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Assessing the adaptability of the live-FISH method to soil *Acidobacteriota*

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Abstract

Acidobacteriota, a widespread and ecologically significant bacterial phylum in soil environments, remains challenging to study due to its members' specific cultivation requirements and resilience limitations under standard laboratory conditions. Traditional Fluorescence *In Situ* Hybridization (FISH) techniques, while valuable for microbial identification, generally require cell fixation, limiting their utility in cultivating and further analyzing live cells. This study aimed to evaluate a modified live-FISH protocol, incorporating newly developed *Acidobacteriota* phylum-specific probes, to enable fluorescent labeling of viable cells within this phylum. In this work, double-labeled DOPE probes targeting *Acidobacteriota* subdivision 1 were developed and tested. Among these, the Acid303 exhibited optimal performance, with strong specificity and signal intensity at low formamide concentrations. Initial viability tests on model gram-positive and gram-negative bacteria revealed variable survival under live-FISH conditions; gram-positive *Bacillus* strains demonstrated higher resilience compared to gram-negative bacteria. However, despite extensive optimization, viable *Acidobacteriota* cells remained elusive, likely due to their sensitivity to the high temperatures and buffer pH used in the hybridization process. Further trials with soil enrichment and filtrate imposed challenges with autofluorescence and cell aggregation, leaving room for further protocol development. Although the live-FISH protocol did not yield viable *Acidobacteriota* cells suitable for cultivation, this study validated the Acid303 as a reliable probe for traditional FISH applications. These findings emphasize the need for further refinement of live-FISH methodologies tailored for sensitive microbial taxa, such as *Acidobacteriota* and suggest potential directions for future research, including the exploration of alternative hybridization environments.

1 Introduction

1.1 Discovery and Cultivation of Novel Bacteria

The discovery of novel bacterial species has been deepening our understanding of the ecology and physiology of microorganisms for centuries. Broader knowledge about the taxonomical bacterial diversity in a given biome gives us insights into the ecology of the microbial community. As these interactions within the biotic community form the backbone of life on Earth, it is important to understand the key players. While cultivation of soil extracts or enrichments can give us insight into some representatives of the microbial community, it is usually not representative of the community as a whole (Joseph et al., 2003). The great plate count anomaly applies for most environments, meaning that only a small fraction of microorganisms can be cultivated under laboratory conditions (Staley and Konopka, 1985). Different bacterial taxa grow at different rates under various conditions. Conditions such as temperature, pH, or salinity can have drastic effects on bacterial behavior as well as growth. Bacteria also behave differently when grown on solid media, such as agar plates, or in liquid culture, such as cultivation in Erlenmeyer flasks (le Goff et al., 2019). Additionally, most bacteria cannot be cultivated under artificial conditions yet (Pham et al., 2012). Recent metagenomic analysis suggests that around 81% of all bacteria and archaea belong to uncultured species (Lloyd et al., 2018). Cultivation independent methods such as metagenomic analysis of soil can be a viable alternative for cultivation-dependent methods. However, ‚Most of the methods used nowadays are outdated and work mostly in the case of bacteria that can be readily cultivated (Chaudary et al., 2019). Thus, novel approaches are required to explore these not-yet-cultivated microbes and their physiological properties.

1.2 *Acidobacteriota* and Their Biosynthetic Potential

Acidobacteriota are gram-negative, typically oval-shaped bacteria with extremely diverse and widespread environmental presence (Quaiser, 2003). They are involved in numerous ecological processes, such as nutrient cycling, decomposition, and soil mineralization (Sikorski, 2022). Based on soil metagenomics, they represent up to 50% of bacterial species present in soils (averaging 20%) and yet, the vast majority of these organisms have not been cultivated and taxonomically identified (Dunbar et al., 2002; Janssen, 2006; Crits-Christoph et al., 2018; Chaudhary et al., 2019). Current consensus stipulates that introducing unusual cultivation conditions (such as changing pH, temperature, introducing compounds naturally occurring in soil etc.) increases the likelihood of successful novel *Acidobacteriota* members´ cultivation (Kalam et al., 2020). However, these can be difficult to maintain and are very labor - and time-consuming.

Acidobacteriota are currently divided into 15 classes containing 26 subdivisions, based on their genomic similarity. Usually, the bacteria belonging to the same subdivision share traits regarding their environmental preference and ecological attributes. So far, only seven subdivisions (1, 3, 4, 6, 8, 10, 23) are represented by taxonomically-defined species and subdivision 1 with the class *Terrigloba* containing the most described species (Dedysh and Yilmaz, 2018; Eichhorst et al., 2018). For the purposes of this study, we focus on subdivision 1, as its members on average grow faster on accessible media compared to representatives of the other subdivisions and were readily available at the time in the laboratory where this study was carried out (Campanharo et al., 2016).

One of the motivators to study *Acidobacteriota* is their biosynthetic potential. Their secondary metabolites (chemical products not directly essential for survival) have the potential to offer a multitude of useful applications, including antibiotics, antifungals, anti-cancer drugs, and more (Banf et al., 2019).

Acidobacteriota have been shown to contain a repertoire of biosynthetic gene clusters (BGCs) in multiple metagenomic studies (Liao et al., 2020; Crits-Christoph et al., 2022; Waschulin et al., 2022,). BGCs are sets of genes that encode a common metabolic pathway synthesis machinery, giving rise to a multitude of diverse chemical compounds. However, in order to extract and analyze these compounds, the respective bacterium has to be ideally isolated, cultivated to larger biomass volumes, and sequenced. Due to the difficulty with which *Acidobacteriota* are typically cultivated, this vast BGC repertoire with its potential remains largely unknown (Crits-Christoph et al., 2018; 2022).

1.3 FISH in Microbial Ecology

Fluorescence *in-situ* hybridization (FISH) is a molecular technique that utilizes 5'-end fluorescently-tagged probes that bind to their respective specific region of a nucleic acid (Rudkin and Stollar, 1977; Langer-Safer, 1982). In this study, the target sequence was located on rRNA of bacterial ribosomes. The probe consists of an oligonucleotide (~15-25 base pairs long) with high level of complementarity to the rRNA target sequence, and a fluorescent tag usually localized at the 5'-end of the oligo sequence (Amann et al., 1990; Chengua C., Wei S., and Peining L., 2016). The fluorescent tag consists of a specific dye whose excitation and emission wavelengths are well-defined. When the probe binds to its target sequence, it creates an amplification effect on the signal coming from other probes in its proximity bound to the same ribosome. This concentration of fluorescent emission causes the signal strength to be higher than from unbound probes. The fluorescence of the unbound probes is dispersed over the background, thus appearing dim in comparison. This fluorescence can be observed in a fluorescent microscope (Pernthaler et al., 2001). The method can infer the number of ribosomal binding sites based on fluorescence intensity (Wagner et al., 1994). Unlike traditional light microscopy, FISH does not rely on differences in morphology, but rather on nucleic acid sequence similarity. Therefore, the only non-sequencing approach to bacterial identification is probe complementarity in FISH by using fluorescent microscopy (Bishop and Neumann, 1970).

Usually, gram-positive species are harder to transform and hybridize with a probe than gram-negative bacteria. The former have a much more prominent peptidoglycan layer than the latter, with some extreme examples such as *Actinomycetota* requiring enzymatic pretreatment with achromopeptidase or proteinase K to make their cell wall permeable for the probes (Wagner et al., 1998).

FISH is usually applied on fixed cells. For cell fixation, formaldehyde or ethanol together with elevated temperatures are used, which are usually lethal to most organisms. So far, FISH has been used only to assess the repertoire of bacterial species or the presence of a targeted species in a sample. However, if the hybridized cells were to survive the entire process, the technique could be useful for identifying and sorting rarely-cultivated bacteria. After the sorting, the surviving bacteria would be grown on agar plates. Due to the prior live-FISH - FACS selection, the number non-target cells that pass through would be significantly restricted. As the fast-growing bacteria usually overgrow the slow ones, this method would increase the number of isolations of the rare and slow-growing soil bacteria.

1.4 Live-FISH-FACS

Live-FISH-FACS (live-fluorescent in situ hybridization – fluorescence-activated cell sorting) technique (further referred to as live-FISH), as described by Batani *et al*. from 2019, is a method of using FISH on bacterial cells aiming to hybridize with the probe and surviving the process. The team applied the method on model bacteria (*Bacillus* sp., *Pseudovibrio* sp., *Ruegeria* sp., and *Amphritea* sp.) and reported sufficient survival for subsequent FACS cell sorting. Live-FISH differs from the basic technique in several

aspects; mostly removing or adjusting steps that are lethal to the organism being hybridized. Unlike traditional FISH, the maximum probe specificity in live-FISH is not given the highest priority, as it only serves as a preliminary sorting mechanism to narrow down the taxa of organisms cultivated. Additionally, it is not only non-target bacteria that can be hybridized by the probe - the soil environments contains a significant amount of unassociated nucleic acid, greatly increasing the chance of off-target probe hybridization. Furthermore, soil particles can cause binding and aggregation of probes, giving off false positive signals.

Despite these drawbacks, Live-FISH could, facilitate successful cultivation of recalcitrant microorganisms from the complex communities (such as the slow-growing soil *Acidobacteriota* in this study). Next, after the living cells have been labeled with a FISH probe, they can be subsequently sorted using FACS, and cultivated and isolated on agar plates. At this stage, any contamination by off-target bacterial taxa would be less severe. These unwanted colonies could be easily removed, as their number would be far lower compared to enrichment plating and, therefore, more manageable.

1.5 Aims of This Study

In this study, an attempt was made to adapt the live-FISH protocol developed by Batani et al., 2019 on model bacteria in order to appropriate this method for soil extracts, targeting *Acidobacteria* subdivision 1. If successful, this would be followed by FACS cell sorting, resulting in more selective cultivation and taxonomical identification.

The principal goal of this work was to explore the viability of using live-FISH to fluorescently label *Acidobacteriota* while maximizing their survival rates. Another goal was to develop a reliable FISH-based analysis method for *Acidobacteriota* subdivision 1 using a probe with high signal intensity, good specificity. Additionally, for the purpose of cell survival in live-FISH, the probe should have had minimal FA concentration.

Various live-FISH strategies were explored, such as changing hybridization temperature, hybridization time, probe sequence, probe type, bacterial species, along with approaches to prove viability using BacLight kit that allows to microscopically differentiate cells based on their viability.

2 Materials and Methods

2.1 Bacterial Strains and Their Cultivations

This study sourced some microorganisms from various natural habitats, such as peatland in Germany, lake in Austria, and an island in Antarctica while others came from well-established culture collections. All the bacteria used in this experiment (strain name + species), their places of isolation with coordinates, growth medium, and growth conditions are listed in table 1.

Table 1: Bacterial isolates used in this study. All cultivations were incubated in semi-dark conditions, liquid media were shaken at 200 rounds per minute (rpm). RT – room temperature.

Strain name	Species	Isolation Source	Coordinates	Growth temp.	Medium	Medium type
P12228	*Kocuria rhizophila*	Antarctica, James Ross Island	63°49'07.1"S 57°49'07.1"W	RT	TSA	Solid
P12266	*Bacillus* sp.	Antarctica, James Ross Island	63°49'07.1"S 57°49'07.1"W	RT	TSA	Solid
SPOG326	*Serratia* sp.	Germany, Schlöppnerbrunnen	50°08'38"N 11°51'41"E	RT	3xR2A	Solid
SLOG1A	*Terriglobus* sp.	Austria, Laxenburg lake	48°04'01.9"N 16°22'25.7"E	RT	3xR2A	Solid
P12204	*Bacillus* sp.	Antarctica, James Ross Island	63°49'07.1"S 57°49'07.1"W	28°C	LB	Solid
KT2440	*Pseudomonas putida*	In-house collection	N/A	28°C	LB	Solid
SPOG32A	*Bryocella* sp.	Germany, Schlöppnerbrunnen	50°08'38"N 11°51'41"E	RT	1/10 R2B	Liquid
SPOG37A	*Edaphobacter* sp.	Germany, Schlöppnerbrunnen	50°08'38"N 11°51'41"E	RT	1/10 R2B	Liquid

2.2 FISH

Bacteria intended for FISH were grown either as colonies on an agar plate or in liquid culture in a 300 mL Erlenmeyer flask containing 50 mL of the growth medium. The sampling for FISH should occur in exponential growth phase to maximize the number of ribosomal binding sites. Visual representation of the traditional bacterial FISH is depicted in Figure 1 below and described later in this chapter.

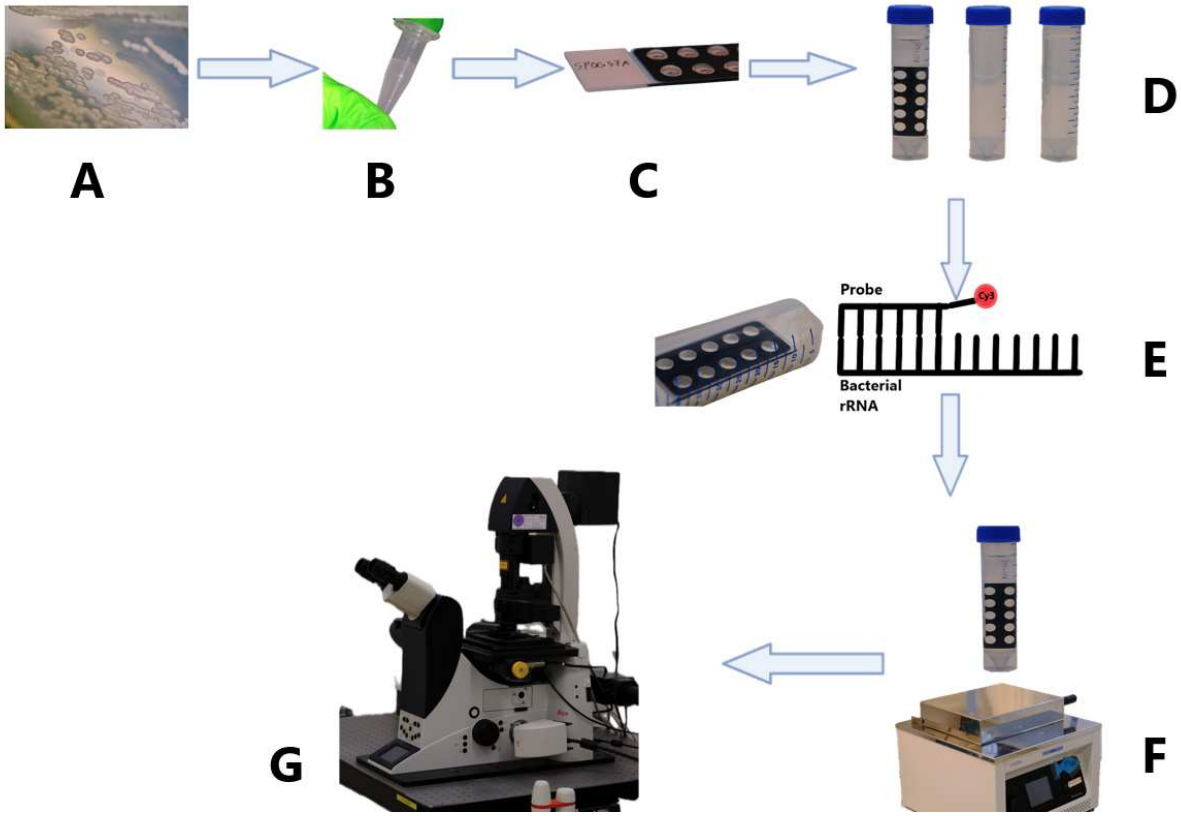


Figure 1: Workflow for FISH technique. Detailed description of this workflow is part of the 2.2 chapter.

For the culture preparation, a loopful of a bacterial colony, approximately 1 µL, was collected and resuspended in 1 mL of 1x phosphate-buffered saline (PBS). In case of liquid culture samples, 1 mL of culture was directly taken from the flask. The resulting cell suspension was vortexed thoroughly until full homogenization. For fixation, the protocol varied slightly between gram-negative and gram-positive bacteria. For gram-negative cultures, the cell suspension was centrifuged at 15,000 g for 5 minutes at room temperature (RT). Following centrifugation, the supernatant was carefully removed, and the cell pellet was resuspended in 1 ml of PBS,. This step was followed by a second round of centrifugation under identical conditions. After discarding the supernatant, 750 µL of 4% paraformaldehyde (PFA) was added to the pellet, which was then topped up to the final volume of 1 mL with 1x PBS, and vortexed to mix. The PFA-treated cell suspension was incubated on ice for 3 to 4 hours. Post-incubation, the sample was centrifuged at 15,000 g for 5 minutes at 4°C. The resulting pellet was washed with 1 mL of ice-cold PBS, a process that was repeated four times to ensure complete removal of the PFA. The final pellet was resuspended in an ice-cold 1:1 ethanol/PBS mixture and stored at -20°C. For gram-positive bacteria, a similar procedure was followed to accommodate the more robust cell wall characteristics of these bacteria. Following the initial centrifugation and PBS washing steps, an additional washing step was introduced to enhance cell permeability. This step involved adding 500 µL of ice-cold PBS and 500 µL of 96% ethanol to the pellet, a modification that increases cell permeability of the gram-positive cell wall. The pellet was then resuspended in an ice-cold 1:1 ethanol/PBS mixture and stored at -20°C for preservation.

Following fixation, 15 µL of the fixed cell suspension was carefully applied to each well on a Teflon-coated FISH slide, which was then placed in a 46°C hybridization oven until the suspension was fully dry,

typically taking 20 to 30 minutes). For dehydration, the slides were sequentially immersed in 50 mL solutions of 50%, 80%, and 96% ethanol, spending 3 minutes in each solution to ensure gradual dehydration. After dehydration, the slides were returned to the 46°C oven for an additional drying period of 15 to 20 minutes. To initiate the hybridization process, 10 µL of hybridization buffer, adjusted according to the probe's required formamide (FA) concentration, and 1 µL of probe were added to each well. To maintain optimal humidity, the remaining hybridization buffer was absorbed into a paper towel placed inside a Falcon tube "hybridization chamber," with the slide was positioned on the top of the dampened paper towel. Hybridization was carried out in the 46°C oven for 2.5 hours. Following hybridisation, the slides were removed from the chamber and immediately transferred to a pre-warmed (48°C) wash buffer, with NaCl concentration adjusted to match the probe's FA requirements. The slides were incubated in a 48°C water bath for 10 to 15 minutes, then briefly rinsed in ice-cold Milli-Q (MQ) water, and dried using pressurized air to remove any residual moisture. To stain the cells, 10 µL of a 1 µg/mL DAPI solution was applied to each well, followed by a 4-minute incubation in the dark to prevent photobleaching. After staining, the slides were rinsed twice in ice-cold MQ water and dried again with pressurized air. Finally, a few drops of CitiFluor AF1 were added on the slide, and a coverslip was gently pressed onto the slide to prepare it for imaging.

2.3 Live-FISH

This protocol was adapted from Batani et al., 2019. In live-FISH, cells from solid cultures were resuspended in PBS, whereas cells from liquid cultures were centrifuged at 13,000 g at RT for 5 minutes, followed by multiple PBS washes to remove any residual medium. Cell survivability was assessed by plating 100 µL of the suspension onto agar plates to monitor growth. For the heat shock step, the cell pellet was resuspended in 1 mL of ice-cold 12 mM $MgCl_2$/3 mM $CaCl_2$ solution (original study used 160 mM and 40 mM, respectively), then centrifuged at 13,000 g for 5 minutes at 4°C. The pellet was subsequently resuspended in 50 µL of ice-cold 7.4 mM $CaCl_2$, to which 4 µL of probe (500 ng/µL) was added, and the mixture was incubated on ice for 15 minutes. After the incubation, cells were subjected to heat-shock by transferring them to at 42°C heating block. An aliquot was taken post-heat shock for another survivability assessment by plating onto agar.

Immediately following heat shock, 500 µL of pre-warmed hybridization buffer (46°C) was added to the cell suspension, and the cells were incubated for hybridization in the 46°C hybridization oven for 2 hours. After hybridization, the cells were centrifuged at 13,000 g for 5 minutes at RT, and the supernatant was removed. The pellet was then resuspended in 1 mL of pre-warmed wash buffer (48°C) and centrifuged at 13,000 g for 15 minutes at 48°C. This washing step was repeated to ensure thorough removal of any residual hybridization buffer. The final cell pellet was resuspended in 500 µL of Ca^{2+} and Mg^{2+}-free 1x PBS and kept on ice. After the final washing step, cell survivability was reassessed by plating on agar o evaluate potential viability retention following the live-FISH protocol.

2.4 Microscopy

Images in Figures 4 and 6 were taken on a Zeiss Axio Observer M1 epifluorescent microscope using Zeiss ZEN version 3.1 Blue Edition software (Carl Zeiss Microscopy GmbH, Göttingen, Germany).

To improve image quality at higher magnification, a Leica TCS SP8 X confocal laser scanning microscope (CLSM) with a 93x HC PL APO glycerol immersion objective with refractive index of 1,30 was used. The CLSM software used for image processing was Leica Application Suite X (LasX) version 3.5.7.23225 (Leica Microsystems GmbH, Wetzlar, Germany).

2.5 Probes

Table 2: Probes used in this experiment. If 0%-50% is stated, the probe can be used with a wider range of FA concentrations. EUB338 was used as a mix with equal ratios of I, II, and III, created by Amann et al., 1990; GAM42a created by Siyambalapitiya et al., 2005.

Probe name	**Specificity**	**Sequence 5' to 3'**	**Optimal Forma-mide %**	**16S rRNA position**
HoAc1402	*Acidobacteriota*	CTTTCGTGATGTGACGGG	10%	338-355
EUB338: I: II: III:	Most *Bacteria*	 GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	0%-50%	338-355
BLS1295	*Bacillota*	GCAGCCTACAATCCGAACTGAGA	30%	1295
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	35%	
NON-EUB	Most *Bacteria*	ACTCCTACGGGAGGCAGC	0%-50%	338-355

The following probes were used for experiments in this study; as general probes EUB338 I-III mix targeting most bacteria and the nonsense probe NON-EUB as the negative control. More specific probes targeting the phylum level include HoAc1402 targeting a wide range of acidobacteriotal subdivisions, BIS1295 targeting *Bacillota* and GAM42a targeting the class *Gammaproteobacteria*.

Initially, the only probe targeting *Acidobacteriota* members available at the time of this study was HoAc1402 (Pankratov et al., 2008). It is a general *Acidobacteriota* probe that should theoretically bind most *Acidobacteriota* species. However, it proved to be unreliable when used as a reference probe – resulting in failure to produce any fluorescence while other controls worked well (not shown). Therefore, to explore the possibilities of live-FISH, a new, reliable, and more specific probe was required.

2.6 New Subdivision 1 Probes

For higher signal specificity, probes targeting the *Acidobacteriota* subdivisions 1, 2, and 3 were ordered. These probes were derived from the qPCR primers designed in a previous study by Hausmann et al., 2018. The reverse complements of the forward primers was used as the basis of the new probes, while the reverse primers' sequences remained unchanged. These probes were named according to the naming convention first proposed by Alm et al., 1996. Probes were ordered with Cyanine 3 (Cy3) fluorescent tag. It was added to both ends of the oligonucleotide for all new probes (DOPE). The probes with their respective sequences are shown in Table 3.

Table 3: New probes used for targeting *Acidobacteriota* subdivisions 1, 2 and 3 and their respective original primers. For primers, Fa/F denotes forward; R denotes reverse. All probes were ordered as DOPE probes, featuring dual Cy3 fluorescent tags.

Primer Name + Direction	Probe Name	Target	Primer Sequence (5'-3')	Probe Sequence (5'-3')
Acid303Fa	Acid303	Subdivision 1	GCGCACGGMCAC ACTGGA	TCCAGTGTGKCCG TGCGC
Acid657R	Acid657	Subdivision 1	ATTCCACKCACCTC TCCCAY	ATTCCACKCACCTC TCCCAY
Acid702Fa	Acid702	Subdivision 2	AGATATCTGCAGG AACAYCC	GGRTGTTCCTGCA GATATCT
Acid805R	Acid805	Subdivision 2	CTGATSGTTTAGG GCTAG	CTGATSGTTTAGG GCTAG
Acid306F	Acid306	Subdivision 3	CACGGCCACACTG GCAC	GTGCCAGTGTGGC CGTG
Acid493R	Acid493	Subdivision 3	AGTTAGCCGCAGC TKCTTCT	AGTTAGCCGCAGC TKCCTCT

Cy3 dye is a fluorescent dye that is excited by a 532 nm laser (excitation maximum 554 nm) and emits a bright fluorescence (emission maximum 566 nm) visualized by TRITC filters. It was chosen as a fluorescent tag for the new probes due to its ubiquity and high fluorescence emission. As each probe's fluorescence varies depending on FA concentration, the optimal concentration had to be determined for each probe empirically. This was accomplished by creating the FA Gradient Curve.

2.7 Qualitative FA concentration Series of New Probes

For each probe, a slide for every FA concentration was prepared (0%-50% in 10% increments). This gradient was purely qualitative for the purpose of deciding on which probe is more suitable for live-FISH. Each slide had one well with each of the following conditions: Target bacterium + tested probe; Target bacterium + EUB338; Target bacterium + non-Cy3 probe; Target bacterium + non-FLUOS probe; non-Target bacterium + tested probe; non-Target bacterium + EUB 338.

Each slide was hybridized for 2,5 hours and washed for 15 minutes. After DAPI staining, each FA concentration was visualized on CLSM at constant conditions (3x zoom, 150% SmartGain). An image of both target probes and DAPI was taken of each concentration – the gradient of Acid303 is in section 3.2

2.8 LIVE/DEAD BacLight Bacterial Viability Kit

The two constituents of ThermoFisher BacLight Bacterial Viability Kit L7007 (ThermoFisher Scientific, Germany) are: SYTO9 green fluorescent dye that stains all cells (regardless of viability), and propidium iodide red fluorescent dye (PI) that only stains cells with compromised cell membrane (Crowley et al., 2016). PI is an intercalating agent in the bacterial chromosome – when it binds multiple residues, its fluorescence exponentially increases, quenching SYTO9 fluorescence at that spot. The excitation/ emission maxima are 480/500 nm for SYTO9, and 490/635 nm for PI (Stocks, 2004).

After hybridization and washing, 15 µL of the hybridized liquid culture was added to each well on a teflon-coated FISH slide. Inside of a laminar flow and in darkness, the slides were dried until the wells were almost dry – this was to ensure that the cells did not die due to desiccation, but the wells were dry enough to ensure bacterial attachment to the slide. Afterwards, 10 µL of the BacLight solution (a 1:1 mix of Component A and Component B) was added onto each slide well and incubated in darkness for approximately 10 minutes. The slides were then washed in ice-cold MQ, dried with pressurized air, and DAPI-stained before microscopy.

2.9 Live-FISH on *Pseudomonas putida* and *Bacillus* sp.

The live-FISH protocol (as described in section 2.3) was applied to *Pseudomonas putida KT2440* and *Bacillus sp. P12204* as control strains. After the application of live-FISH protocol, each bacterial culture was pipetted on three separate FISH slides. One slide of each bacterial strain was incubated for 3 hours at 46°C; 5 hours at 37°C; and one of each for 24 hours at 28°C.

2.10 Live-FISH on *Bryocella* sp. and *Edaphobacter* sp.

Live-FISH protocol modified for the salt concentration was applied to both species with the exception of the adjusted hybridization step employing 12 hours and 24 hours at 28**°**C. These gentler conditions to help increase the survival of the less resilient representatives of *Acidobacteriota,* such as the ones used in this study. Hybridization was carried out in the dark and BackLight kit was applied afterwards.

2.11 Soil Enrichment and Filtration

Peat soil was sourced from Isle of Skye, Scotland, coordinates 57,43061° N, 6,77815° W by the author of this study. This soil was proven to contain *Acidobacteriota* from subdivision 1 by plating and colony isolation on VL55+XPC (Xylan-Pectin-Cellulose) agar plates followed by standard PCR protocol and 16S sequencing (primers 616V and 1492R). In a 300 mL Erlenmeyer flask, 25 g of peat soil was mixed with VL55 + XPC liquid medium (50 mL) and kept on shaker at 200 rpm at RT. After two weeks, the enrichment was supplemented with additional 50 mL of VL55 + XPC liquid medium. After a total of 3 weeks the enrichment was taken off the shaker, larger soil particles were left to settle, and the liquid was carefully transferred by pipetting into the upper part of a filtration unit. The liquid culture was filtered using a 5 µm filter into a sterile Schott bottle. After filtration, the process was repeated with 2 µm, and 1 µm filters. All filters were MF-Millipore MCE 47 mm nitrocellulose membranes.

A part of the final filtrate was PFA-fixed, hybridized with Acid303 probe, and analyzed using CLSM as a classic FISH control. Another part was stored at 4°C for possible live-FISH on soil filtrate in case the control FISH proved successful.

2.12 FA melting curve of Acid303 on *Edaphobacter* sp.

The protocol from part 2.7 was repeated for the quantitative FA gradient with some adjustments. Here, SPOG37A was used instead of SPOG32A, as the cells do not tend to aggregate as much, and the sample can be quantified with higher precision. A slide was prepared for multiple FA concentrations (0%; 10%; 20%; 30%; 40%). After hybridization, DAPI was applied to all wells. *Pseudomonas putida* KT2440 was used as the non-target organism control. Slide layout is shown below.

SPOG37A	SPOG37A + EUB338	SPOG37A + NON-Cy3	SPOG37A + NON-EUB	SPOG37A + Acid303	SPOG37A + Acid303
P. putida	*P. putida* + EUB338	*P. putida* + NON-Cy3	*P. putida* + NON-EUB	*P. putida* + Acid303	*P. putida* + Acid303

For wells where visible signal was detected, 10 pictures were taken from a randomized position in the well for each FA concentration and exported in black-and-white TIFF images. These images were then imported into daime (version 2.2.3) for analysis. The daime software was able to assess the fluorescence emitted by cells. This fluorescence was then be quantified and plotted. The images were segmented (removal of unclear signals, such as non-cell fluorescence and cells at the edge of the picture), the resulting values were visualized in a line graph.

3 Results

3.1 Live-FISH With Probe EUB338 on Representative Bacteria

This preliminary experiment was carried out using a modified protocol developed by adapting Batani et al., 2019. The adjustments mostly involved lowering the salt concentrations before and after heat shock, the protocol used is described in part 2.3. It served as a demonstration of survival and hybridization of both gram-positive bacteria (*Kocuria sp.* P12228; *Bacillus sp.* P12204) and gram-negative bacteria (*Serratia sp.* SPOG326; *Terriglobus sp.* SLOG1A). Small portion of the cells (100 µL) was plated on agar after each major step of the protocol in multiple dilutions, as seen in Figure 3. The universal probe EUB338 was tested here due to its reliability.

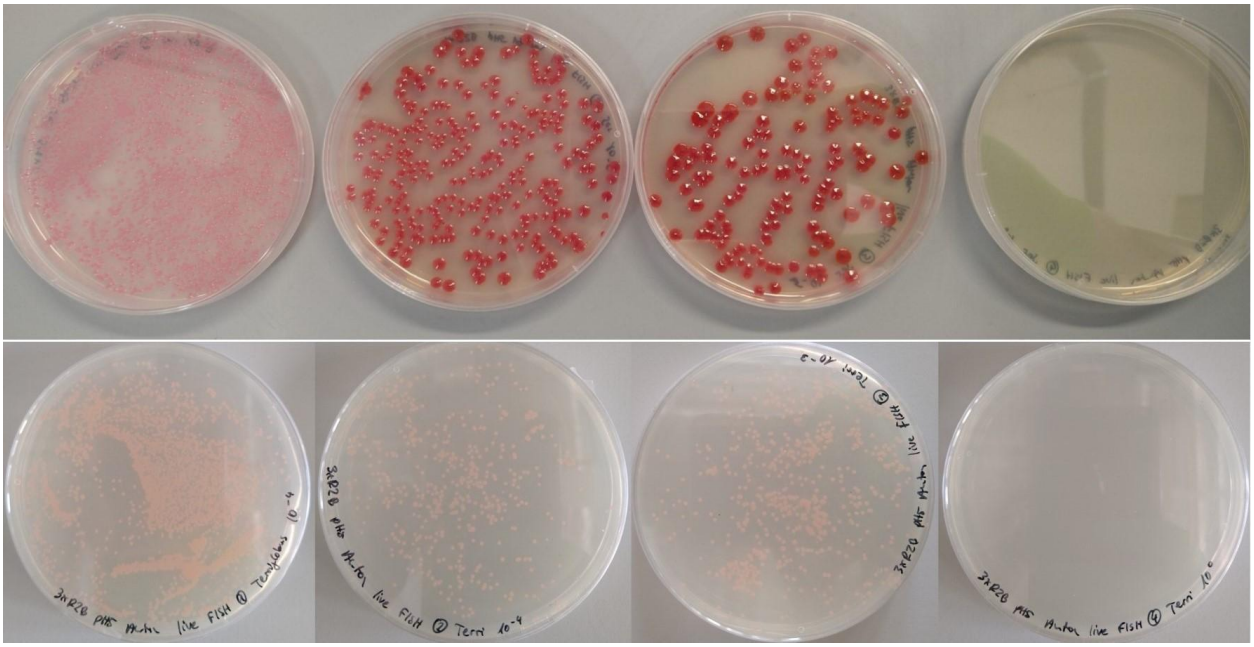


Figure 3: *Serratia sp.* SPOG326 (upper row) and *Terriglobus sp*. SLOG1A (lower row) survival. Images depict the agar plating results after each important protocol steps, which flow from left to right as follows: **A:** pre-experiment control, where the cells are numerous and not stressed, dilution 10^{-4}; **B:** after washing, where some cell loss occurred during the multiple washing steps, dilution 10^{-4}; **C:** after heat shock, where the cells suffered heat damage. Cell number reduction is apparent, note the dilution decrease to 10^{-3}; **D:** after hybridization, no viable cells were detected, even at dilution 10^{0}. Agar medium used was 3xR2A.

Survivability of the two gram-negative bacteria, *Serratia* sp. SPOG326 and *Terriglobus* sp. SLOG1A, was high after the washing step and before the heat shock. Nevertheless, a clear reduction in colony formation (Figure 3B) was observed based on visible inspection of the agar plates compared to the pre-experiment control (Figure 3A), for both a dilution of 10^{-4} was used. After the heat shock, it is apparent that a high number of cells did not survive (Figure 3C). Here, a dilution of 10^{-3} was used instead of 10^{-4}used in the previous step, yielding a similar number of colonies. However, survival tests done after hybridization yielded no viable cells on the test plates, even at zero dilution (Figure 3D).

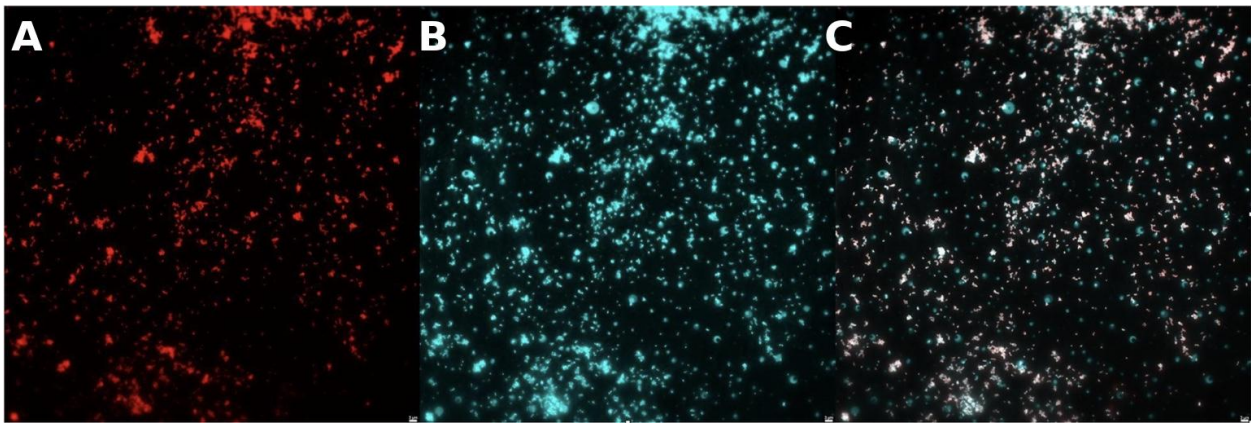


Figure 4: Live-FISH results of *Terriglobus* sp. SLOG1A. A: EUB338 probe; **B:** DAPI; **C:** overlay of both fluorescent signals. Size bar of 5 µm is on the bottom-right.

As a contrast to their survival tests, gram-negative bacteria fared well in terms of hybridization. Good fluorescence signals were obtained from *Terriglobus sp.* SLOG001A using the EUB338 probe (Figure 4A). The probe signals also overlapped well with the DAPI signal (Figure 4C). The circular and semi-circular shapes are artifacts from the FISH process.

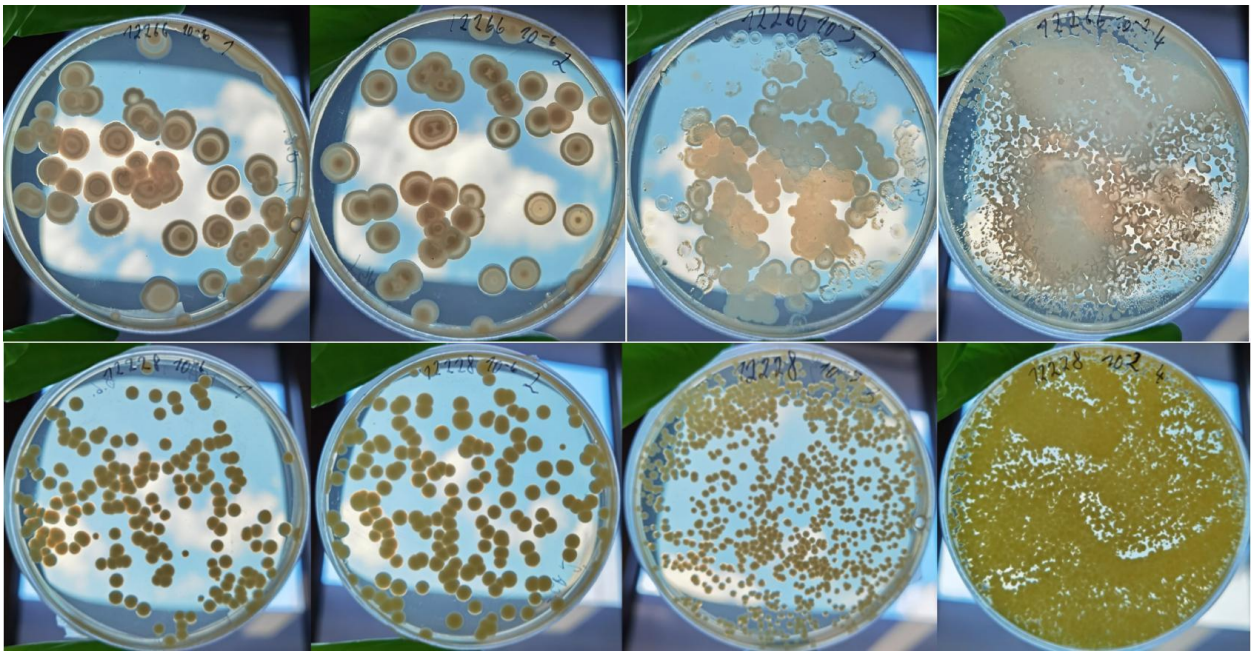


Figure 5: *Bacillus sp.* P12204 (upper row) *and Kocuria sp.* P12228 (lower row) survival assessment. Images depict the agar plating results after each important protocol steps, which flow from left to right as follows: **A:** pre-experiment control where the cells are numerous and not stressed, dilution 10^{-6}; **B:** after washing, where some cell loss occurred during the multiple washing steps, dilution 10^{-6}; after heat shock, where the cells suffered heat damage. Cell number reduction is apparent, note the dilution decrease to 10^{-5}; after hybridization, which left plenty of survivors at dilution 10^{-2}. Agar medium used was TSA.

The representatives of gram-positive bacteria mentioned above fared much better in terms of survivability. Even at 10^{-2} dilution, the agar plates were full of growth of each respective bacterium. Notable is the change of colony morphology after the heat shock step.

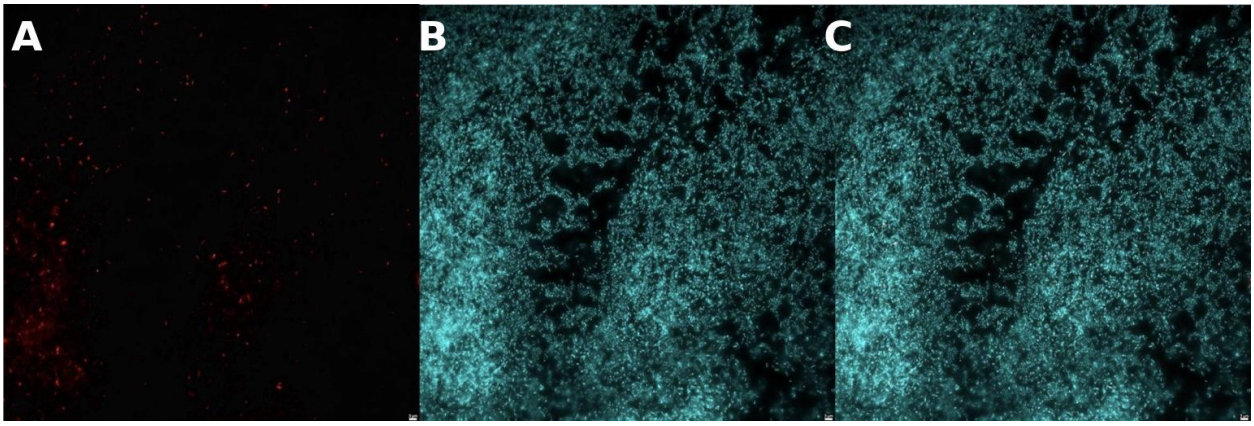
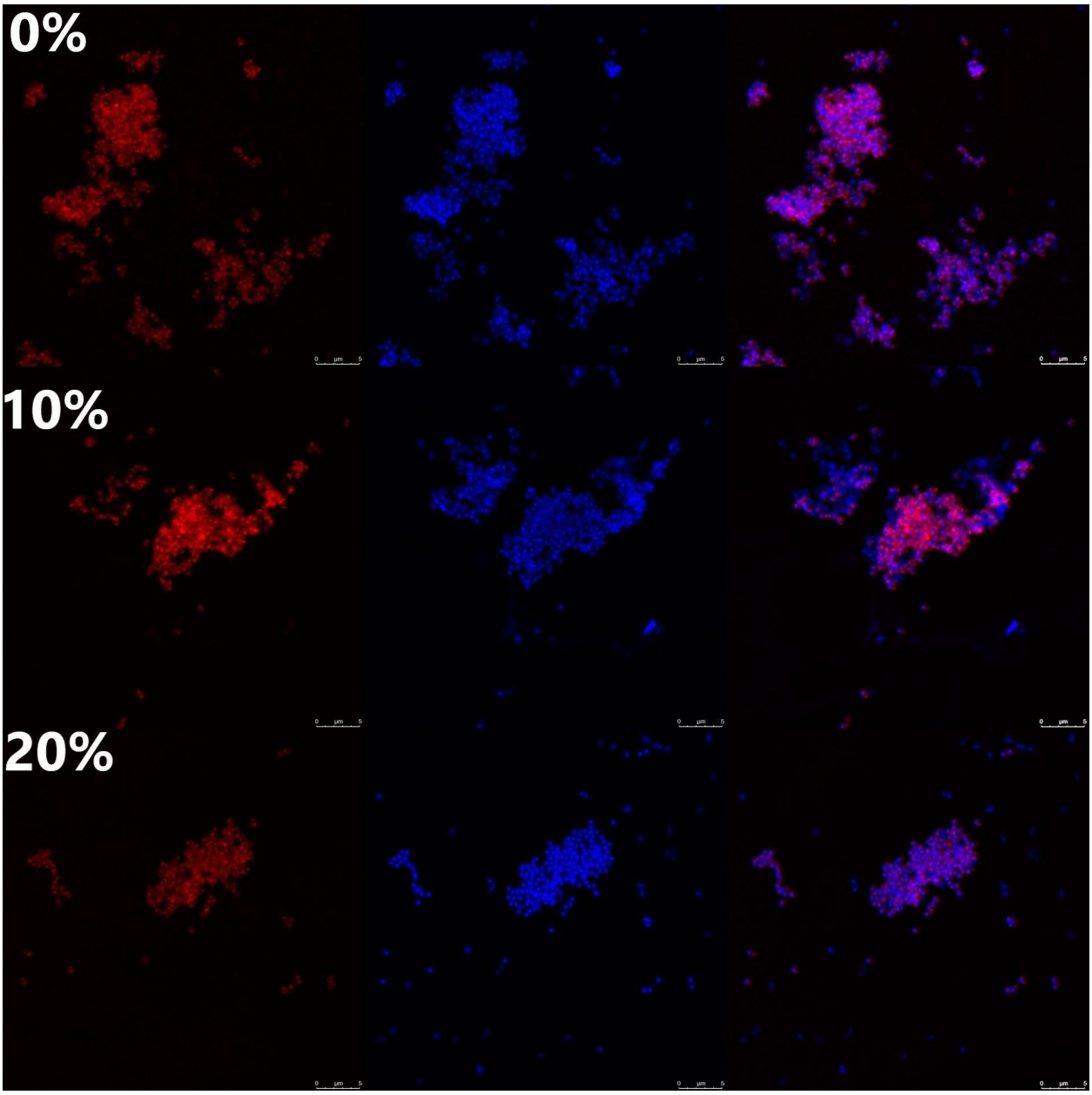


Figure 6: Live-FISH results of *Bacillus sp. P12204.* A: EUB338 probe; **B:** DAPI stain; **C:** overlay of the two. Size bar of 5 µm is on the bottom-right.

In comparison to well hybridized gram-negative bacteria, almost no probe signal from the gram-positive bacteria could be detected, with exception of a few cells and background noise. This was the case despite the massive amounts of cells present in the sample, as clearly visible from the DAPI signal in B section of Figure 6.

3.2 Qualitative FA Concentration Series of New Probes

In qualitative FA gradient, the strength of specific signals was assessed at different FA concentrations of hybridization buffer. The expected pattern of increasing/decreasing fluorescence across the FA range suggests the FA concentration where the hybridization is optimal. As this first FA gradient was only qualitative, the probe (Acid303) was tested only on its target organism and the picture at each concentration had no replicates. The goal was to visually roughly identify at which point the fluorescence starts to fall off in order to use the lowest possible FA concentration of hybridization buffer. This was to ensure minimal FA lethality on cells, especially during longer cultivations. Additionally, it removed another variable related to survival.


0%
10%
20%

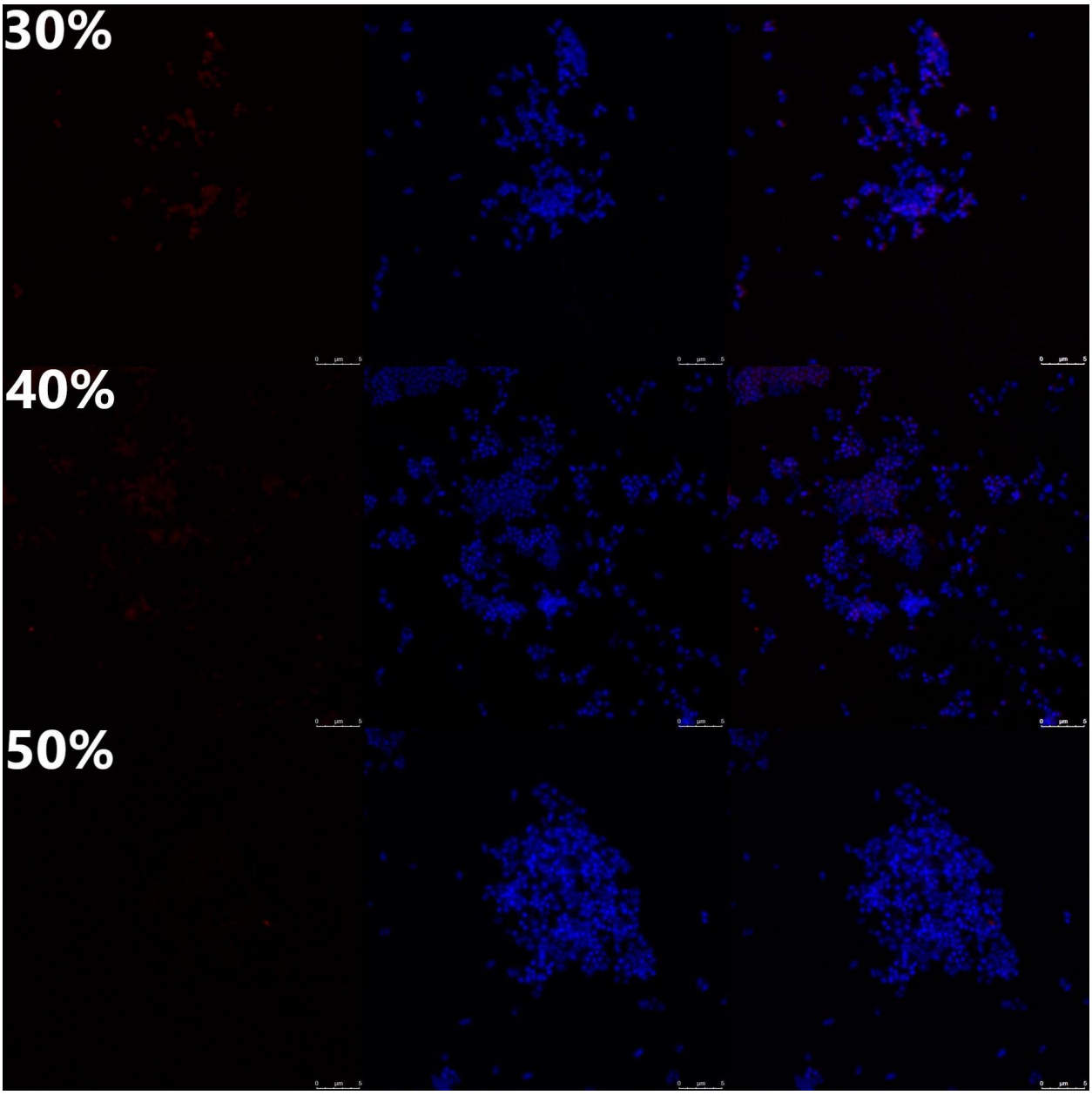


Figure 7: Qualitative FA Gradient on SPOG32A. The specific probe (Acid303) is depicted in the left column, DAPI stain is in the center column, while on the left is their overlay.

Qualitative FA gradient indicated that the best FA concentration of hybridization buffer was between 0% and 10%. Above 10% FA, the signal gradually faded until being imperceptible beyond 40% FA. This was an optimal result for the purposes live-FISH, as 0%FA could be used. The stringency would suffer with no FA, but cell survival was given a higher priority.

3.3 BacLight Kit Test on *Bryocella* sp. and *Edaphobacter* sp.

The BacLight Kit solutions (A and B) were mixed in a 1:1 ratio. This solution was added to the cell suspension at the rate of 3 µL BacLight per 1 mL of cell suspension. The working solution was applied on FISH slide wells containing fixed SPOG32A culture. It was then washed gently with MQ, dried, CitiFluor was added, and the bacteria observed with CLSM.

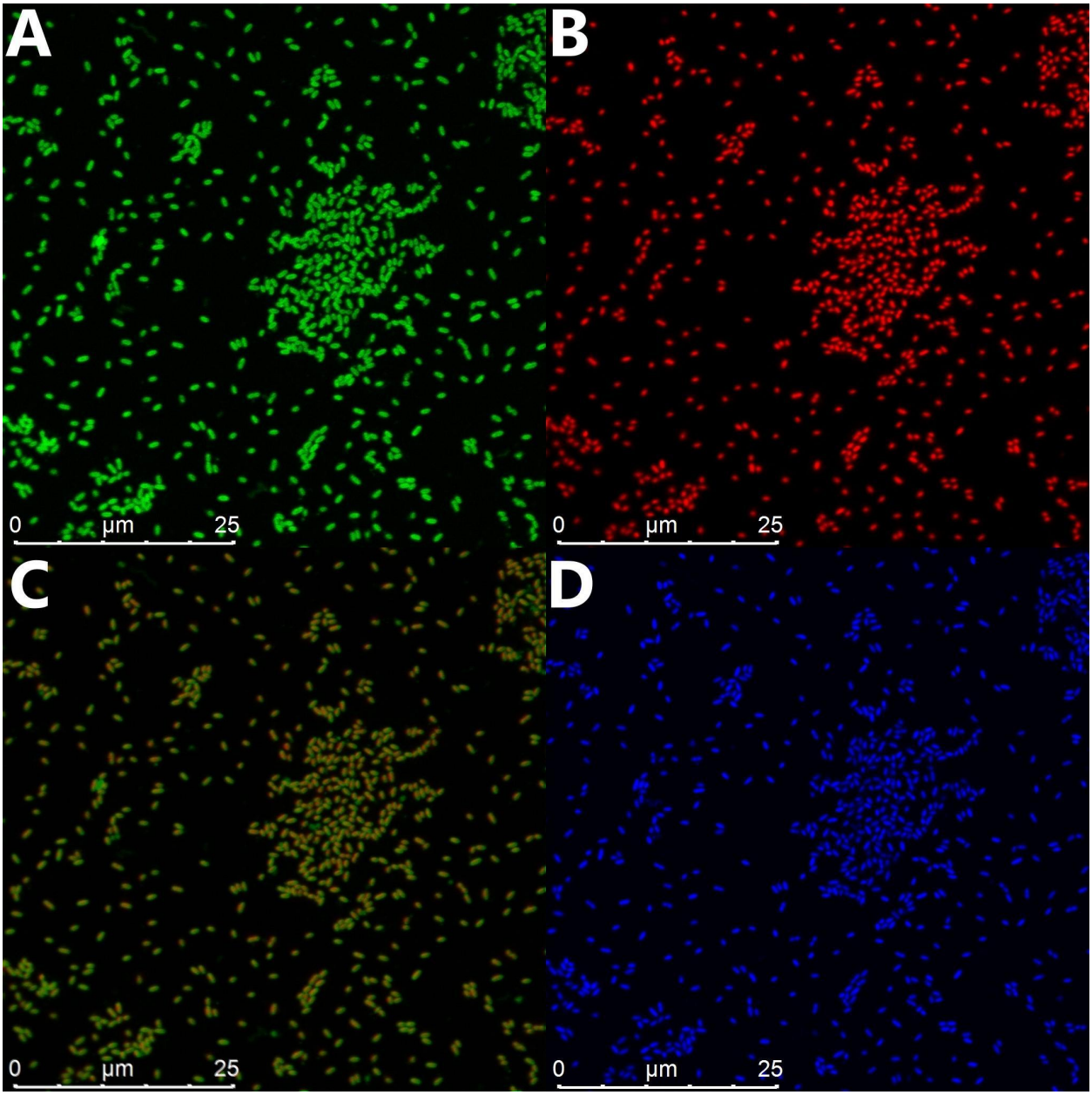


Figure 8: BacLight kit example with SPOG32A strain. The cells were fixed to get a negative BacLight control. **Part A** shows green SYTO9 fluorescence and represents all cells. **Part B** contains only the red fluorescence of PI – dead cells. **Part C** is an overlay of **A** and **B**. **Part D** is DAPI control. Scale bar is in the bottom left corner.

The cells were clearly visible and each dye had remarkable intensity. It was also confirmed that all cells emitted the red PI signal alongside the green SYTO9 signal (Figure 8). DAPI staining also confirmed that SYTO9 stained all cells.

With the negative control established, a positive control experiment was carried out. Here, SPOG37A served as the test member of *Acidobacteriota*. Instead of being fixed, 20 µL of the cell culture was transferred to each well on a FISH slide. This slide was left to almost completely dry in a laminar flow. After most of the water evaporated, the BacLight working solution was added to each slide. The working solution consisted of 3 µl of BacLight diluted in 1 mL of 0,9% NaCl. The slide was left to incubate and then was gently washed away with MQ. The slide was dried, CitiFluor and coverslip applied, and visualized using the CLSM.

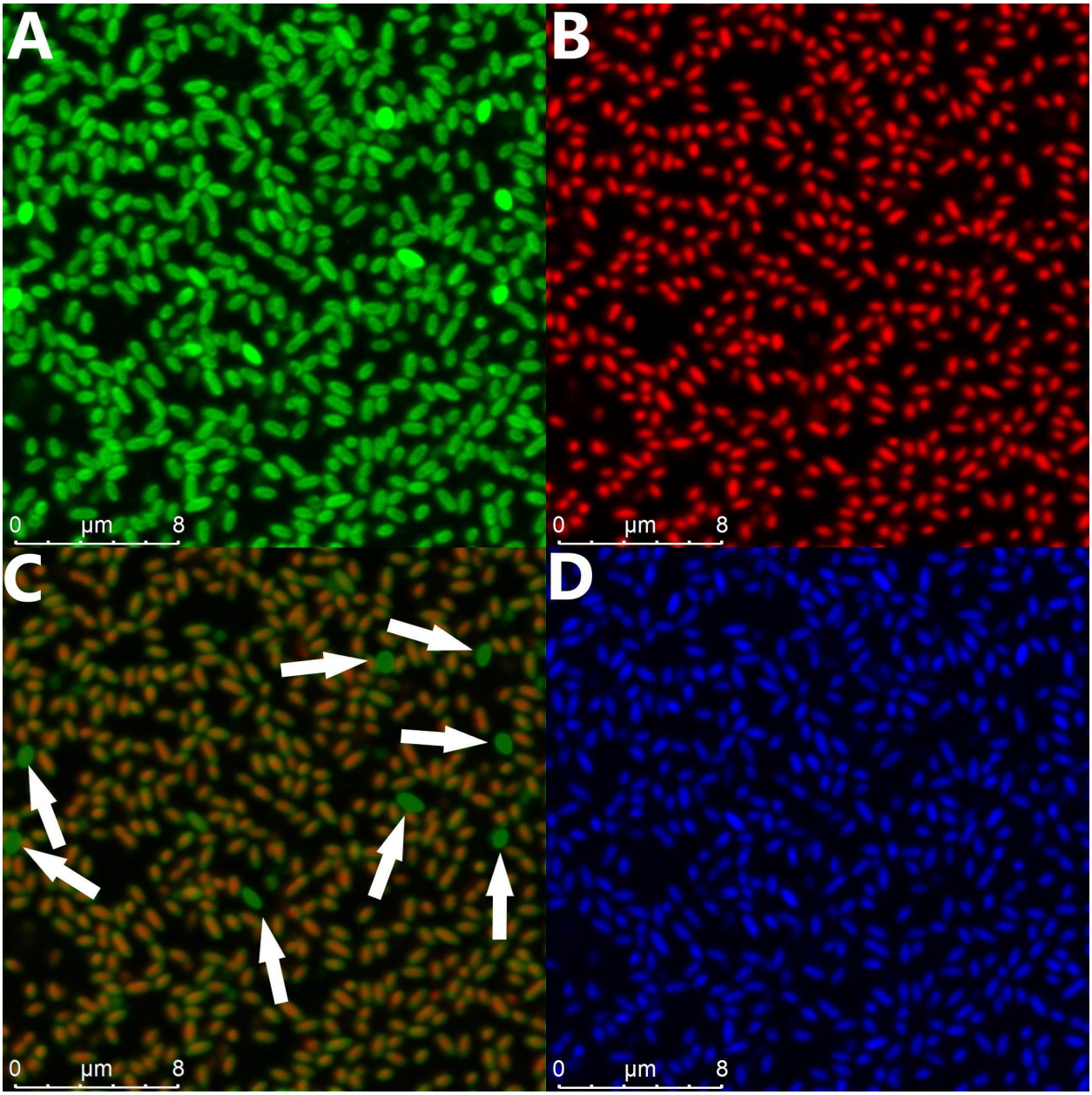


Figure 9: BacLight kit test on pure culture of SPOG37A. A: SYTO9; **B:** PI; **C:** their overlay, white arrows point at the cells that are SYTO9 positive and PI negative - alive; **D:** DAPI. Scale shown on the bottom left corner. Cells were sampled from the logarithmic liquid growth phase.

As is apparent from Figure 9, the vast majority of SPOG37A cells did not survive the protocol. However, the few acidobacterial cells that did survive proved that the procedure can be viable with some optimization. Additionally, it provided a benchmark on how the surviving cells should look compared to the dead cells.

3.4 Live-FISH on *P. putida* and *Bacillus* sp.

In the live-FISH test of *P. putida* and *Bacillus* sp., both species were sampled from their logarithmic growth phase and hybridized with their respective probes (BLS1295 targeting *Bacillus* sp., and GAM42a targeting *P. putida*). Three hybridization conditions were chosen: 24 h at 28°C; 5 h at 37°C; and 2 h at 48°C. The last of these conditions served as a simulation of normal FISH with fixed cells, where higher hybridization but lower survival rates were expected. The 24 h at 28°C condition was expected to result into higher survival but lower or more specific hybridization. It was hypothesized that 5 h at 37°C would be a good compromise.

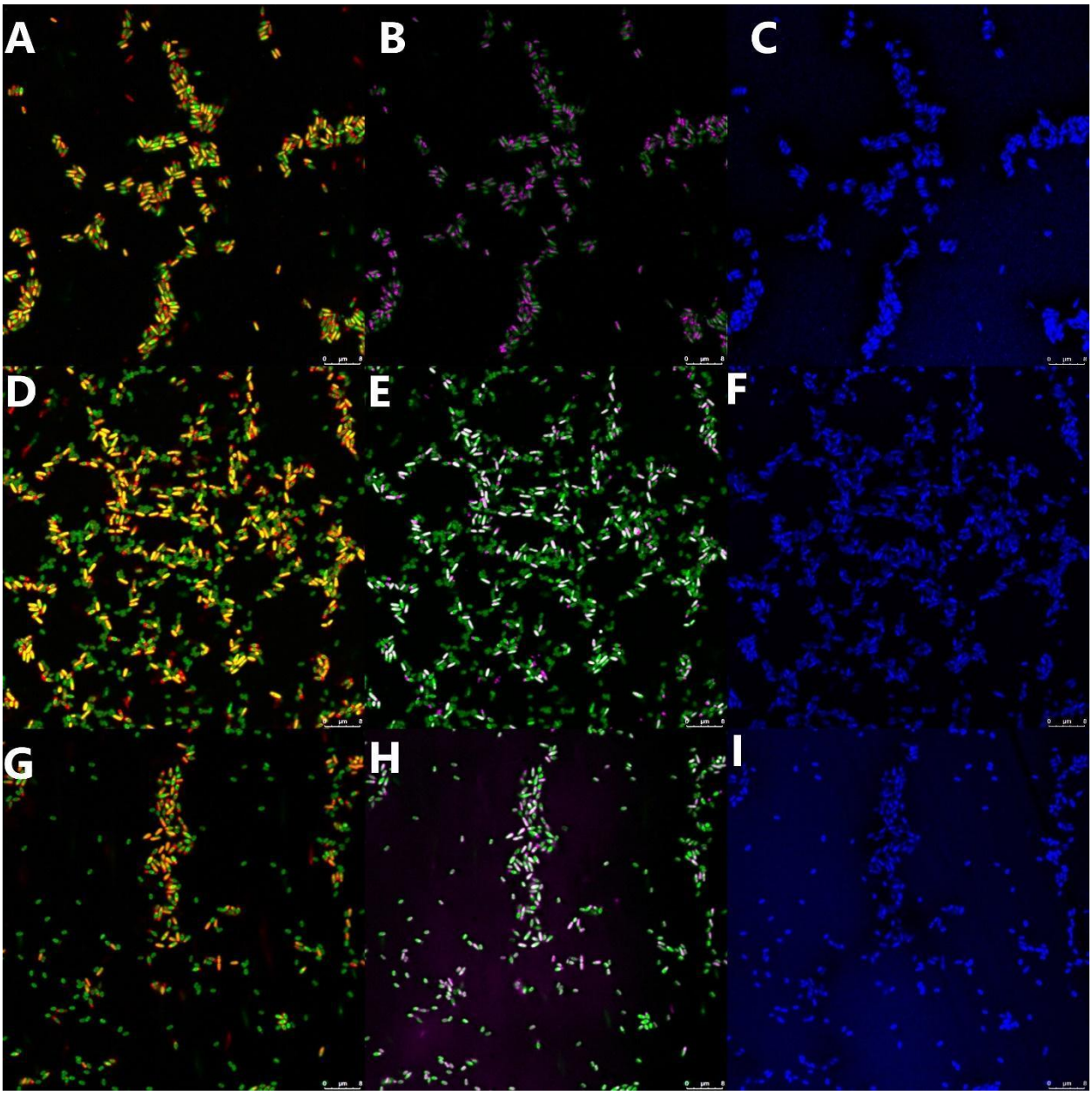


Figure 10: Example showing *Bacillus* sp. P12204 live-FISH. Green signal: SYTO9; red signal: PI; purple: target probe; blue: DAPI. **A-C**: 2 h 46°C incubation; **D-F**: 37°C 5 h incubation; **G-I**: 24 h 28°C incubation. Columns **A-G**: BacLight kit viability of the cells; **B-H**: hybridization efficiency; **C-I**: all biomass present. Scale is in the bottom right corner.

The results of the hybridization in Figure 10 aligned with the expectations. Incubation 2 h 46°C had high hybridization rates but low survival, 37°C 5 h had an average survival and hybridization rates, while 28°C 24 h had higher survival but relatively low hybridization rate. However, these results only applied for *Bacillus* sp., where the survival check by agar plating resulted into dozens of viable colonies. However, *P. putida* had minimal survival, where only the 28°C 24 h condition resulted in a few colonies while having slightly better hybridization rates (not shown). These results showcase the difference in adaptation

between gram-positive and gram-negative bacteria in regards to harsh conditions and cell wall permeability.

3.5 Live-FISH on selected *Bryocella sp. and Edaphobacter sp.*

The live-FISH protocol was modified to reflect the delicate nature of *Acidobacteriota*. The temperature was set at 28°C and two timepoints (12 h and 24 h) were sampled. After applying the BacLight kit, the cells were visualized.

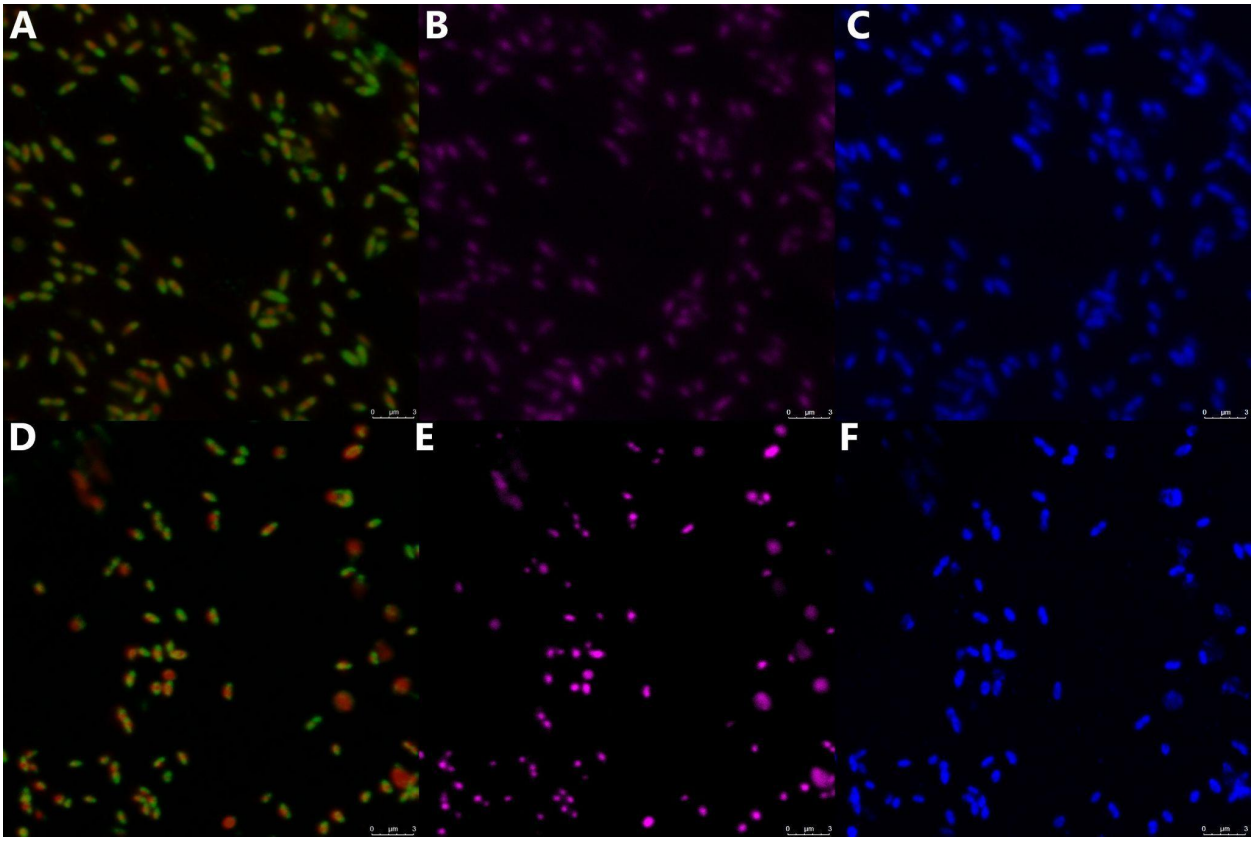


Figure 11: live-FISH on SPOG32A. Pictures **A**, **B**, and **C** were taken after 12 h incubation at 28°C; **D**,**E**, and **F** are after 24 h incubation at 28°C. **A** and **D** show the overlay of SYTO9 and PI; **B** and **E** show the hybridization of the Acid303 probe; and **C** and **F** are DAPI controls.

From Figure 11, it is apparent that hybridization worked quite well, where majority of the cells were hybridized. However, there was no survival of cells in BacLight microscopy images. This was confirmed by plating the live-FISH culture on 1/10 R2A agar - not a single colony appeared.

Next, the same procedure was applied on SPOG37A. However, some slide wells contained higher cell density to obtain bigger sample size for the purpose of survival and hybridization assessment.

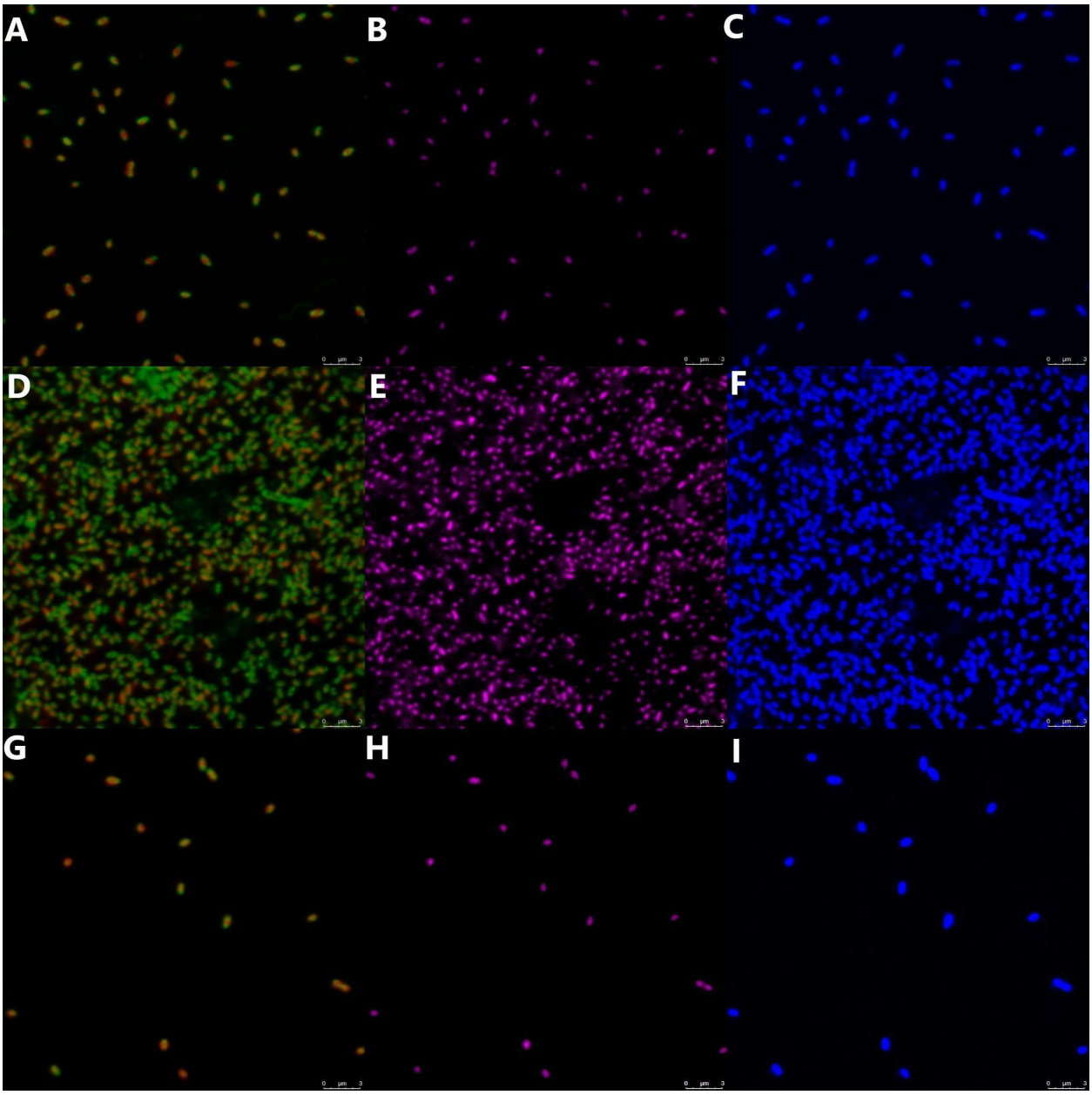


Figure 12: live-FISH on SPOG37A. A-C: 12 h incubation at 28°C; **D-F:** 24 h incubation at 28°C with higher cell density; **G-I:** 24 h at 28°C with fewer isolated cells. **A,D**, and **G** are the overlays of SYTO9 and PI; **B,E**, and **H** show Acid303 probe hybridization; and **C, F**, and **I** show DAPI control.

Just as in the case of SPOG32A, the live-FISH on SPOG37A resulted in good hybridization but universal mortality. This is the case in both 12 h and 24 h hybridization times. A shorter hybridization could trade a slightly lower hybridization rate for a higher survival.

At this magnification, the need for digital zoom results in a blurriness that is apparent from Figures 11 and 12. The principal cause is the size of the cells - these members of *Acidobacteriota* measure between 0,6 µm to 0,9 µm, making them smaller than some viruses.

3.6 FISH on Soil Enriched with VL55+XPC

After 3 week of shaking cultivation, the VL55-XPC - enriched peat soil was filtrated through 5 µm, 3 µm, and 1 µm filters. A standard FISH was done on each of these three filtrates. The 1 µm FISH filtrate was hybridized with Acid303 probe and dyed with DAPI, and visualized using CLSM.

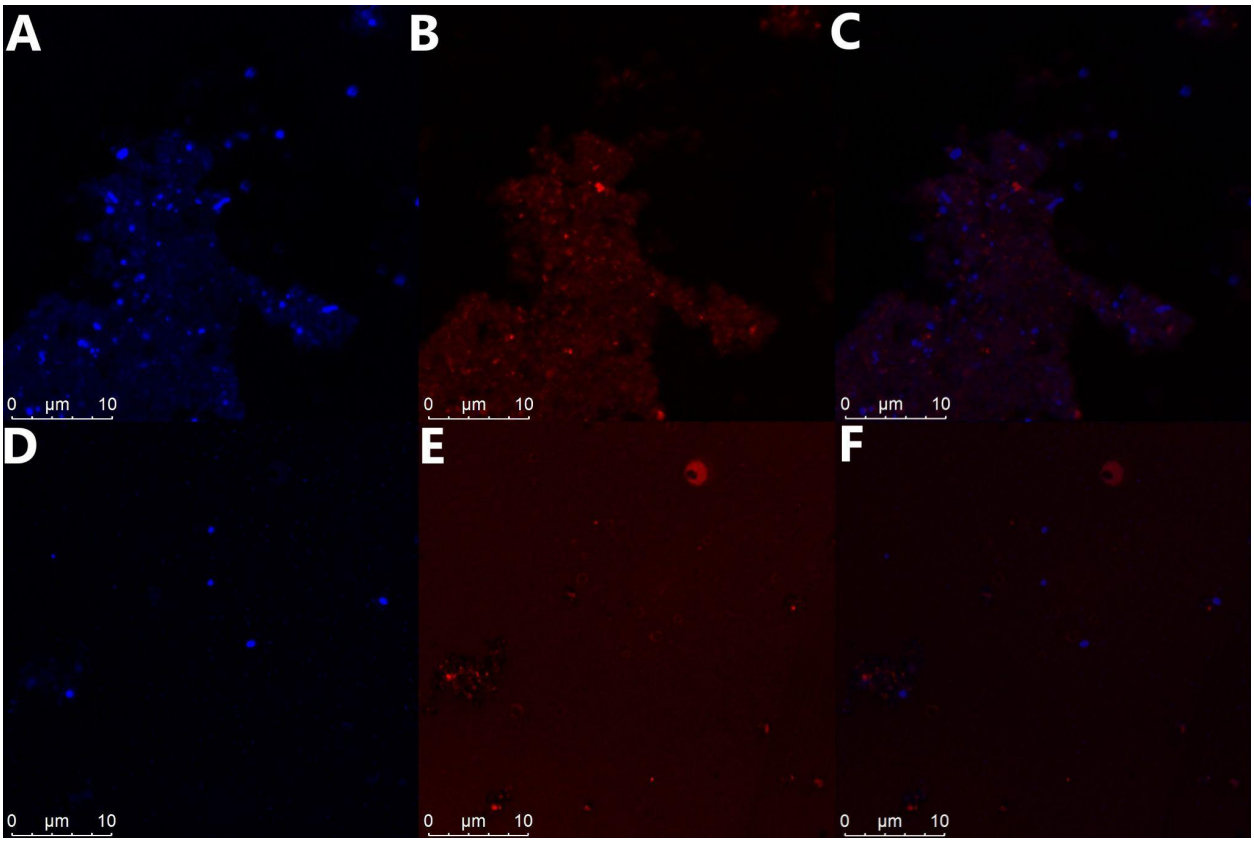


Figure 13: FISH on 5 µm filtrate of VL55+XPC soil enrichment. A and D: DAPI signal; **B and E:** specific probe Acid303; **C and F:** overlay.

As apparent from Figure 13, there was no overlap of DAPI and Acid303 signal, indicating that if DAPI successfully dyed actual cells, the specific probe failed to hybridize with its intended target. Most of the Cy3 signal in the sample is most likely the result of autofluorescence and non-specific binding. In the E picture, there is an fluorescent artifact in the upper-right corner as well as a general low-level background probe signal. The cause of this phenomenon could not be identified.

3.7 Melting Curve Analysis of Acid303 on *Edaphobacter* sp.

To establish the optimal FA concentration of the hybridization buffer of Acid303 while controlling for the specificity, the melting curve (or quantitative FA gradient) of Acid303 on SPOG37A was carried out. For probe + target organism, the expected result was a plateaued curve that has an exponential fall-off, indicating the best FA concentration for this particular probe. Importantly, the probe + non-target curve has to retain low levels of fluorescence, showing that the probe is specific to the target organism.

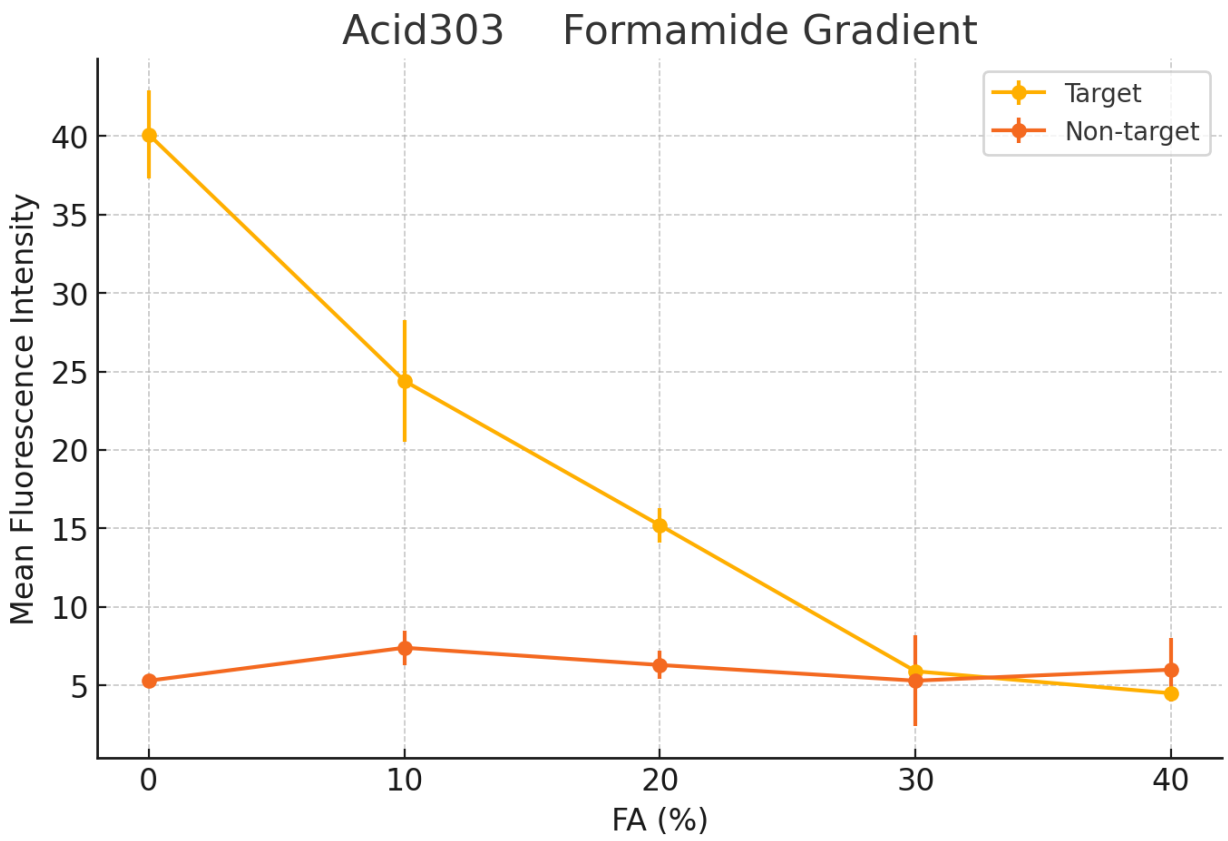


Figure 13: Melting curve of SPOG37A and *P. putida* hybridized with Acid303 probe. The x-axis represents the increase in FA content of the buffer while y-axis represents the fluorescence in Mean Fluorescence Intensity units.

The orange slope represents the fluorescence intensity change of non-target organism *P. putida* across the FA concentrations. There was barely any visible change of fluorescence throughout the range. In contrast, the target organism SPOG37A (in yellow) started off with a very high signal at 0% FA and a gradual decrease was observed until 30% FA, where the fluorescence intensity of target and non-target organisms floated around the same value. No visible fluorescence was observed after 30% for either sample, which correlates with the graph in Figure 13. In general, the intensity of the observed fluorescence correlates with the results of the melting curve.

4 Discussion

The initial live-FISH protocol of this study was derived from the one published by Batani et al., 2019. However, some adjustments were made, such as lowering the salt concentrations – unlike this study, original paper worked mostly with marine-derived bacteria that have higher resistance to high osmotic pressure. Other modifications included the changes to temperature, hybridization temperature, and other factors that could negatively influence *Acidobacteriota* survival.

4.1 LIVE/DEAD BacLight Bacterial Viability Kit

Bacterial viability kits are used to determine the viability of each bacterial cell in a population. It relies on the principle that the cell membrane is semi-permeable when the cell is healthy. Compromised cell membrane almost always means that the cell is dead (Grégory et al., 2001). The BacLight kit contained two vials called Component A and Component B. Component A was composed of 1,67 mM SYTO9 & 1,67 mM PI while Component B is composed of 1,67 mM SYTO9 & 18,3 mM PI. The manufacturer recommended varying the ratios of Components A and B to achieve optimal balance of SYTO9 and PI signals. In this study, 2:1; 1:1; and 1:2 ratios were assessed. From these, 1:1 was found to offer the optimal balance SYTO9 and PI signal strength. This is also the recommended ratio by manufacturer as well as most commonly used in practical applications of the Backlight kit (Ivanova et al., 2019). As PI quenches the fluorescence of SYTO9, there is almost no overlap of the green and red signal. This helps to resolve cells that are no longer viable. However, not all cells that lack the red PI fluorescence are viable. By varying the focal plane of the microscope, some cells that are slightly off the focal plane appear to only emit green SYTO9 fluorescence with no red PI fluorescence. However, these cells are also dead – even though the PI fluorescence is not detected, the PI quenching effect causes a dark area to appear within SYTO9 fluorescence. To remedy this, a z-stack of merged pictures would bring all cells within the plane of focus, making the assessment easier. It was difficult to involve the Backlight kit in the live-FISH procedure. The reagents could not be added to the hybridized cells after the live-FISH protocol. The prolonged exposure would not only decrease the cell viability but also eliminate the control over incubation length, decreasing the specificity of the signal. To combat these limitations, the live-FISH sample was pipetted on the FISH slide with the BacLight working solution. This way, the cells were exposed to the chemicals in the kit for only a short period, making the cell loss minimal. Before the BacLight solution was added, the live-FISH culture was let dry almost completely in laminar flow so that most bacteria attach on the slide but do not die from desiccation. On these attached bacteria, the solution could be added and washed away in controlled quantity and for specific time.

4.2 HoAc1402 and New Probes

Apart from survival test of bacteria undertaking the life-FISH protocol, HoAc1402, a general *Acidobacteria* probe was tested in this study. This probe should theoretically bind most respective species. However, it proved to be unreliable when used as a reference probe – sometimes it failed to produce any fluorescence while other controls worked well. This is most likely due to the probe binding on the end of the sequence. If the sequence is truncated due to environmental factors, the probe cannot bind well and this sometimes results in little to no signal (Juretschko et al, 2002; Loy et al., 2007). For the purpose of live-FISH, a new probe was required to eliminate HoAc1402's unreliability as a variable. The new probes were based on primers developed for a study by Hausmann et al. in 2018 where the team was interested in 16S real-time quantitative PCR. The primers were designed with high specificity in mind, where for each acidobacterial subdivision (1-3) there was a set of complementary primers, one

forward and one reverse (section 2.6, table 3). As *Acidobacteriota* subdivision 1 species were the only ones available among the three subdivision, only the first two probes were tested.

4.3 Live-FISH

4.3.1 Live-FISH with EUB338 on Representative Bacteria

Gram-negative bacteria were found to be significantly stressed while applying live-FISH protocol. The lethality of this protocol is clearly visible in Figure 3, where a high number of cells survived until the hybridization step, after which no cells could be found on a survival check agar plate, even at no dilution. This absolute lethality was most likely caused by the long incubation at high temperatures and potentially toxic chemicals. On the other hand, gram-positive bacteria's survival (Figure 5) could be attributed to their more robust peptidoglycan layer in the cell wall that offers better protection against environmental factors. However, the stress the bacteria had to undergo is obvious – after the heat shock, the morphology of the bacterial colonies dramatically changed. The colonies appeared to be smaller, paler, and less defined. This could have been caused by the cells prioritizing shock response metabolite production over division and growth (Räsänen et al., 2001; Pavlovsky et al., 2015). The difference in morphology is pronounced after the last step and the much smaller number of viable cells reflects the extreme conditions of the hybridization process. At the time of these first experiments, the BacLight kit was not available. Therefore, no definitive answer could be established whether the cells that survived were also hybridized. However, it showed that some bacteria, especially the more resilient gram-positive bacteria, can survive the live-FISH procedure. Further optimization of the method could be used for future cultivation effort targeting so called rare actinobacteria. *Actinomycetota* are well-established secondary metabolite producers. Most known *Actinomycetota* have been already well studied and their secondary metabolites identified. These metabolites include many of the commonly used antibiotics, and as such have a significant impact on pharmaceutical and healthcare industries. However, the potential of the rare actinobacteria is largely unexplored, mainly due to the difficulty of cultivation and isolation under the standard laboratory conditions. Many of them require unknown specific growth conditions which is why standard methods of cultivation fail (Ramasamy et al., 2022). Novel cultivated rare actinobacteria could lead to the identification of novel secondary metabolites, such as novel antibiotics (Parra et al., 2023). Rare actinobacteria could be a better choice for live-FISH due to their hardiness compared to *Acidobacteriota* – the survival rate of live-FISH could be higher and, therefore, the amount of cells recovered for subsequent FACS could be sufficient for further cultivation efforts.

4.3.2 Live-FISH on *P. putida* and *Bacillus* sp.

To establish a baseline for the live-FISH methodology, *P. putida KT2440* and *Bacillus* sp. P12204 were used as representative organisms of gram-negative, and gram-positive bacteria, respectively. While *Bacillus* sp. P12204 showed a good rate of survival and some hybridization, *Pseudomonas* cells were mostly dead, with only two colonies growing on the test TSA agar plate. *Bacillus* sp. showed much better survival rates, most likely due to its thick peptidoglycan layer that offers some protection against unfavorable environmental conditions (Pasquina-Lemonche et al., 2020). The 5 h 37°C condition seems promising, as that temperature can be withstood by a number of bacteria indefinitely and it is closer to the optimal probe hybridization temperature (46°C). This also supports the hypothesis that gram-positive bacteria could be a better target for live-FISH as it is much easier to modify hybridization rate than survival rate, as the tools for increasing the former are well-tested and the procedure can be fine-tuned. Conversely, survival is affected by many factors and it is hard to pinpoint the exact variable that has to be changed for survival rate to increase. Interesting factor to note is that the cells had decent survival

despite the high level (35%) of formamide present during the hybridization process. Further experiments in this area could focus on creating a mock community of various Gram-positive bacteria and testing the specificity of probe hybridization.

4.3.3 Live-FISH on *Bryocella* sp. *and Edaphobacter* sp.

As it was well-established that *Acidobacteriota* are more sensitive to environmental factors than *Bacillus* spp. and *Pseudomonas* spp., the protocol was adequately adjusted. Except for the heat shock, the temperature during the *Acidobacteriota* live-FISH never exceeded 28°C. Additionally, the survival check by plating on agar was done only once – *Acidobacteriota* colonies take considerably longer to grow until visible (approximately 6 days) compared to model bacteria used here such as Bacillus sp. (1-2 days) which is why perpetual survival assessment on agar plate was not feasible to accomplish during the time of this study. Instead, the procedure's survival assessment mostly relied on the BacLight kit. A discrepancy between BacLight and the actual cell survival may have occurred, mainly due to the relatively small number of cells visually assessed. However, for the purpose of this study only a rough estimate was required, as the goal was not quantifying the cell survival but merely assessing whether at least some cells survived the procedure. Therefore, even if a small fraction of cells survived the procedure (e.g. 1-10%) there would still have been the possibility to sort these with FACS and cultivate them. Unfortunately, even adjusted protocol proved to be lethal for acidobacterial cells. This is clearly visible as the survival of the bacteria is minimal to none depicted in Figures 10 and 11. As the temperature was rather mild at 28°C and the cells were shown to survive the heat shock step in satisfactory quantities, the main contributing factor of high lethality was most likely the high pH conditions of the buffers in which the cells were hybridized and washed, ranging from pH 7 to 8. As for the lethality variables such as chemicals found in hybridization and wash buffers, it is difficult to change the composition without disturbing the stringency of the hybridization process. Even though the *Acidobacteriota* phylum is known for the preference of its members for acidic soils, multiple reports have found that there are numerous members with strong preference for neutral or even alkaline soils (Dunbar et al., 1999; Dunbar et al., 2002), especially members of subdivision 4 (Ward et al., 2009). The live-FISH technique would be more viable for these recalcitrant species that tolerate or thrive in neutral or alkaline pH and would be not greatly affected by the alkaline pH of FISH buffers.

4.4 FA Concentration series of New Probes

This preliminary FA gradient served as a qualitative indicator to assess which probe is more suitable for live-FISH. Acid657 showed constant low-level fluorescence across the FA concentration spectrum (not shown). This result may indicate that the probe might not bind its target site efficiently. As the signal was low and non-specific, no further experiments involved Acid657. However, Acid303 started off with strong fluorescence at 0% FA (Figure 7). This fluorescence was the same if not higher at 10% FA. From this point, a gradual fluorescence drop-off was observed until at 30-40% FA no visible fluorescence was detected. Afterwards, an off-target bacterium (*Pseudomonas* sp. KT2440) was tested at 0% and 10% FA (not shown) and was found to have low to none hybridization with Acid303, indicating that this probe binds *Acidobacteriota* subdivision 1 with at least phylum-level specificity. As no *Acidobacteriota* species of subdivision other than 1 was available, the subdivision specificity could not be tested. Future assessment of the probe should be carried out on a broader range of genetically-related off-target bacteria, such as various *Acidobacteriota* subdivisions. This would confirm the specificity of the probe to subdivision 1, making it a more specialized alternative to HoAc1402. The qualitative FA gradient suggested that Acid303 is the superior live-FISH probe candidate not only due to the strong gradient of fluorescence emission, but also because the fluorescence peaked between 0-10% FA. By selecting 0% FA

concentration for the live-FISH experiments, the variable of FA cell lethality was removed while maintaining good hybridization rates and relatively good specificity. Additionally, due to the absence of barely any signal above 40% FA, the 50% FA slide was removed from the subsequent quantitative FA gradient.

4.5 Melting Curve Analysis of Acid303 on *Edaphobacter* sp.

The gradient has a noticeable fall-off in signal for target bacterium (SPOG37A) with the strongest fluorescence at 0-10% range. This would suggest that the optimal FA concentration for this probe would be 10%, as the presence of FA increases specificity to its target sequence. The probe binding to *P. putida* was very low across the FA spectrum, around the same level as the background fluorescence. The line resembled linear gradient rather than an exponential one, which was surprising. However, the accuracy of every datapoint was limited by the tendency of the bacteria to aggregate – daime software removes aggregated cells and cells that are on the edge of the image. This might have contributed to the sharp decline of fluorescence emission beyond 0% FA. Nevertheless, the positive control probe EUB338 showed very high fluorescence across the range of FA concentration (not shown), while no observable signal was detected in NON-EUB negative control. This suggests that the Acid303 probe could be effectively used in a system where *Acidobacteriota* and *Gammaproteobacteria* coexist without causing false-positive signals to be produced. However, to truly ensure that this probe binds *Acidobacteriota* subdivision 1 specifically over other subdivisions, the probe needs to be tested with representatives of these subdivisions with quantification of their genomic differences. Unfortunately, this experiment could not be carried out due to time constraints and the unavailability of *Acidobacteriota* representatives of other subdivisions at the time. Non-aggregating representatives of subdivision 1 would make a great candidate for the refinement of FA gradient curve, resulting in better specification of the optimal FA concentration for the Acid303 probe.

4.6 FISH on Soil Enrichment Filtrate

Every live-FISH procedure had been preceded by a FISH control. This was to ensure that live-FISH results could be interpreted in a controlled manner. The FISH control of 1 µm soil filtrate showed inconclusive results (Figure 13), where the DAPI signal was not in overlap with the probe signal. These results suggest that the probe could have been bound to non-specific targets on soil particles instead of acidobacterial cells (Klauth et al., 2004), or that there were no *Acidobacteriota* subdivision 1 within the observed samples. However, the latter point is unlikely, as a member of *Granulicella* genus was isolated from the peat on a VL55+XPC agar. The filtration step was designed in a cascade (from 5 µm to 1 µm, sampling 5, 3, 2, and 1 µm filtrate) to sequentially remove bigger particles in order not to clog filters with a smaller pore size. Final size of 1 µm was selected because it removes larger bacteria while passing the vast majority of *Acidobacteriota* (size approx. 0,7 µm) through (Kielak et al., 2016). Each of the filtrates (5, 3, 2, and 1 µm) was hybridized and tested for specific probe signal that aligned with DAPI. However, with decreasing filtrate pore size, the autofluorescence increased as well, making it impossible to distinguish any bacteria-specific signal. As peat slurry contains very fine soil particles (Kalantari, Behzat, and Bujang, 2009), some of these (<1 µm) are filtered together with the *Acidobacteriota*. Unfortunately, soil particles cause a lot of non-specific probe binding, resulting in a significant amount of autofluorescence within the sample (Khalili et al., 2019). This is also apparent from the Acid303-hybridized peat samples. The autofluorescence issue is mostly absent in soil samples with larger particles, such as lake sediment – the soil can be simply centrifuged away, leaving behind the supernatant that contains the bacteria and little debris (Lindahl and Bakken, 1995). While procedures such as Nicodenz ultracentrifugation could have been used, this process is usually quite lethal to bacteria less resistant to physical stress, *Acidobacteriota*

being one example. Additionally, as *Acidobacteriota* tend to bind soil particles (George et al., 2011), there was most likely a significant loss of bacterial cells during the filtration process. Additionally, any aggregated bacteria incorporated into the soil matrix may be inaccessible for the probe. Possible future improvements of this step include sonication to disrupt the aggregation of soil particles and wash steps to isolate whole bacteria from soil debris. However, the protocol for these procedures would have to take into account the poor survival rate of *Acidobacteriota* under laboratory conditions (Costa and Curamae; 2021) to prevent further losses of viable bacterial cells. Due to these drawbacks, live-FISH was not attempted on soil filtrate, as this process alone requires more optimization before the autofluorescence is removed and any conclusive results can be achieved.

4.7 Protocol Adjustments and Future Developments

The original protocol, published by Batani et al., was designed with marine bacteria in mind. Therefore, some aspects had to be changed to reflect the different osmotic conditions in which soil bacteria usually survive. Before the heat shock, when the cells are resuspended in ice-cold MQ containing salts, 160 mM $MgCl_2$/40 mM $CaCl_2$ was lowered to 12 mM and 3 mM, respectively. Additionally, right before the heat shock when the probes are added to the bacteria, the $CaCl_2$ concentration was lowered from 100 mM to 7,4 mM. In these conditions, the survival of the bacteria tested was very high, as the osmolarity was similar to a 0,9% NaCl physiological solution. Further live-FISH experiments could experiment with these salt concentrations to balance osmotic pressure with the heat shock transformation efficiency. Next, the plating of the bacteria on a plate was initially done after each step in 4 dilutions that reflected the amount of cells that was expected to survive, as shown in section 3.1. Later, in section 3.4, the bacteria were only plated at no dilution, just to see whether any cells at all survived and to roughly correlate these findings with the results of the BacLight kit. In the *Acidobacteriota* live-FISH, only one agar plate was plated at no dilution for the same reason. Further protocol adjustments concerned the temperature and length of incubation. Low temperature hybridization (<30°C) lowers the thermally-induced lethality but promotes pH-related lethality. Thus, a compromise I necessary and future studies could focus on testing the variations of temperature and pH. Recently, it was announced by a Danish team at the 16th Symposium on Bacterial Genetics and Ecology (Nita et al., 2023) that they managed to use a novel method of microdroplets to achieve higher cultivation rates of rare soil *Acidobacteriota*. Also, they implemented a modified hybridization methodology that apparently facilitates higher survival rate of *Acidobacteriota* species. Although still unpublished, their conference abstract reports successful live-FISH and FACS on *Acidobacteriota s*ubdivision 1. Furthermore, it seems that one of the main obstacles in *Acidobacteriota* live-FISH is the high pH (7-8) of the buffers. Its purpose is to maintain high stringency of the probe binding its target. The high pH, however, causes poor survival of *Acidobacteriota* subdivision 1 that generally require lower pH for survival, such as the ones presented here. This hypothesis was tested by streaking a loopful (1 µL) of SPOG37A on a R2B plate of pH 7,3. There was no observable growth of biomass after 10 days. After 24 hours of cultivation at room temperature, a loopful of cells from this plate was inoculated on a fresh R2B plate with pH 5. After 10 days, there was noticeable growth of small bacterial colonies, indicating that this strain strongly prefers pH 5 and does not thrive at neutral or alkaline pH. Additionally, the long cultivations (12 hours and 24 hours, with similar hybridization rates) seemed to be very detrimental to the overall survival of the tested representatives of *Acidobacteriota*. Therefore, it is necessary to optimize the FISH procedure employing buffers with lower pH while remaining as much efficiency as possible. No such cases of lowering the pH of FISH buffers were found in literature, most adjustments concerned increasing the pH. If such optimization was successful, these buffers could then significantly enhance the survival of *Acidobacteriota* in the live-FISH protocol. No

papers were found in literature where a low-pH FISH buffer was successfully tested. Therefore, the possible results of this protocol adjustments remain hypothetical.

5 Conclusion

In summary, this thesis to some extent confirms the original paper's (Batani et al., 2019) claim that more robust bacteria can survive the highly stressful conditions of live-FISH. However, gram-negative bacteria, and especially *Acidobacteriota,* failed to survive the hybridization process under all experimental conditions applied within this study. The biggest obstacle is the temperature of the hybridization stage and the high pH of the hybridization and wash buffers.

Even though the experiment did not result in successful live-FISH of *Acidobacteriota* from soil, it gives us insight into how the methods can be optimized and potentially applied on a different group of microorganisms. Additionally, Acid3030Fa probe was tested and seemed to be a reliable hybridization tool for *Acidobacteriota* subdivision 1. The probe that can be further used not only for live-FISH, but also for regular FISH, offering us a viable subdivision 1 alternative to the HoAc1402 probe.

Future improvements could focus on varying the pH of FISH buffers together with length of cultivation while the temperature should stay below 30°C to minimize heat-related cell death.

Zusammenfassung

Die Untersuchung von *Acidobacteriota*, einem weit verbreiteten und ökologisch bedeutsamen Bakterienstamm im Boden, ist aufgrund der spezifischen Kultivierungsanforderungen seiner Mitglieder und der eingeschränkten Widerstandsfähigkeit unter Standard-Laborbedingungen nach wie vor schwierig. Herkömmliche Fluoreszenz-In-Situ-Hybridisierungsverfahren (FISH) sind zwar für die Identifizierung von Mikroorganismen wertvoll, erfordern jedoch in der Regel eine Zellfixierung, was ihren Nutzen bei der Kultivierung und weiteren Analyse lebender Zellen einschränkt. Ziel dieser Studie war es, ein modifiziertes Live-FISH-Protokoll zu evaluieren, das neu entwickelte, für das Phylum der *Acidobacteriota* spezifische Sonden enthält, um die Fluoreszenzmarkierung lebensfähiger Zellen innerhalb dieses Phylums zu ermöglichen.In dieser Arbeit wurden doppelt markierte DOPE-Sonden für die Unterabteilung 1 von *Acidobacteriota* entwickelt und getestet. Unter diesen zeigte die Acid303 eine optimale Leistung mit starker Spezifität und Signalintensität bei niedrigen Formamid-Konzentrationen. Erste Lebensfähigkeitstests mit gram-positiven und gram-negativen Modellbakterien ergaben ein unterschiedliches Überleben unter Live-FISH-Bedingungen; gram-positive *Bacillus*-Stämme zeigten eine höhere Widerstandsfähigkeit im Vergleich zu gram-negativen Bakterien. Trotz umfangreicher Optimierungsmaßnahmen konnten jedoch keine lebensfähigen *Acidobacteriota*-Zellen gefunden werden, was wahrscheinlich auf ihre Empfindlichkeit gegenüber den hohen Temperaturen und dem Puffer-pH-Wert zurückzuführen ist, die im Hybridisierungsprozess verwendet werden. Bei weiteren Versuchen mit Bodenanreicherung und Filtrat traten Probleme mit Autofluoreszenz und Zellaggregation auf, so dass Raum für die weitere Entwicklung des Protokolls blieb. Obwohl das Live-FISH-Protokoll keine lebensfähigen *Acidobacteriota*-Zellen ergab, die für die Kultivierung geeignet waren, hat diese Studie die Acid303 als zuverlässige Sonde für herkömmliche FISH-Anwendungen validiert. Diese Ergebnisse unterstreichen die Notwendigkeit einer weiteren Verfeinerung von Live-FISH-Methoden, die auf empfindliche mikrobielle Taxa wie *Acidobacteriota* zugeschnitten sind, und weisen auf mögliche Richtungen für die zukünftige Forschung hin, einschließlich der Erforschung alternativer Hybridisierungsumgebungen.

Table of Abbreviations

FA	**Formamide**
ddH2O	**Double-distilled water**
MQ	**Milli-Q water**
EtOH	**Ethanol**
FISH	**Fluorescence In-Situ Hybridization**
RPM	**Revolutions per minute**
PFA	**Paraformaldehyde**
PBS	**Phosphate-buffered Saline**
RT	**Room temperature**
UV	**Ultraviolet**
CLSM	**Confocal Laser-Scanning Microscope**
TSA	**Tryptone Soy Agar**
R2A	**Reasoner's 2 Agar**
FITC	**Fluorescein isothiocyanate**
Cy3	**Cyanine 3**
DAPI	**4,6-diamidino-2-phenylindole**
DOPE	**Double labeling of oligonucleotide probes**
TRITC	**tetramethylrhodamine**
XPC	**Xylan-pectin-cellulose**
FACS	**Fluorescence-activated cell sorting**

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ChatGPT v4 and v4o was used for minor edits of the language.

Addendum

10x Phosphate Buffered Saline (PBS), pH 7,3

Following ingredients were dissolved in 500 mL of MQ: 40g NaCl; 1 g KCl; 9 g Na_2HPO_4 x $2H_2O$; and 1,2 g KH_2PO_4; pH adjusted to 7,3 and autoclaved. From this stock, 1 part was mixed with 9 parts MQ to form 1x working concentration.

4% Paraformaldehyde (PFA)

In a Falcon tube, 8,5 mL of 23,5% formaldehyde and 41,5 mL of 1x PBS were mixed to form 50 mL 4% PFA working solution.

5M NaCl

In 100 mL of MQ, 29,2 g NaCl added and stirred until dissolved.

1M Tris-HCl, pH 8

In 100 mL of MQ, 12,1 g Tris-HCl was added, solution was stirred until everything dissolved and pH was then adjusted to 8 using NaOH pellets.

0,5M EDTA, pH 8

In 100 mL of MQ, 18,6 g of EDTA sodium salt was added. As EDTA only dissolves at pH 8, the pH was adjusted immediately with NaOH pellets to pH 8 and then the solution was stirred until EDTA dissolved.

10% SDS

In 10 mL of MQ, 1 g of SDS was added, the solution was vortexed and left in a 48°C water bath until SDS dissolved and then vortexed again.

50%, 80%, and 96% EtOH

In 3 Falcon tubes, 96% ethanol was diluted using MQ for each respective concentration (50%; 80%, and no dilution of 96%) to make up 50 mL.

PBS:EtOH

In a Falcon tube, 25 mL of 1xPBS and 25 mL of 96% EtOH were mixed to make up 50 mL in total.

FISH buffers

Hybridization buffer - all units in µl, final volume 1 mL

FA%	0%	10%	20%	30%	40%	50%
5M NaCl	180	180	180	180	180	180
1M Tris/HCl	20	20	20	20	20	20
ddH2O	799	699	599	499	399	299
FA	0	100	200	300	400	500
10% SDS	1	1	1	1	1	1

Wash buffer - all units in mL, final volume 50 mL

FA%	0%	10%	20%	30%	40%	50%
5M NaCl	9	4,5	2,15	1,02	0,46	0,22
1M Tris/HCl	1	1	1	1	1	1
0,5M EDTA	0	0	0,5	0,5	0,5	0,5
MQ	up to 50 mL	up to 50 mL	up to 50 mL	up to 50 mL	up to 50 mL	up to 50 mL

Live/Dead BacLight kit

Solution A: SYTO9 1,67 mM/Propidium iodide 1,67 mM; in DMSO

Solution B: SYTO9 1,67 mM/Propidium iodide 18,3 mM; in DMSO

Working solution: 1:1 mix of A:B; 4 µL per 1 mL of MQ.

Live-FISH buffers

Hybridization buffer - final concentrations: 20 mM Tris-HCl; 0,01% SDS; 0/35% FA

FA	0 µL (Acid303); 350 µL (other probes)
10% SDS	1 µL
1M Tris-HCl	20 µL
5M NaCl	180 µL
MQ	add up to 1 mL
	1 mL

Wash buffer final concentrations: 20mM Tris-HCl; 5mM EDTA (if FA in HB >20%); 0,01% SDS; 0,08M NaCl.

0,5M EDTA	50 µL (if FA in HB >20%)
10% SDS	5 µL
1M Tris-HCl	100 µL
5M NaCl	80 µL
MQ	add up to 5 mL
	5 mL

TSA solid medium (1 L)

In 1 L of MQ, 15 g tryptone; 5 g enzymatic digest of soybean meal; 5 g NaCl; 15 g Agar were mixed together. The medium was available as a ready-made with pH already at 7,4. From this mix, 40 g was used per 1 L of MQ. The thoroughly dissolved ingredients were autoclaved and plated with 25 mL per plate and let to solidify.

1 L 3xR2B solid medium

In 1 L of MQ, 1,5 g casein hydrolysate, 0,25 g enzymatic digest of animal tissue; 0,5 g casein hydrolysate; 0,5 g yeast extract; and 0,5 g dextrose were dissolved. The pH was adjusted to 5, the medium autoclaved, and 25 mL poured per plate and let to solidify.

1 L LB solid medium

In 1 L of MQ, 10 g Peptone; 5 g Yeast extract; 5 g NaCl; and 12 g Agar were dissolved, the pH adjusted to 7, autoclaved, and 25 mL was poured per plate and let to solidify.

1 L VL55+XPC liquid medium

In 960 mL, 1,95 g MES (2-Morpholinoethanesulfonic acid); 10 mL 20mM $MgSO_4x7H_2O$; 10 mL 30 mM $CaCl_2$ x $2H_2O$; 10 mL 20 mM $(NH_4)_2HPO_4$; 1 mL Selenite-Tungstate solution; 1 mL Trace element solution SL10; pH was adjusted to 5. After autoclaving, 1,5 mL of Vitamin solution was added, as well as 200 mg of sterile Xylan, Pectin, and Cellulose each.

Selenite-Tungstate solution

In 1nL of MQ, 0,5 g NaOH; 0,003 g $Na_2SeO_3x5H_2O$; and 0,004 g $Na_2WO_4xH_2O$ were dissolved. Solution was then filter-sterilized and kept at 4°C.

Trace Element Solution SL10

In 1 L of MQ, 10 mL 7,7M HCl; 1,5g $FeCl_2x4H_2O$; 70 mg ZnCl; 100 mg $MnCl_2x4H_2O$; 6 mg H_3BO_3; 190 mg $CoCl_2x6H_2O$; 2 mg $CuCl_2x2H_2O$; 24 mg $NiCl_2x6H_2O$; and 10 mg $Na_2MoO_4x2H_2O$ were dissolved. Solution was then filter-sterilized and kept at 4°C.

Vitamin Solution

In 1 L of MQ, 17 mg Cobalamin; 13 mg 4-aminobenzoate; 3 mg Biotin; 33 mg Nicotinic acid; 17 mg Hemicalcium D-(+)-panthotenate; 50 mg Pyridoxamine-HCl; 33 mg Thiamine-HClx$2H_2O$; 10 mg D,L-6,8-thioctic acid; 10 mg Riboflavin; and 4 mg Folic acid were dissolved. Solution was filter-sterilized after mixing.

0,89% NaCl MQ, pH 5

In 1 L of MQ, 8,9 g NaCl was dissolved; pH adjusted to 5; and autoclave-sterilized.