou et al., 2015; Ben-Amor et al., 2005; Foladori et al., 2015; Gözdereliler et al., 2013; Grégori et al., 2001; La Ferla et al., 2017; Liu et al., 2019; Matos and Lopes da Silva, 2013; Maurice et al., 2013; Mohd et al., 2019; Peris-Bondia et al., 2011; Song et al., 2019; Wang et al., 2019)
Pump activity	Ethidium bromide (EB)	Cells with malfunctioning efflux pumps accumulate intracellularly the fluorophore	NA	NA
Glucose intake	2-NBDG	This analog of glucose is specifically uptake by the phosphoenolpyruvate phosphotransferase system	Animal microbiota	(Tao et al., 2019)
Membrane potential	Cationic dyes: Carbocyanine ($DiOC_n(3)$) Rhodamine 123 (Rh123) Anionic dyes: $DiBAC_4(3)$	Cationic dyes: accumulate inside viable cells Anionic dyes: accumulate inside dead cells	Animal microbiota; Microbial fuel cells; Soil	(Maurice et al., 2013; Mohd et al., 2019; Pepè Sciarria et al., 2019)
pH gradient	cFSE	A pH-dependent fluorescent probe	Plant microbiota	(Chitarra et al., 2000)
Metabolic activity (e.g. respiratory and enzymatic activities)	Respiratory activity: CTC Enzymatic activity: Fluorescein diacetate (FDA) or derivates	A non-fluorescent substrate that upon cell entrance is enzymatically transformed into a fluorescent substance that is accumulated	Aquatic environments; Dairy products; Soil; Wastewater treatment plants	(Bunthof and Abee, 2002; Espina, 2020; Hoefel et al., 2003; La Ferla et al., 2017; Lentendu et al., 2013; Ziglio et al., 2002)

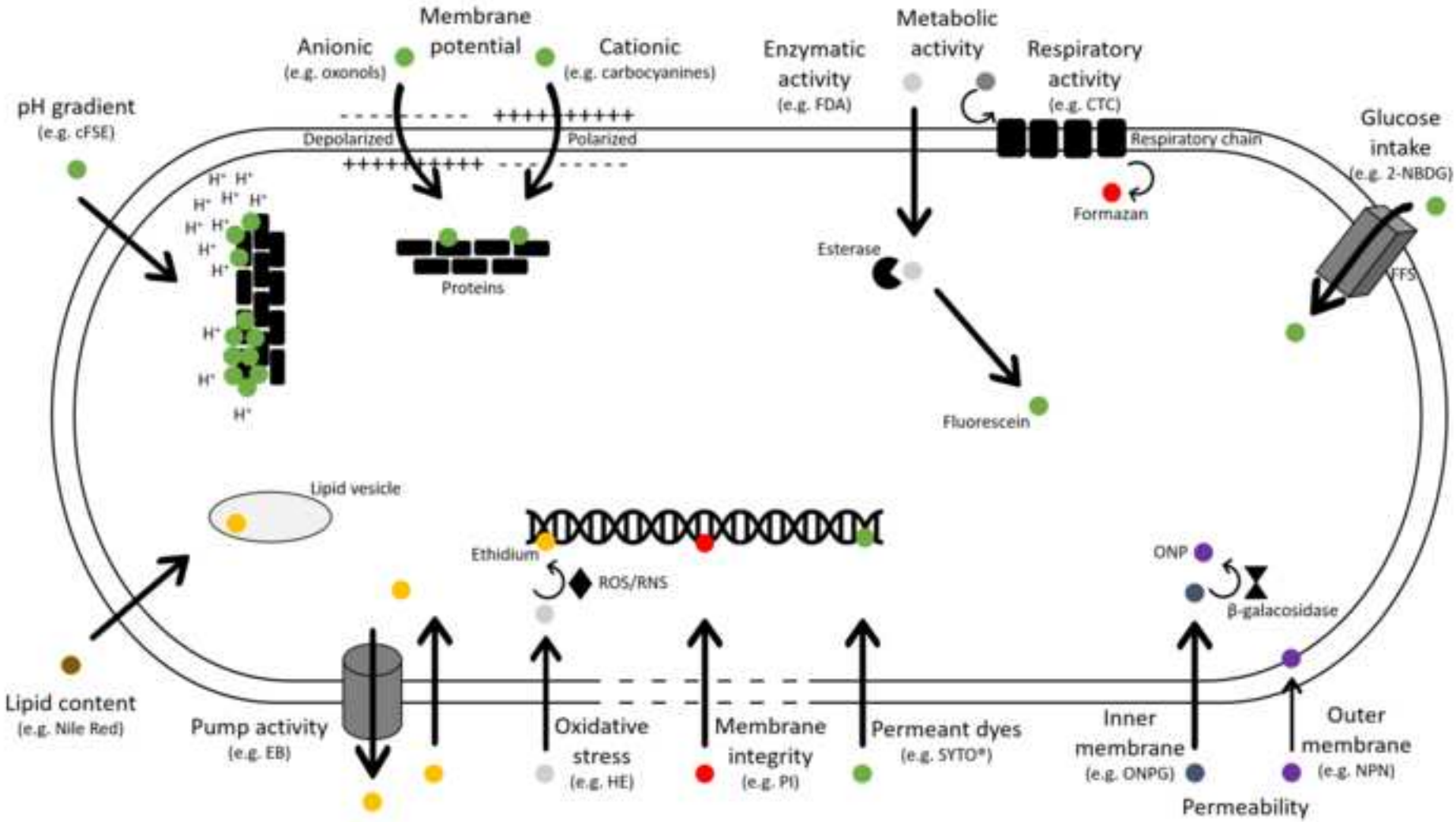
Oxidative stress	CellROX™ Green Hydroethidine (HE)	The fluorophore suffers oxidation by reactive oxygen and/or nitrogen species, forming ethidium that intercalates with DNA	Dairy products	(Fallico et al., 2020; Hickey et al., 2018)
Lipid content	BODIPY™ Nile Red	Binds to non-polar lipid droplets and stored lipids (polyhydroxyalkanoates) inside cells	NA	NA
Gram character	SYTO™ dye + Hexidium iodide Wheat germ agglutinin conjugated with a fluorophore	Hexidium iodide only stains Gram-positive bacteria Wheat germ agglutinin only binds to Gram-positive bacteria	Animal microbiota; Dairy products	(Duquenoy et al., 2020; Holm and Jespersen, 2003)

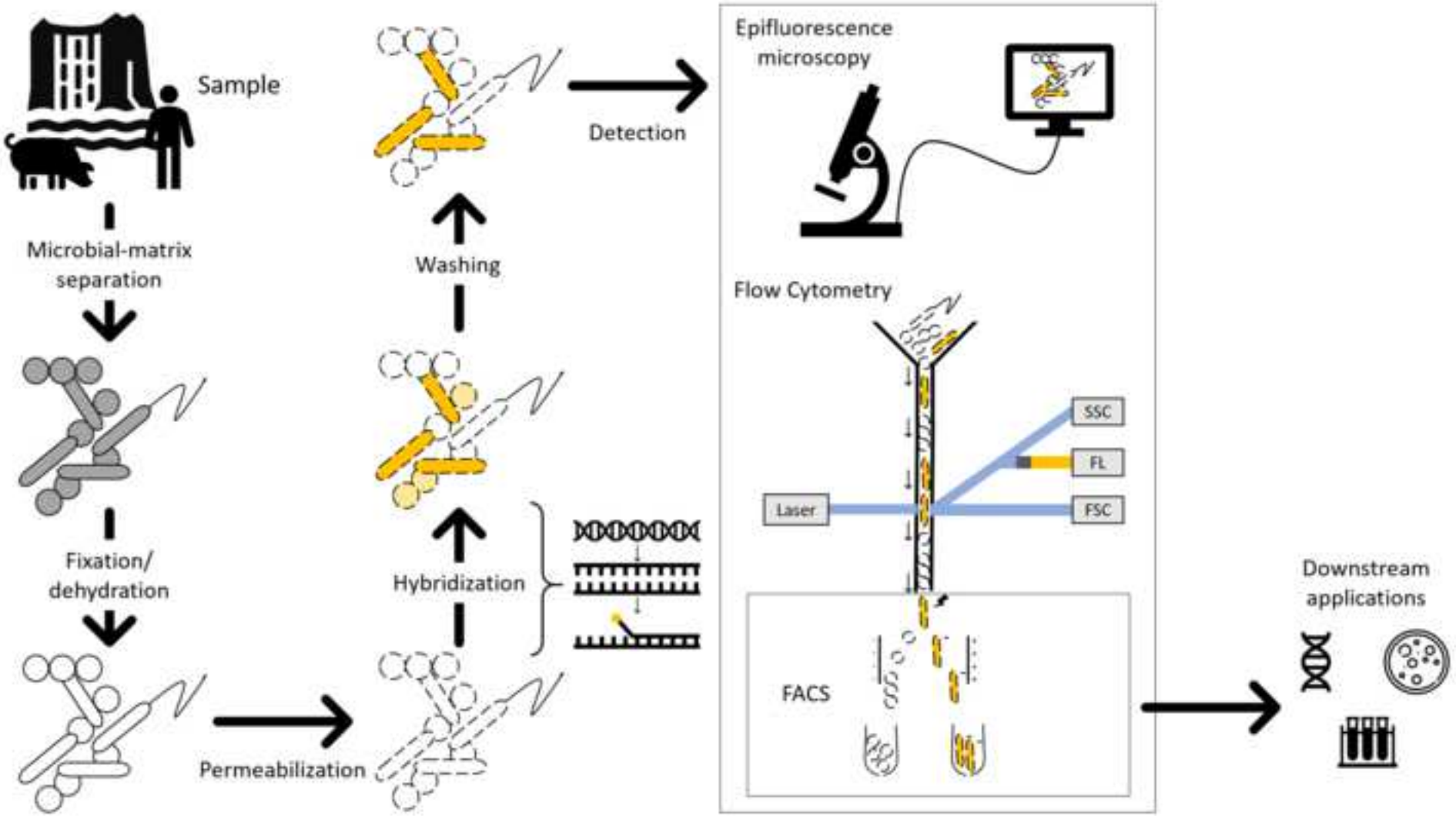
DAPI – 4',6-diamidino-2-phenylindole; $DiBAC_4(3)$ – bis-(1,3-dibutyl barbituric acid)-trimethine oxol; CTC – 5-cyano-2,3-ditolyl tetrazolium chloride; cFSE – 5(6)-carboxy-fluorescein succinimidyl ester; 2-NBDG – 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; BODIPY™ – boron-dipyrromethene. * – some referenced examples are not developed in the manuscript.











Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: