SIMBA

Sustainable Innovation of Microbiome Applications in the Food System

Deliverable 3.1

Protocols for analysis of biofilm formation in water and soil substrates

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Summary

Biofilms are densely populated layers of microorganisms growing on a variety of surfaces. Through the excretion of extracellular polymeric substances, biofilms can colonize almost any surface ranging from rocks, sand grains, leaf surfaces, roots, skins of fish and mammals and many others. Studies of biofilm communities have employed specific natural environments, recreation of natural environment, or complete artificial laboratory settings; the latter have broader applications and can be applied to microbes isolated from different habitats. The possibilities of using artificial settings is connected with a broad range of techniques that can be applied to the study of microbes from different origins: clinical, soil or water. Despite a wealth of information is available on biofilms in general, their potential use in facilitating crop growth in saline agriculture, protecting seaweed surfaces against colonization by unwanted harmful species and protecting fish scales against putative pathogens is less well defined.

At the University of Parma and at the Netherlands Institute for Sea Research different types of biofilms have been studied for several years by using various techniques. This document contains different protocols for biofilm maintenance and analysis thereof that will be applied within the SIMBA project to further facilitate the use of these unique types of microbiomes for sustainable food production.

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1. Objective

The general objective of this deliverable is to define the most common methodologies employed to analyse microbial biofilm which are connected to the work of WP3, WP2 and WP6. Within WP3, some of these techniques will be used to:

- Cultivate strains and consortia obtained from partners in conditions ideal for biofilm formation
- Create standard operating procedures (SOPs) for microbiological analysis on biofilms and for ESEM analyses. Analyses will include transcriptomics and proteomics methods to address the expression of specific diagnostic genes and functions, to be evaluated as biomarkers of effectiveness.
- Analyse selected strains and consortia in field conditions to establish a ranking for biofilm formation properties; challenging inhibiting factors will be tested to assess the resilience of strains and consortia

2. Background

Bacteria in nature are not only found as single planktonic cells, but often form heterogeneous communities involved in a complex matrix [1]: the so called “biofilms”. Biofilms have been studied especially in aquatic and clinical environments. The development of communities, often involved in pathogenic settings, follows different steps [2] that include microbial sensing and signalling, surface colonization, biofilm development and dispersal. On the contrary, biofilms growing in aquatic environments are characterized by two attachment phases: a reversible one (guided by transitory physicochemical attraction) and an irreversible one (mediated biologically with the contribution of exopolymeric substances). Interestingly, the latter can assume a variety of different structures: cone–, mushroom- and column-shaped clusters of cells [3]. Studies of biofilm communities have been conducted on specific natural environments [4,5] or the recreation of natural environments [6,7], and artificial laboratory settings [8–10]; the latter have broader applications and can be applied to microbes isolated from different habitats. Often, the only analysis of natural biofilms is achieved through Next Generation High Throughput Sequencing techniques. Detailed analyses of the processes taking place in biofilm development and maturation requires an artificial settings where biofilms are grown and maintained under laboratory conditions after which a broad range of techniques can be applied as explained below. These experiments are widely used for the study of microbes from different origins: clinical, soil or water.

In marine environments, biofilms covering both natural and man-made surfaces can protect the residing microbial community from different temporary stressors such as dehydration, competition and limited access to nutrients [11]. In 2019 Zhang et al. identified more than 7,300 biofilm specific microbial species [12]. A catalogue of core genes found in biofilms revealed 97.3% of bacterial genes, 2.5% of eukaryal origin and 0.2% of archaea [12]. Especially genes encoding proteins involved in biofilm formation (i.e. extracellular polysaccharide biosynthesis), stress response (DNA repair,

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oxidative stress and antibiotic resistance, heat shock and osmotic stress) and microbial interactions were abundant amongst the core biofilm genes [12]. Cyanobacteria and to a lesser extend diatoms play an important role in photosynthesis driven biofilms in a wide variety of habitats [13,14]. Interestingly, biofilms of these phototrophic bacteria have been used to purify waste water, for bioremediation and antifouling (due to their capacity to produce several bioactive substances) [15,16].

Coastal microbial mats are stratified biofilms, also consisting of Bacteria, Eukarya and Archaea that are found on sandy beaches worldwide. An important feature of these microbial mats is their ability to stabilize the sand due to the excess production of photosynthetic generated sugars, and to fertilize the barren sand by fixing nitrogen, carbon and active sulphur cycling. Stabilization and fertilization are essential to facilitate plant growth. On the Dutch barrier island of Schiermonnikoog, this resulted in the formation of a highly diverse and rich saltmarsh vegetation that covers large parts of the beach (Figure 1). The saltmarshes consist of salt tolerant plants (halophytes) and depend for growth on an equal salt tolerant microbiome, especially around the roots, the rhizosphere, where microorganisms provide the plants with among other nitrogen and other essential growth substrates. In addition to facilitating salt marsh formation, coastal microbial mats have an important role in natural coastal protection [13].

Figure 1. Saltmarsh vegetation at the Dutch barrier island of Schiermonnikoog.

For the SIMBA project, both the initial microbial mat members as well as the transformed saltmarsh microbiome are of importance to facilitate saline agriculture. Currently, saline agriculture focused on obtaining salt tolerant varieties of known crops (Potatoes, Tomatoes, wheats and others) whereas their microbiome is largely neglected. Here, we want to learn from the saltmarsh and microbial mat microbiomes and use isolates plus consortia of these to facilitate plant growth.

One of the challenges in biofilm research is to study natural biofilms under controlled laboratory conditions. Generally, this results in a rapid change of the natural community structure and diversity and loss of geochemical features typical for microbial mat communities. NIOZ is following two approaches. First, the development of so-called minimal microbial mats in an experimental setup mimics the natural conditions. Instead of growing in liquid culture as done so often, the minimal

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mats are grown on natural sandy sediment that allows the formation of an anoxic subsurface, essential for a functional sulphur, carbon and nitrogen cycle.

The second approach in microbial mat analysis for sustainable saline agriculture focusses on the enrichment of beneficial organism that is low in energy requirements when applied to the field.

The cheapest energy source is of course the sun. However, traditional photosynthetic microorganisms like cyanobacteria only grow well at the surface and suffer from light competition with chlorophyll containing plants that use light of the same wavelength. NIOZ is trying to enrich phototrophic microorganisms that use sun light as an extra energy source but at a wavelength ranging from 500 to 600 nm which is not used by chlorophyll. Especially, in the sandy substrate of a beach, light can penetrate up to 3 mm retaining 1% of the total light intensity [17]. A protocol for the enrichment of phototrophic bacteria other than cyanobacteria from the microbial mats is explained below.

By applying a WGS approach also an atlas of soil microbes is now available [5]. In this work, although an overwhelming diversity of bacterial communities has been discovered, the most prominent taxa around the world were found to be: *Proteobacteria, Actinobacteria* and *Acidobacteria*. Of particular interest for the SIMBA project are PGPMs, plant growth promoting microbes, which are microorganisms whose presence in soil helps growth of crops and helps them to cope with stressful conditions [18–21]. One of the objectives of WP2 within the SIMBA project is to identify new microbial consortia which are composed of a combination of different PGPMs. Through literature studies optimal consortia were identified and described in deliverable D2.1 “List of suitable PGPMs accompanied by operational procedures for application on soil or plants”.

Deliverable D3.1 describes the main steps in forming, characterisation and analysis of biofilms, with references to literature and websites. A general scheme of the workflow is depicted in Figure 2.

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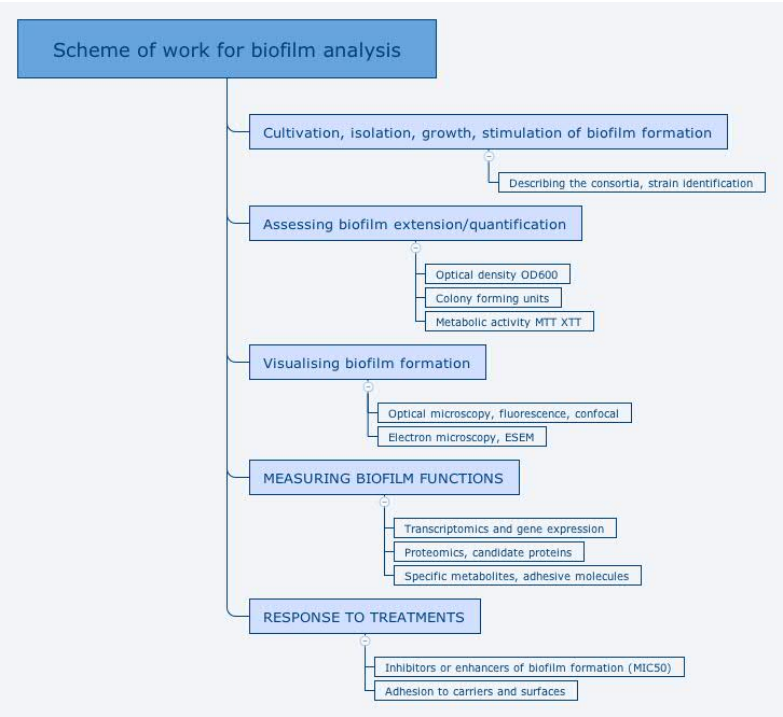

Figure 2. Workflow for biofilm characterization and analysis.

Deliverable D3.1 does not describe in detail analytical techniques based on chemical or physical properties of the biofilms; the authors have chosen to focus on methods available in the respective laboratories, concerning isolation, identification, enumeration of microorganisms and functional properties of microbiomes in biofilms.

3. Methodologies

3.1. Isolation of biological material, maintenance and propagation

Microorganisms residing in soil have often been isolated because of their plant growth promoting properties. In order to do this, bacteria growing in the rhizospheric soil were identified by serially diluting the sample, plating in rich media (i.e. Nutrient Agar, Luria Bertani, TSA) [22–24] and incubating for few days at 28°C. Details on soil sampling, growth conditions and inoculum size can be found in several papers [25]. Alternatively, culturing can also be done in specific media to isolate microbes having specific stress resistance (i.e. salt tolerance) [26,27]. Long term storage is mostly done at -80°C in 20-50% glycerol [22,27].

Marine microorganisms have been isolated from mudflats, marine sediments, or by submerging carbon steel compounds, glass, polymers, woods, macrophytes in water for a variable amount of time [4,12,28–30]. Marine isolates can be resuspended in sterile artificial seawater (sea salts 35 g L^{-1}) and inoculated on Marine Agar and grown at 20°C [5], or 2216 marine medium (Difco) [6] and conserved in 15-25% glycerol at -80°C [5,7]. For their propagation often used media are: Zobell broth

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(pastone 4 g L^{-1}; yeast extract 1 g L^{-1}; sea salts 30 g L^{-1}) at 22° C with shaking (150 rpm) [5], Minimal medium or rich medium as defined in [6], carbon-reduced VNSS medium (0.5 g L^{-1} peptone, 0.25 g L^{-1} yeast, 0.25 g L^{-1} glucose)[8].

In the past, identification of the isolates was carried out by sequencing the 16S rRNA gene [4,30], but now metagenomic analysis allows us to characterize all genetic information in the genome and is the preferred tool (see for example [5,12]; see also 3.5).

Coastal microbial mats are freshly harvested from the beach and maintained at -20°C without the addition of cryo-preservatives. A study is on the way as part of WP6 to determine the optimal conditions for long term cryo-preservation of these complex microbial communities. Initial results indicated that high concentrations of glycerol had a negative effect on reviving the photosynthetic communities due to the stimulation of heterotrophic growth which resulted in total anoxia of the system. Better results are obtained with either methanol or DMSO as cryo-protectant.

3.2. Growth of biofilms

3.2.1. Growth of biofilm in static conditions

Once microbes are isolated, other techniques are used to study biofilm formation. Below is a list of growing conditions that can be employed in the lab.

- Growth of biofilm on glass surfaces

Strains can be grown either on a glass slide submerged in media [28,31], or on petri dishes having a glass bottom [4]. After incubation, cells can be stained (i.e. with DAPI 4–6-diamidino-2-phenylindole), and surface colonization is visualized using a fluorescence microscope and eventually quantified (using ImageJ [32]).

- Growth of biofilm on polystyrene microtiter plates

Although microtiter plates cannot mimic the natural environment and its variables in all depth, this technique is still a standard in biofilm studies [33,34], and consists of evaluating growth in 96-well microtiter plates of planktonic cells versus biofilms. The number of planktonic cells can be determined by measuring absorbance at 600 nm, whereas the biofilm formed at the bottom and walls of the wells is assessed by staining with Crystal Violet [5,10,11]. A step by step protocol can be found in [31]. An interesting variant is the Calgary Biofilm Device which consists of a polystyrene lid with 96 pegs that can be fit into a standard 96-well microtiter plate, a system that allows transferring pegs between microtiter plates allowing the simultaneous comparisons of different growth and exposure conditions [35,36]. The latter system allows to easily estimate cell numbers that adhere to the surface by applying mild sonication and plating the recovered cells in the appropriate media [31,35].

- Growth of biofilms on unconventional materials

Growth of marine biofilms can also be tested on unconventional materials. Indeed, marine microbes can easily colonize whatever surface they encounter [37], and this aspect can be tested

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in the lab. Wood [38], stainless steel, PVC and plastics [39–42] have all been investigated. Protocols to grow bacterial communities on natural substrates such as algae (often *Ulva australis*) have also been developed [7,43].

Soil microbes can be grown on various substrates [44]. Of particular interest is char, a product derived from the pyrolysis of biomass waste of plant material, which has been shown to improve soil quality in agricultural settings [45,46]. The same material has been used to deliver PGPMs and is employed in WP2 as a possible carrier. Growth of microbes on this surface has been described and their effects on plant growth has been tested [47,48]; Partner UNIPR is optimizing a protocol for its functionalization and the delivery of the PGPM consortia identified in WP2. The protocol will be described in D2.2 "Tools for delivering microbial consortia and monitoring plant performance".

- Protocol for growing artificial microbial mats.

NIOZ developed a unique method to grow microbial mats that maintain most of the functional complexity of a natural mat. These minimal mats are essential for studying microbial ecology such as the effect of different levels of microbial diversity on functioning, the role of invasive species and the enrichment of beneficial species that cannot be isolated by traditional methods.

Minimal mats are formed in clear, translucent polypropylene boxes, commonly used in plant biology (SacO2 microbox: https://saco2.com). Initial experiments failed due to minimal oxygen diffusion through the wall that prevented the sediment sublayer from becoming anoxic. We therefore added glassware (Type Pokal: IKEA) that fit inside the box and did not allow oxygen to penetrate to the lower region of the mats (Figure 3). The boxes (type, O95/114+OD95/114) are covered with a lid that contains an integrated white, L-type filter that allows a gas exchange of 9.87 GE/day (almost 10 volume replacements per day). Lids with higher gas exchange capacities resulted in a faster drying out of the microbial mats. Addition of 50 ml of Milli-Q water in the box outside the glass maintains optimal humidity inside the box and prevents dehydration of the mat.

The glasses are filled in the following order. First 5 ml of BA+ medium (see appendix) containing 5% v/v glycerol was covered with 20 grams of beach sand (the sand was pre-filtered over a 300 µm mesh) to make a dense slurry. Glycerol is essential to stimulate fast aerobic heterotrophic growth in order to quickly consume the available oxygen and to generate an anoxic subsurface that allows sulphate reduction and other anaerobic processes. The BA+ medium is a brackish medium (10.4 ‰), similar to the salt concentration in the natural saltmarshes. Subsequently 100 grams of sand were added and 50 ml of Milli-Q water poured outside the glass in the box. The box was closed and autoclaved for 20 minutes at 120ºC. After cooling down and reaching room temperature, the sand was inoculated with a mixture BA+ medium containing 10 grams of a natural microbial mat obtained from the North Sea beach of Schiermonnikoog (53.491657 N, 6.141256 E). The mat was freshly sampled and incubated for 48 hrs at -20ºC to eradicate larger meiofauna species such as nematodes and beach insect larvae. The final volume of added liquid was 50 ml. Boxes are tightly closed and ready for growth. Minimal mats with reduced diversity are generated by inoculating with various dilutions of the original mat sample. Since true

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dilutions cannot be made, we rather speak about serial resuspensions. A side view of a growing minimal mat is presented in Figure 3 and top views of different serial resuspension are shown in Figure 4). Standard incubation occurs at 23ºC under a 16-8 h light-dark regime with top illumination. In order to prevent cyanobacteria/algal growth from the side and subsequent aeration of the subsurface, the boxes are shielded by covering the sides with black thick paper. The mats can continue to grow for up to 6 months without the need for adding water to the top to replenish evaporated water. However, the minimal mats were regularly checked, and limited amounts of sterile water was added to keep the top moist.

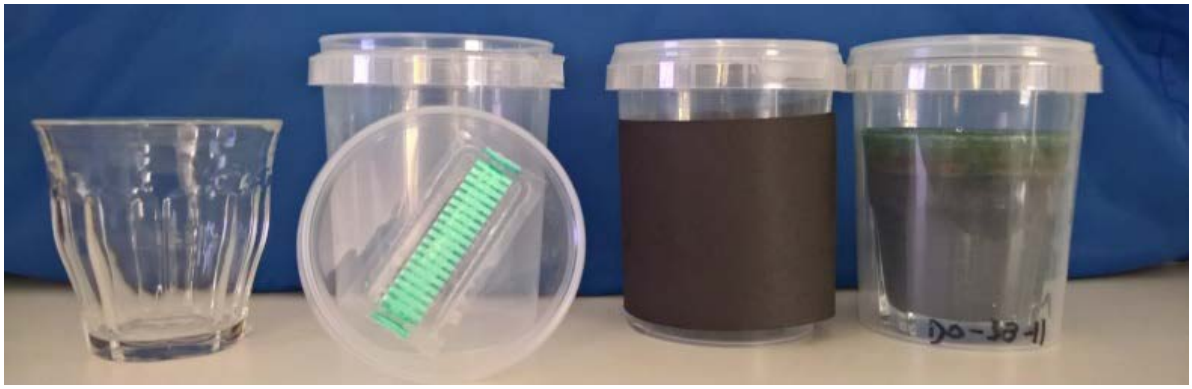

Figure 3. Schematic setup of an artificial mat consisting of an inner glass, a translucent polypropylene box with cover that allows gas exchange but prevents microbes or spores to go in or out. A black paper cover around the box prevents cyanobacterial growth on the sides. At the right a full-grown minimal mat is shown that was incubated for 6 months at 23C under 16:8-hour light:dark illumination.

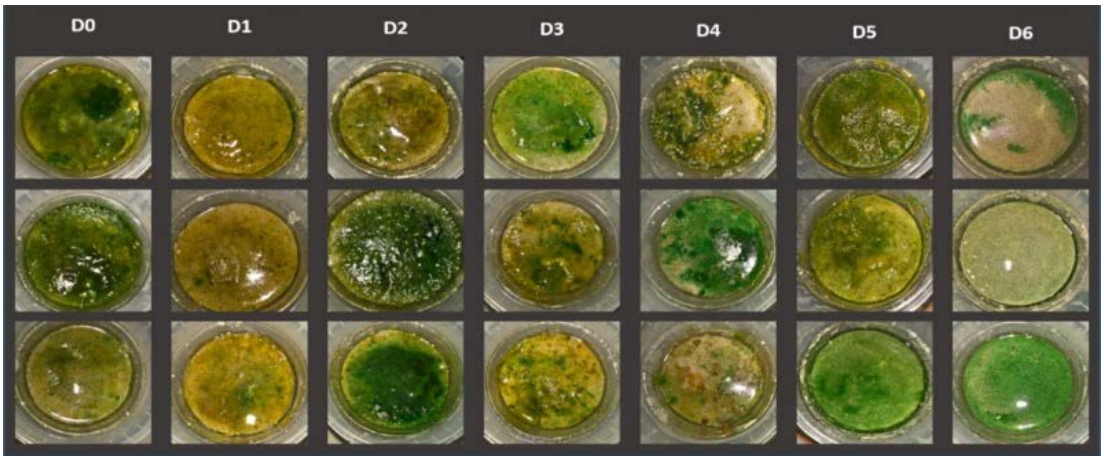

Figure 4. Top view of minimal microbial mats at dilutions ranging from undiluted (D0) to a near 6-fold dilution (D6). Clear differences in density of green photosynthetic microorganisms can be observed with only a view photosynthetic species still being present at the highest dilution.

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3.2.2. Growth of biofilm in dynamic conditions under continuous flow

Laboratory scale bioreactors represent a mode of growth that closely resembles the one of natural biofilms. In these systems, the continuous flow of media promotes growth of only attached cells, while washing out planktonic species. The most common designs are: CDC reactor, drip-flow reactor and flow reactors associated to the use of CLSM [49–51]. Microfluidics has also been widely employed in biofilm studies and is based on laminar flow within channels less than 100µm wide. This set up has been applied to a variety of studies, like effects of antibiotic, quorum sensing signalling, bacterial persistence [52].

3.2.3. Manipulating microbial mats to favour non-photosynthetic phototrophic microbial mat members

Light is a major driver in both marine and freshwater microbial ecosystems. Light is also one of the drivers of stratification in microbial mats, together with oxygen and sulphide concentrations. The upper, green layer is formed by photosynthetic diatoms and cyanobacteria. Products of their photosynthetic metabolisms, oxygen and excessive amounts of sugars are the substrate for photoheterotrophic and fermenting bacteria located deeper in the mats. These photoheterotrophs can be found at up to 3 mm depth and influence the deeper layers of the mats. Several of these phototrophic microorganisms contain rhodopsin like membrane proteins that function as a light driven proton pump. Indeed, metagenomic analysis revealed several types of rhodopsins found in bacteria and archaea (Bolhuis, unpublished results). In contrast to chlorophyll that absorbs light in the range of 400 to 450 nm and of 630 to 670 nm, bacterial and archaeal rhodopsins absorb light between 500 and 600 nm [53]. Enrichment of bacterial and archaeal rhodopsin containing microorganisms is established using the same artificial microbial mats as described above using light at various selective wavelengths.

LED panel construction.

LED panels were constructed in-house using High Power Epiled Chips (https://www.epileds.com.tw/) emitting light at 500nm, 530nm, 595nm and 740nm on 20mm Star PCB. Per panel, 10 LEDs were mounted on rectangular extruded aluminium heatsinks measuring 200mm (L) x 86mm (W) x 22mm (H) and connected to the power supply in 5 element serial connections. Each lamp was further cooled using two 80mm x 80mm PC cooling case fans, and a single 19V transformer was used to provide energy for LED panels (Figure 5).

Incubation of microbial mats at different wavelengths

Triplicate artificial microbial mat containing microboxes (as described above and in Figure 3) were placed in large, closed plastic containers to prevent contamination with ambient light in the 21ºC controlled climate room. The containers have a dimension of 600mm (L) x 400 mm (W) x 280mm (H) and the LED panels are mounted directly over the microboxes with a distance of approximately 200

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mm between LED and microbial mat surface. Lighting of the LEDs is synchronized using a single timer to illuminate the mats under a 12:12 h light dark regime. The artificial minimal mats are illuminated with different LEDs that emit light at distinct wavelengths. As a control, one triplicate set is incubated under white light. The containers were finally covered with aluminium foil to prevent any interference related to external light sources (Figure 5). All samples were finally incubated at 21°C and 12h-12h light-dark conditions.

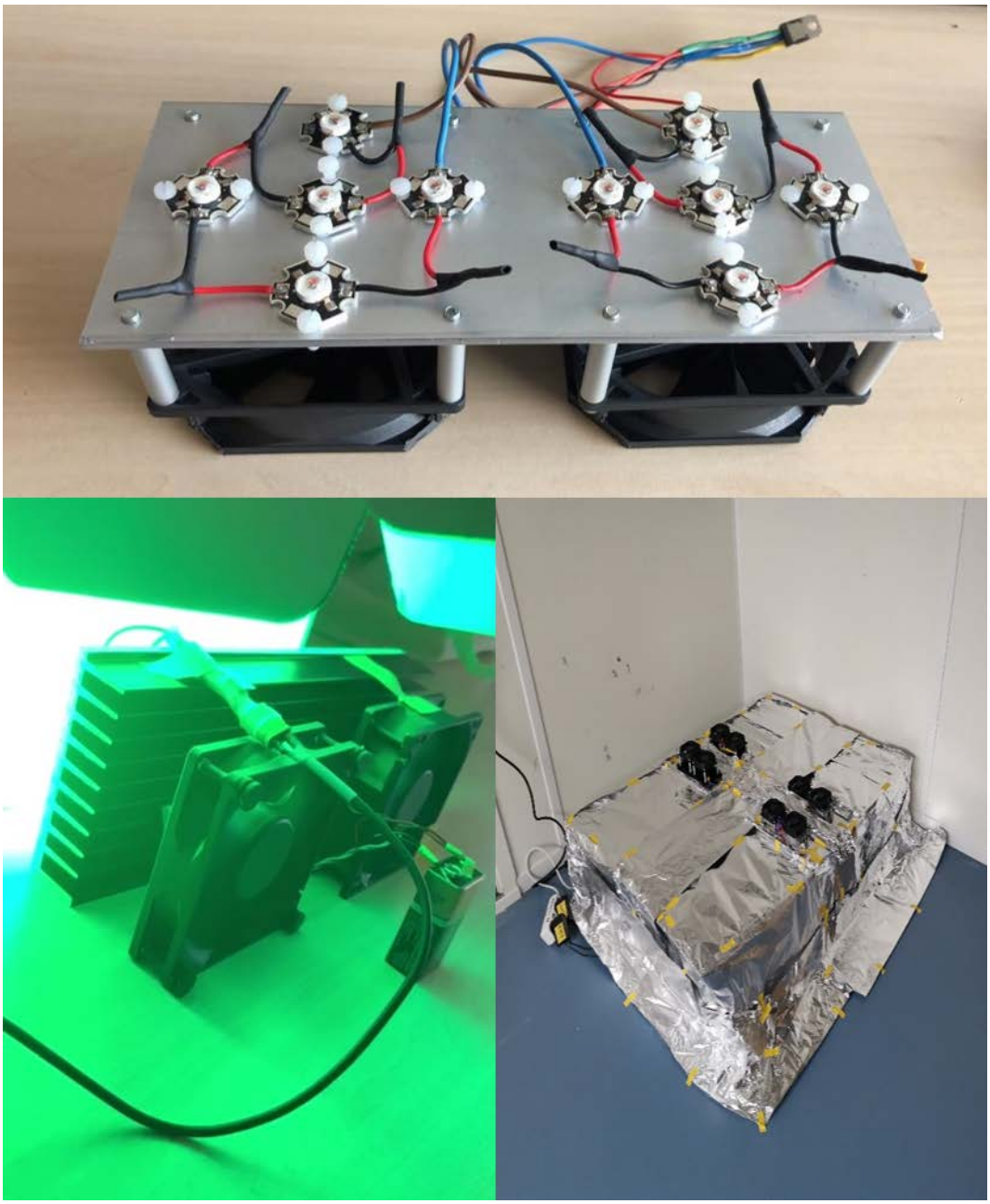

Figure 5. Top panel, Design of the LED panels with cooling units. Bottom left panel, a green light emitting LED panel. Bottom right, the complete setup with four different LED panels each emitting a different wavelength and each illuminating three replicate artificial microbial mats.

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3.3. Quantification methods

3.3.1. Microbiological and Molecular methods

Most bacteria adhere to solid surfaces at the air-liquid interface. In this case, the biomass attached to these substrates can be detached by mild sonication and cell viability can be estimated by determining the Colony Forming Units on agar plates (for a detailed protocol see [31]). Alternatively, the concentration of cellular suspensions can be estimated by using fluorophores in combination with flow cytometry [36]. Moreover, qPCR can be applied to estimate cell numbers thanks to the amplification of a targeted DNA fragment during the gene amplification (a technique that could be applied to specifically quantify the different species living within a biofilm), and a detail protocol to apply this approach can be found in [54] or by propidium monoazide qPCR (PMA-qPCR) [55]. Finally, biomass in cyanobacteria and diatom dominated biofilms can be estimated by measuring the chlorophyll content [56].

3.3.2. Chemical methods

Different dyes and fluorochromes can bind biofilm components. Crystal Violet is the most frequently used dye in microtiter plate assays and it can give information on total biomass (see 3.2.1) [31]; safranin can be similarly employed [34].

Cellular physiology can be assessed with the use of colorimetric methods. Here, metabolically active cells can convert, thanks to dehydrogenase enzymes, a certain compound into a coloured product whose concentration can be estimated spectrophotometrically. The most used compounds are: XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2Htetrazolium-5-carboxanilide inner salt), TTC (2,3,5-triphenyl-2H-tetrazolium-chloride) and the redox indicator resazurin (Alamar Blue) [57–59].

3.3.3. Physical methods

Methods estimating the biofilm biomass and its thickness have also been developed. Simply biomass can be calculated by the difference in slide weight displayed before or after the colonization, or by Electrochemical impedance spectroscopy [60–62]. Biofilm thickness instead has been measured by ultrasonic time-domain reflectometry or by analysing fluid transport properties [36,61,63].

3.4. Visualizing biofilms

3.4.1. Imaging techniques to study biofilm biomass and cell viability

Biofilm visualization can be achieved using different techniques. A brief description of the possible methods along with articles describing sample preparation and imaging is given below; often the choice among them is driven by availability rather than suitability. An overview can also be found in [36]

- Light microscopy

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Light microscopy is the cheapest, simplest, and fastest method to observe cells that adhere to a surface. A method correlating biofilm mass and light absorption has been described [64]. Alternatively, dyes can be used to increase image clarity (see 3.3.2).

- Confocal Laser Scanning Microscopy CLSM

CLSM is considered the most powerful technique that can describe the spatial structure of a biofilm, along with its functions [65]. Acquisition of images at different depths, combined with specific image analysis, allows the creation of a 3D image which can give information on volume, thickness and roughness of the microbial community investigated. In CLSM, different fluorescent probes can be used, even simultaneously, increasing the power of this technique. A detailed step by step protocol on how to prepare a sample can be found in [60]. In addition, microbial complexity can be assessed by using specific fluorescent oligonucleotides recognizing a specific sequence of the 16S rRNA, which coupled with fluorescence in situ hybridization (FISH) can give information on the relative abundance of microbial species. Efficiency of this technique can also be increased by using peptide nucleic acid (oligonucleotide analogues in which the sugar-phosphate backbone has been replaced by a pseudopeptide skeleton) as recognition elements and by replacing the fluorochrome with more sensitive molecules (i.e. enzymes) [66,67]. Interestingly, with the advent of the CLAS-FISH, a combination of labelling and spectral imaging FISH, several microbial taxa can be now identified in a single image [68–70], in a technique which has been tested also on marine biofilms [71].

- Transmission Electron Microscope, TEM

In general, Electron Microscopy gives the possibility to observe biofilm ultrastructure and its environment. TEM is used to image cell or its structures (i.e. proteins or nucleic acids) by electrons at high magnification and resolution, thanks to stains containing high atomic weight substances. The procedure requires also post-fixation and dehydration steps [34].

- Scanning Electron Microscope, SEM

Images produced by SEM are based on scattering and absorption of electrons released from a sample coated with heavy metals (often gold); this allows the creation of a 3D image of a cell, and to study its morphology, the spatial structure around it and the presence of a matrix (EPS) [36,72]. Sample preparation is similar to the one for TEM and can be found in [60]. The main limitation of this technique is that samples needs to be fixed and dehydrated, procedures that can give artefacts.

- Environmental Scanning Electron Microscope, ESEM

ESEM allows imaging of a biofilm in its natural state as it does not need any pre-treatments, high vacuum, and dehydration. The images have high resolutions and depict biofilms as it was originally [73–76]. With this technique it is possible to image the structure of a biofilm on a surface, its coverage and thickness.

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3.4.2. Most common fluorophores used in biofilm studies

Most common fluorophores employed are [65]: DAPI and SYBR-green, which stain the DNA, Calcofluor, staining the cell walls of yeast, FM1-43 staining cell membranes, and Syto-9 staining living and dead bacteria cells (both Gram positive and negative). The latter can be combined with Propidium Iodide, a fluorophore staining dead cells, a mixture allowing to study distribution of viable bacteria (spatial if applied to imaging with a microscope). So far, specific staining of the extracellular matrix is difficult due to its complexity. Indeed, the latter (a mix of polysaccharides, proteins and extracellular DNA) can change even between different strains. So far only fluorescent lectins binding sugars can be used to stain certain extracellular polysaccharides [52].

3.5. Omics of biofilms

The advent of high-throughput technologies has also been applied to biofilm studies, allowing to describe in even more details these microbial communities [4,5,12]. Moreover, these technologies can also provide information on cellular activities and metabolic capacities within the community [52].

Cells growing in a biofilm display different gene expression patterns than planktonic cells. To understand better the gene network promoting these different types of growth transcriptomic analysis has been employed, and unique features of biofilms have been described. To achieve this, microarrays, RT-qPCR and recently RNA-Seq have been employed [77–82]. These global approaches can give very interesting results, for example they have been useful to understand how plants influence microbial growth [83], they have identified components supporting colonization, especially when this is done in extreme environments [12,79,84]. Unfortunately, RNA seq cannot describe the heterogeneity between cells within a biofilm. To overcome this, microfluidics or microdissection combined with RT-qPCR have been successfully used [85]. It could also be of interest to compare gene expression between two different species of biofilm forming bacteria or to understand how a microbial community reacts to certain chemicals. In all these cases, planktonic cells are of no interest and only biofilms need to be considered.

Proteomic approaches have also been employed productively. With this respect, the two most common approaches are Two-dimensional differential gel electrophoresis [86] or shot gun proteomic investigations. The latter is a high-throughput technique which is able to identify in a single run more than thousand proteins with (iTRAQ) or without (label free) the use of isobaric tags [87–90]. Proteomics have been used to describe the characteristics of growth within a biofilm, and factors promoting this process, of a single species [91–93], or of communities [94,95].

Finally, biological activities can be estimated directly by measuring metabolites present within the cell or secreted in the EPS. This metabolomic approach relies mostly on the LCMS identification of different chemical compounds, and their comparison with identified compounds in specific databases [96–99].

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3.6. Substances influencing biofilm growth

The best-known signalling molecules involved in coordinating cell growth within a biofilm are acyl-homoserine lactone (Quorum sensing system), c-di-GMP and the stress related molecule nitric oxide. Many environmental signals can alter the cell-to cell communication by modifying negatively the input given by these molecules (i.e. low concentration diffusible molecules, the plant hormone ethylene, substances produced by microalgae and cyanobacteria) [30,100–103]. Other molecules on the contrary have a positive impact on biofilm formation or on the structures. For example, presence of Ca^{2+} or malic acid produced by plants stimulate bacteria aggregation [28,104], light availability positively affects cyanobacteria growth [105], while species intermixing increases biofilm growth [106].

Negative effects have been studied in more details. The effect of a certain inhibitor on biofilm formation can be tested in a conventional way by determining, through a series of serial dilutions, the Minimal Inhibitory Concentration or MIC_{99}. A step by step protocol can be found in [107]. This technique can be applied to planktonic cells. The influence of a molecule on a biofilm can be tested by measuring the Minimal Biofilm Inhibitory Concentration (the minimal concentration preventing biofilm from growing) or the Minimal Bactericidal Eradication Concentration MBEC, which allows to estimate the concentration at which a molecule is able to kill cells within a preformed biofilm. For a protocol see for example [108]. The effect of the possible interactions between two molecules can be estimated by calculating the fractional inhibitory concentration (FIC) index: if FIC <0.5 molecules work synergistically, if the index is >4 molecules are antagonistic, while the values in between show that no clear association can be derived [109].

4. Dedicated websites

Scientific communities sharing research, events and news can be found at https://naturemicrobiologycommunity.nature.com. Website dedicated to antimicrobial studies are: http://www.baamps.it/ and http://sing.ei.uvigo.es/antimicrobialCombination.

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5. Conclusion

This deliverable contains a broad overview of the main techniques used to study biofilm forming microorganisms isolated from soil or water, a work supported by more than 100 references. This guide can be used by researchers within the SIMBA project to characterize these communities of cells. For example, within the WP3, UNIPR will employ this guide to generate standard operating procedures (SOPs) for microbiological analysis on biofilms, analyses that will be focussed also on the definition of biofilm biomarkers. Moreover, a ranking for biofilm formation properties will be defined; along with the evaluation of the resilience of strains and consortia to environmental assaults.

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Moreover, we established protocols for the development of stable minimal microbial mat communities with different diversities that can be maintained over several years and used to study different ecological relevant questions. Future research includes using minimal mats at different dilutions to be added to the soil of halophytic plants in order to facilitate their growth with an optimized microbiome. The enrichment for phototrophic prokaryotes from microbial mats will be analysed by means of high throughput metagenomics. A pilot study using only two wavelengths (500 nm & 530 nm) revealed distinctive microbial populations after 3 months of incubation. The current experiment will run for at least 4 months to provide sufficient biomass for DNA extraction and initiating isolation procedures for novel strains. Later in this project, these strains and enrichments will be added to crops at different salinities to test their ability to facilitate growth and or salt tolerance of the different crops.

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6. Document Information

EU Project	*No 818431*	**Acronym**	SIMBA
Full Title	Sustainable Innovation of Microbiome Applications in the Food System		
Project website	www.simbaproject.eu		

Deliverable	**N°**	D3.1	**Title**	Protocols for analysis of biofilm formation in water and soil substrates
Work Package	**N°**	3	**Title**	Marine microbiomes for sustainable high quality food production

Due date of deliverable:	31-10-2019
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Nature of deliverable:	R[2]

Authors (Partner)				
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[1]Dissemination level (DELETE ACCORDINGLY): **PU:** Public, **CO:** Confidential, only for members of the consortium (including the Commission Services), set out in Model Grant Agreement, **CL:** Classified, information as referred to in Commission Decision 2001/844/EC

[2] Nature of deliverable (DELETE ACCORDINGLY): **R:** Report, **DEM:** Demonstration, pilot, prototype, plan design, **DEC:** Website, patent filing, market studies, press & media, videos, **Other:** Software, technical diagram, etc., **Ethics:** Ethics deliverable

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Issue Date	**Revision N°**	**Author**	**Change**
09/10/2019	1	UNIPR+NIOZ	First version
29-10-2019	2	UNIPR+NIOZ	Revised version

7. Appendix

List of step by step protocols mentioned in the deliverables:

- Crystal Violet staining: Merritt JH, Kadouri DE, O'Toole GA. Growing and analyzing static biofilms. Curr Protoc Microbiol 2011. doi:10.1002/9780471729259.mc01b01s22.
- Determination of CFUs: Merritt JH, Kadouri DE, O'Toole GA. Growing and analyzing static biofilms. Curr Protoc Microbiol 2011. doi:10.1002/9780471729259.mc01b01s22.
- Determination of cell number by RT-qPCR: Klein MI, Scott-Anne KM, Gregoire S, Rosalen PL, Koo H. Molecular approaches for viable bacterial population and transcriptional analyses in a rodent model of dental caries. Mol Oral Microbiol 2012;27:350–61. doi:10.1111/j.2041-1014.2012.00647.x
- Preparation of sample for CLSM: Chandra J, Mukherjee PK, Ghannoum M a. In vitro growth and analysis of Candida biofilms. Nat Protoc 2008;3:1909–24. doi:10.1038/nprot.2008.192
- MIC_{99} determination: Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008;3:163–75. doi:10.1038/nprot.2007.521.
- MBEC determination: Billings N, Ribbeck K. Minimal Bactericidal Concentration for Biofilms (MBC-B). BIO-PROTOCOL 2014;4. doi:10.21769/bioprotoc.1115.

Culture medium: BA+/G

Samples were incubated using brackish medium BA+ (brackish medium with addiction of NO_3^- source). The main difference between BA+ and marine medium is the lower BA+ final concentration of NaCl, $MgCl_2$, KCl, $MgSO_4$ (https://ccy.nioz.nl/Media for detailed information and comparison), which is closer to the natural brackish conditions that favour the development of coastal microbial mats. In order to speed up the process,

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glycerol (G) at 1% final concentration was also provided as further carbon source.

BA+ composition. The medium was supplied with 1% glycerol (BA+G)

Minerals	Stock Solutions (g/L)	Quantity (mL Stock/L Media)	Final Concentration (mM)
NaCl	250	33.3	142
$MgCl_2 \cdot 6H_2O$	200	3.3	3.3
KCl	50	3.3	2.14
$MgSO_4 \cdot 7H_2O$	350	3.3	4.62
$CaCl_2 \cdot 2H_2O$	50	3.3	0.99
Na_3-citrate	0.6	8.3	0.027
Na_2-EDTA$\cdot 2H_2O$	0.1	8.3	0.002
Trace metal mix (A5 + Co)	See recipe below	1	-

Adjust to 900 mL with MQ water and autoclave

Minerals	Stock Solutions (g/L)	Quantity (mL Stock/L Media)	Final Concentration (mM)
$NaNO_3$	150	8.3	13.29
$K_2HPO_4 \cdot 3H_2O$	4	8.3	0.15
Na_2CO_3	20	2.3	0.44
Fe NH_4-citrate	6	0.8	-
Vitamin B12 (Cyanocobalamin)	0.02	1	-

For solid medium use 7 g/L of agarose. Sterilize the agarose separately in 550 mL of MQ water. In this case the mineral solution is filled up to 400 mL

Trace metal mix A5 + Co

Trace metals	Quantity (g/L)	Concentration in the final media (mM)
H_3BO_3	2.86	0.047
$MnCl_2 \cdot 4H_2O$	1.81	0.009
$ZnSO_4 \cdot 7H_2O$	0.22	0.0007
$Na_2MoO_4 \cdot 2H_2O$	0.39	0.0016
$CuSO_4 \cdot 5H_2O$	0.08	0.0003
$Co(NO_3)_2 \cdot 6H_2O$	0.05	0.0002

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Abbreviations:

AFM Atomic Force Microscopy

CLAS FISH combination of labelling and spectral imaging FISH

CLAS-FISH combination of labelling and spectral imaging FISH

CLSM Confocal Laser Scanning Microscopy

DAPI 4–6-diamidino-2-phenylindole

DMSO Dimethylsulfoxide

EPS Extracellular polymeric substances

ESEM Environmental Scanning Electron Microscope

FIC fractional inhibitory concentration

FISH fluorescence in situ hybridization

iTRAQ Isobaric Tags for Relative and Absolute Quantitation

LCMS liquid chromatography-mass spectrometry

MBEC Minimal Bactericidal Eradication Concentration

MIC Minimal Inhibitory Concentration

PGPM Plant Growth Promoting Microbes

PMA-qPCR propidium monoazide qPCR

PVC polyvinyl chloride

qPCR quantitative polymerase chain reaction

RT-qPCR reverse transcription- quantitative polymerase chain reaction

SEM Scanning Electron Microscope

SOPs standard operating procedures

TEM Transmission Electron Microscope

TSA Tryptic soy agar

TTC (2,3,5-triphenyl-2H-tetrazolium-chloride)

WGS Whole Genome Sequencing

WP2 Work package 2

XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2Htetrazolium-5-carboxanilide inner salt)

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