Finanziato dall'Unione europea NextGenerationEU
Ministero dell'Università e della Ricerca
Italiadomani PIANO NAZIONALE DI RIPRESA E RESILIENZA
SUS-MIRRI.IT

Biofilms and microscopic observations

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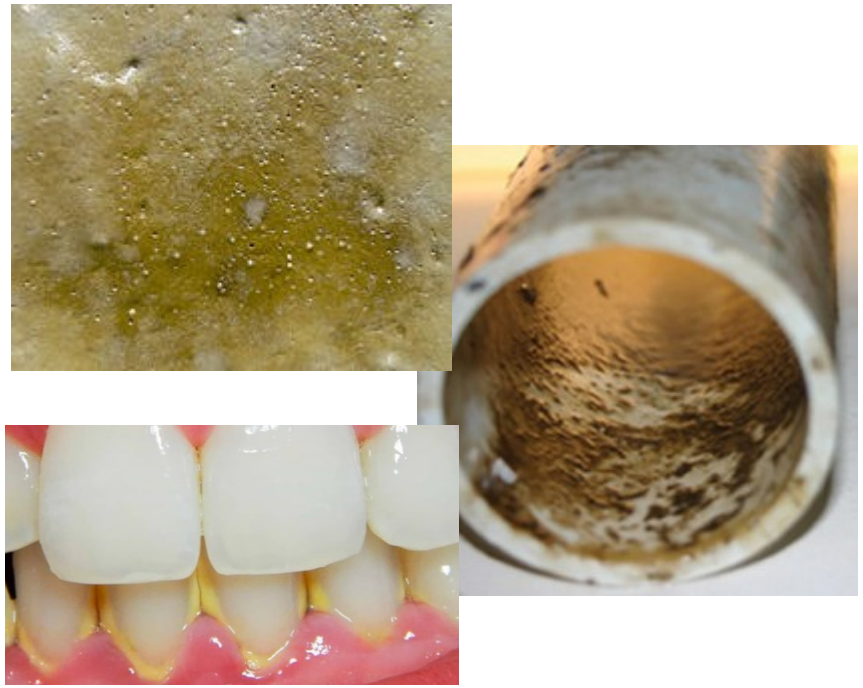
Training course SUS-MIRRI.IT

What is a BIOFILM?

Definition

- A biofilm is a **highly organized** and **irreversibly associated** (not removed by gentle rinsing) structure consisting of **multi-cellular** microbial community enclosed in a self-produced **extracellular polymeric matrix** attached on a surface.
- Biofilms are formed whenever there is free flow of fluid, microorganism and a solid surface as one of the basic **survival strategies** employed by bacteria
- May form on a wide variety of surface: e.g. living tissues, medical devices, industrial piping systems, **soil**.

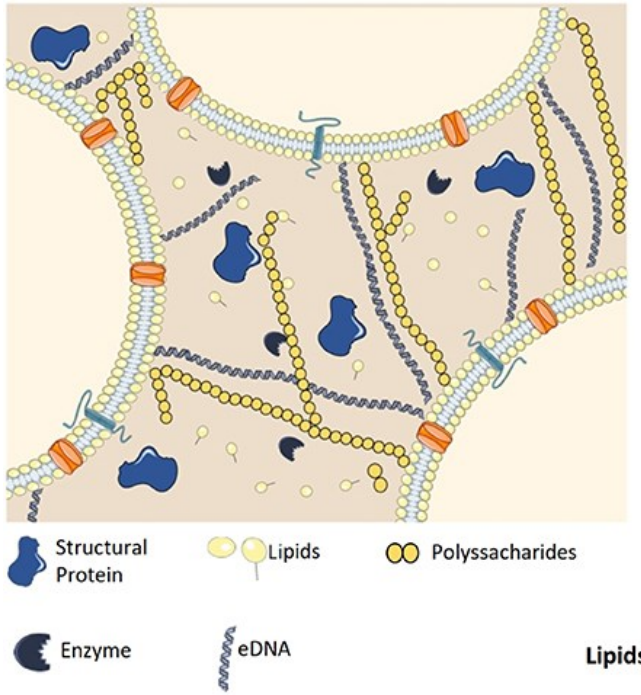


https://doi.org/ 10.3201/eid0809.020063

What is a BIOFILM?

The EPS (Extracellular Polymeric Substance)

- Natural polymers of high molecular weight secreted by microorganisms into their environment.
- Components of EPS can be of different classes: **polysaccharides, lipids, nucleic acids, proteins, lipopolysaccharides, and minerals.**
- **50-90%** of a biofilm's total organic matter.
- Determines the **physicochemical properties** of a biofilm.



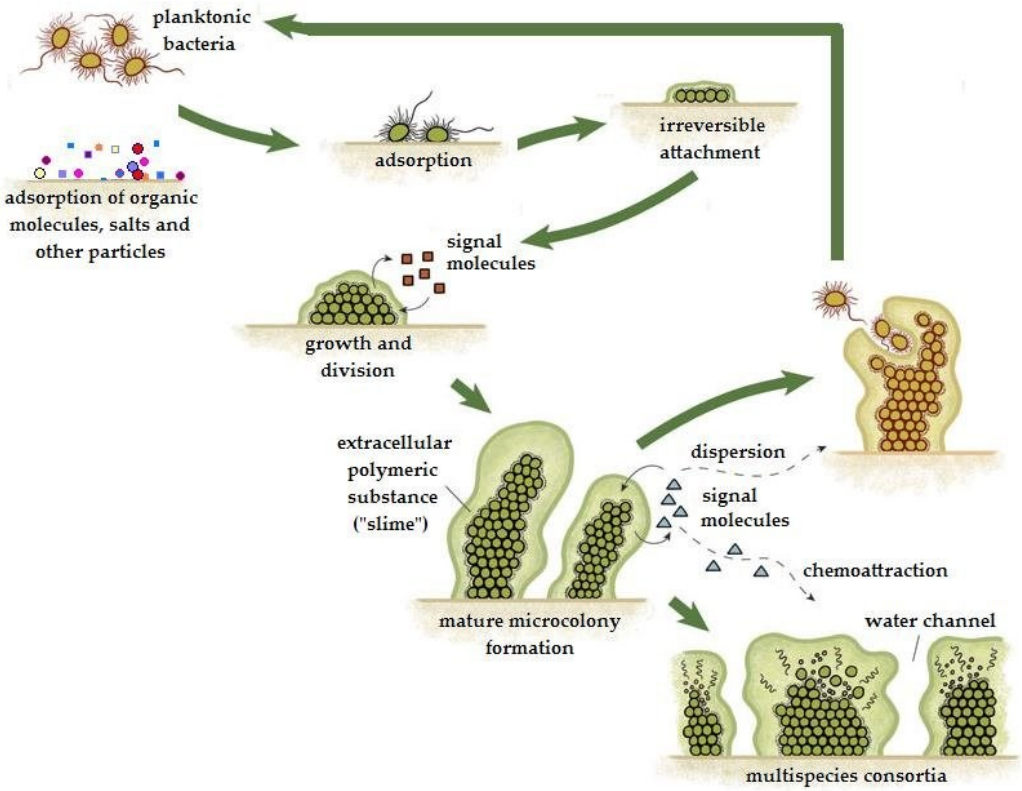
Polyssacharides	Cohesion of the structure Nutrient source Water retention Protective barrier Sorption of organic compounds and inorganic ions
Enzymes	Enzymatic activity Nutrient source
Structural Proteins	Cohesion of the structure Nutrient source Protective barrier Sorption of organic compounds and inorganic ions Electron donar or acceptor
eDNA	Cohesion of the structure Nutrient source Exchange of genetic information
Lipids and biosurfactants	Nutrient source

https://doi.org/10.3389/fmicb.2020.00952

What is a BIOFILM?

Ultrastructure

1. **Attachment**: **initial attachment** of free-floating microorganisms to a surface (weak, reversible adhesion).
2. **Adhesion**: colonists irreversibly **anchor** themselves more permanently using cell adhesion structures (e.g. pili, flagella) and form micro colonies and excrete EPS.
3. **Maturation I**: A biofilm is formed, and cells form **multi-layered clusters**.
4. **Maturation II**: Three-dimensional growth (microcolonies and water channels)
5. **Detachment**: The biofilm reaches a critical mass and disperses planktonic bacteria, ready to colonize other surfaces.



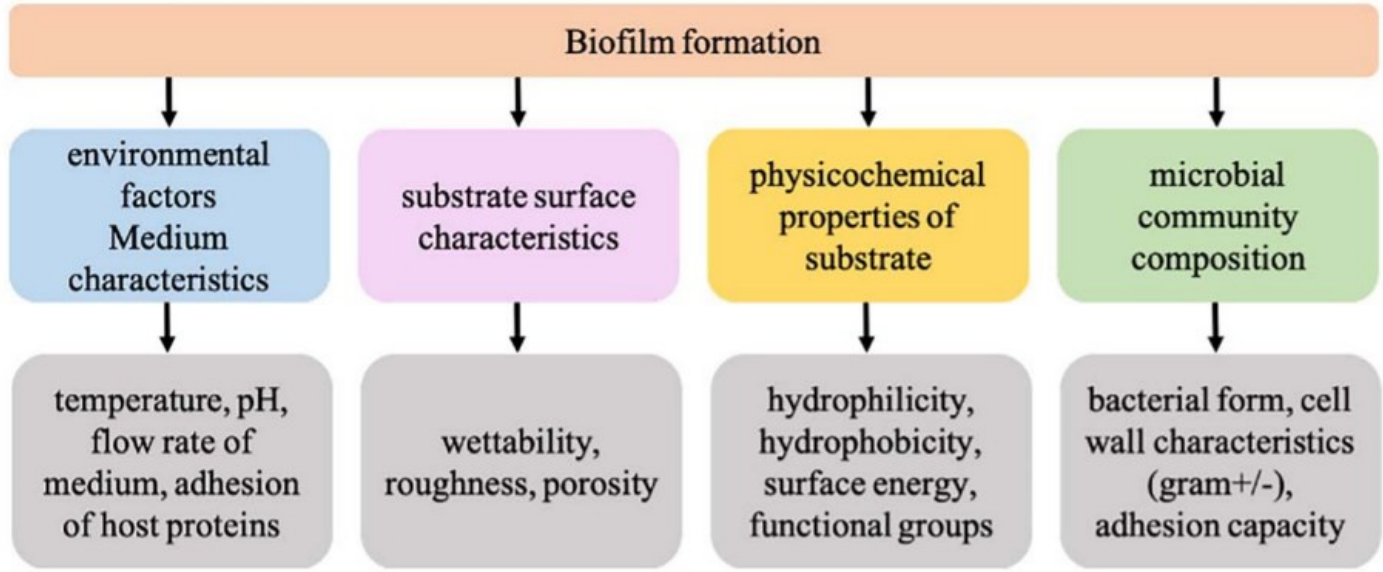
https://doi.org/10.1038/s41579-022-00767-0

What is a BIOFILM?

Factors affecting biofilm formation

Causes:

- Recognition of **attachment** sites
- **Nutritional** cues
- Sub-inhibitory **antibiotic** concentrations



Factors influencing the formation of biofilms on a substrate.

https://doi.org/10.1016/j.cofs.2015.02.003
https://doi.org/10.3389/fmicb.2021.687118

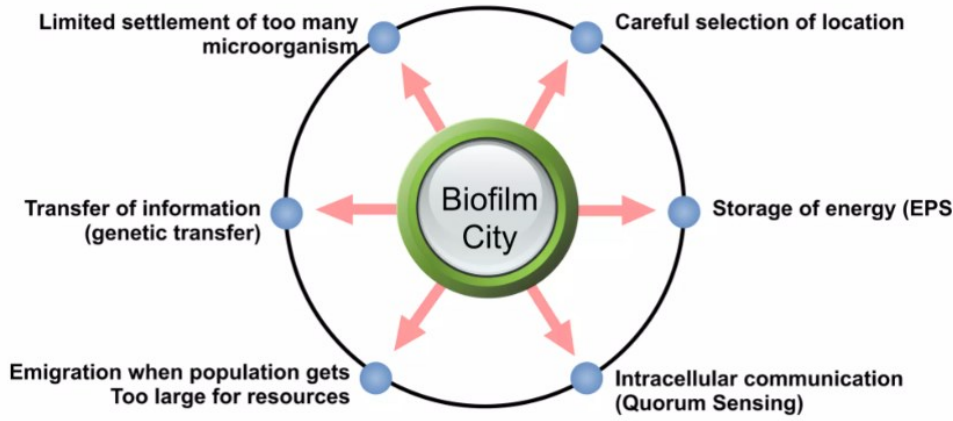
What is a BIOFILM?

Advantages for the bacteria

Biofilm formation allows otherwise unicellular organisms to assume a temporary communal lifestyle.

- **Protection** from environmental threats (e.g. anitibiotics, dessiccation)
- **Metabolic improvement**: Trapping of nutrients and metabolic cooperation
- **Organized internal compartmentalization** which helps the bacterial species in each compartment with different growth requirements (e.g. aerobic-anaerobic)
- Easier communication: **Quorum sensing**
- **Exchanging genetic materials**: may acquire new traits

AUTOPOIESIS - COMMUNITY - SYNERGY - HOMEOSTASIS

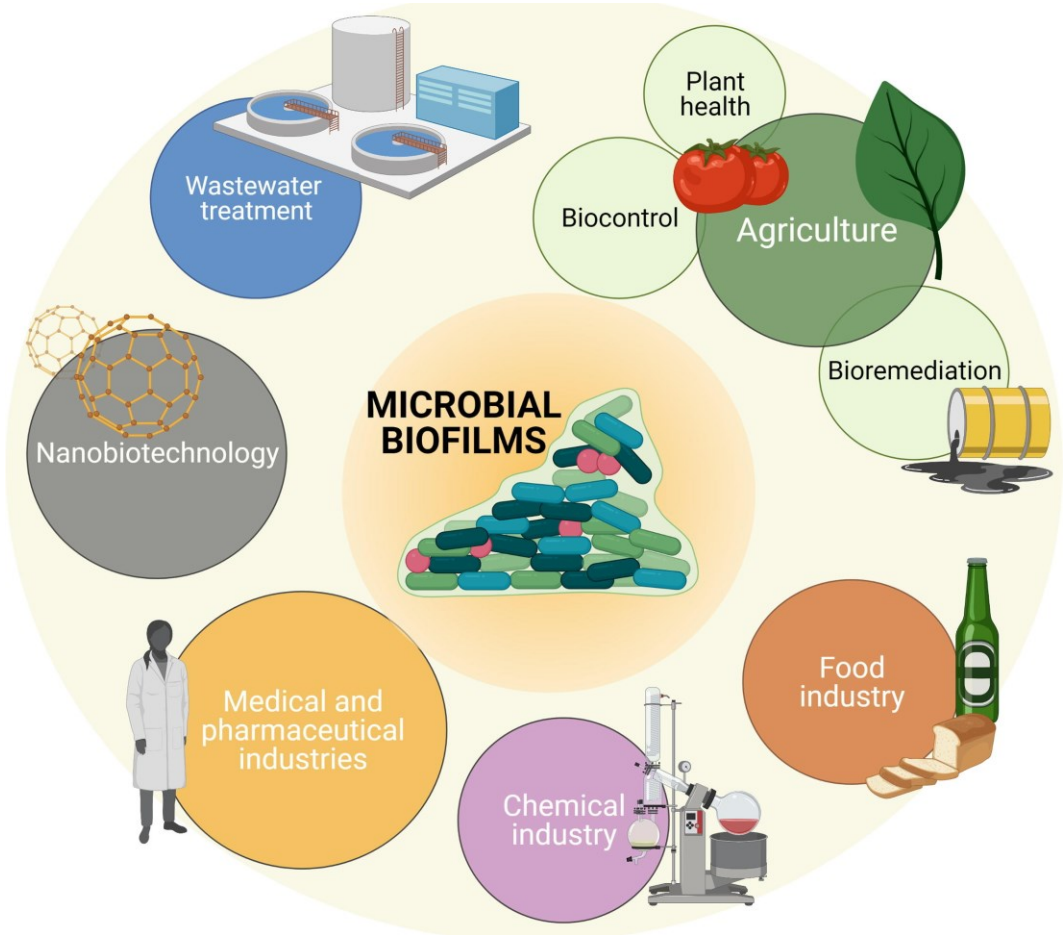


https://doi.org/10.4103/2231-0762.151956
https://doi.org/10.1021/acs.est.0c00716

Beneficial biofilms and their applications

Used in a variety of applications

- **Water purification and bioremediation** (elimination of recalcitrant and toxic compounds)
- **Productive biofilms**: developing biofilm systems to produce value-added compounds (e.g. surfactant, proteins, PHA)
- **Quorum quenching** (e.g. prevent the membrane biofouling)
- **Bioaugmentation**
- **Phototrophic biofilms**
- **Agricultural applications**

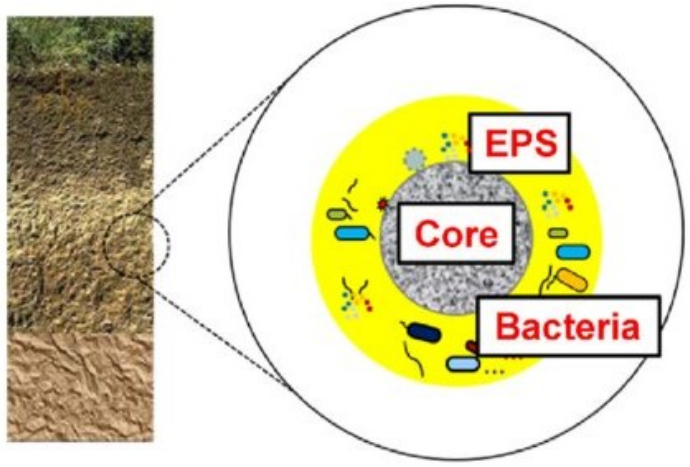


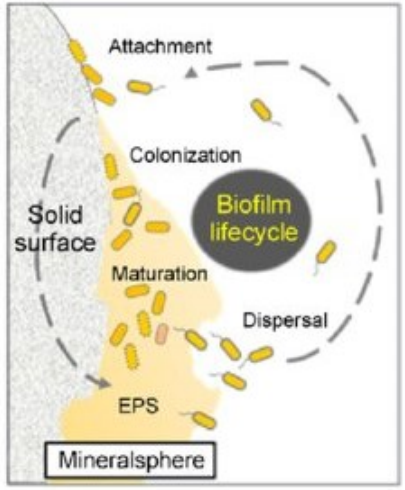
https://doi.org/10.1007/s10811-017-1172-9
https://doi.org/10.1016/B978-0-12-819001-2.00005-X

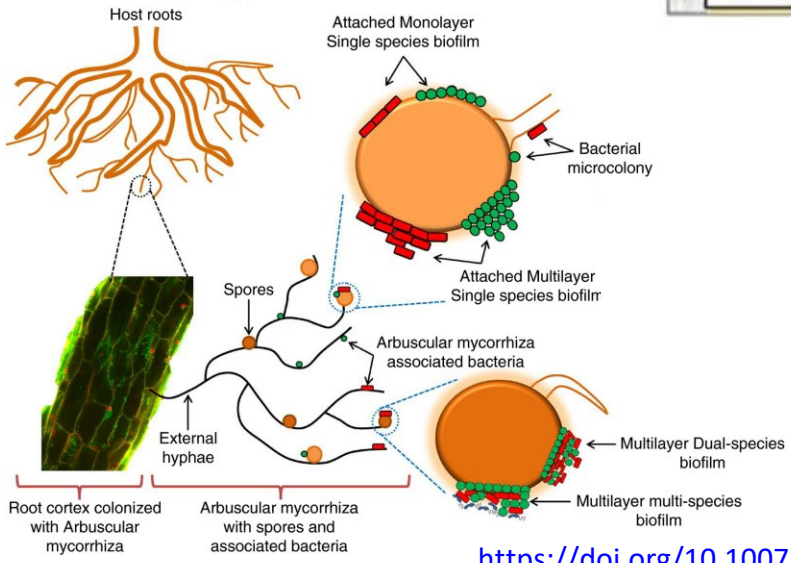
Agricultural applications

Biofilms in the PLANT-SOIL continuum

- **Continuous surface films** (5-15 um thick) - 1% of total soil volume and 10–6 % of the soil surface area
- **EPS** accounting for 80-90% of biofilm's dry mass
- Soil **hotspots** - Engine driving many key biogeochemical cycles
- The enriched environment around plant roots allows establishment of **interactions** between soil bacteria and the roots
- A healthy soil microbiome regulates different enzymatic processes and biogeochemical cycles, protects plants from pests and diseases, stimulates plant development, maintains root health, aids in nutrient absorption, and boosts environmental sensitivity.





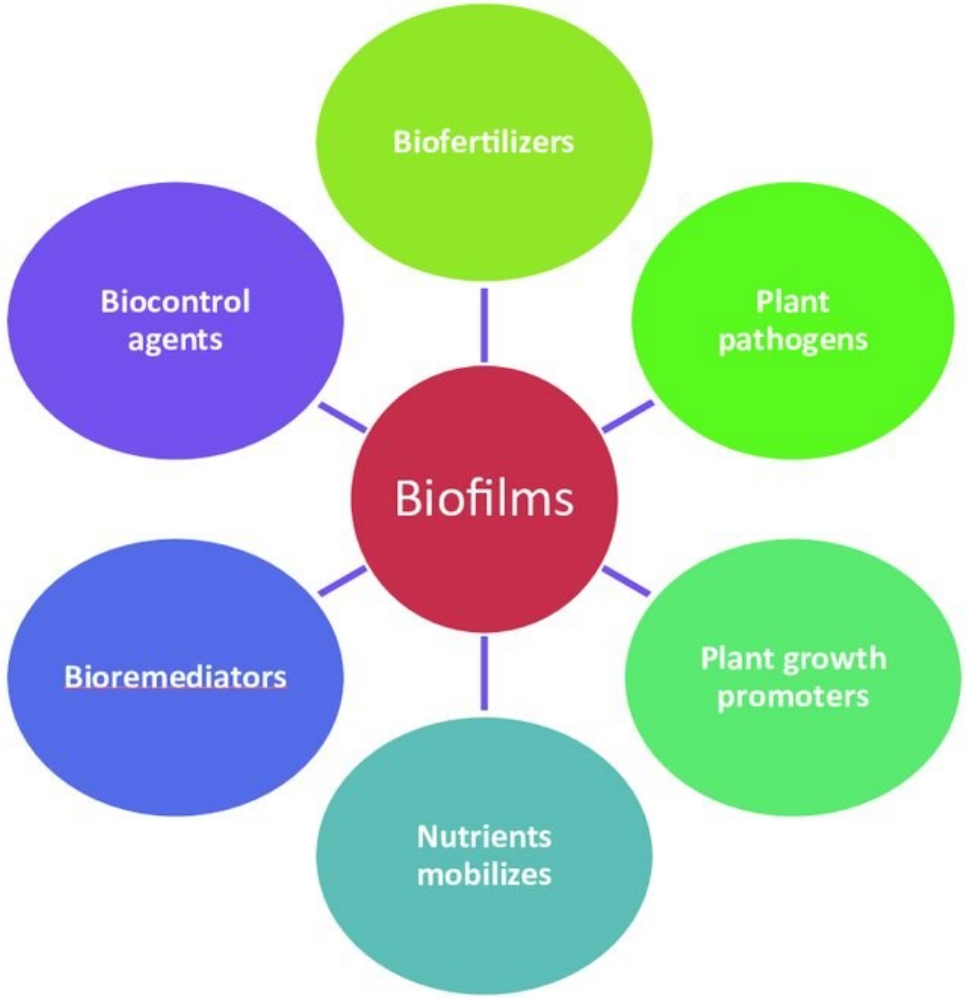


https://doi.org/10.1007/s42832-019-0017-7

Agricultural applications

Biofilm functions relevant for agriculture biotechnology

- Studies on application of biofilms in agriculture are at their **infancy**
- Understanding bacterial-fungal association/interactions has immense potential for their utilization as **multi-species inoculants**
- Research need to develop **multi-species biofilms** (Trichoderma as a matrix with agriculturally important bacteria (Azotobacter chroococcum, P. fluorescens, and B. subtilis))



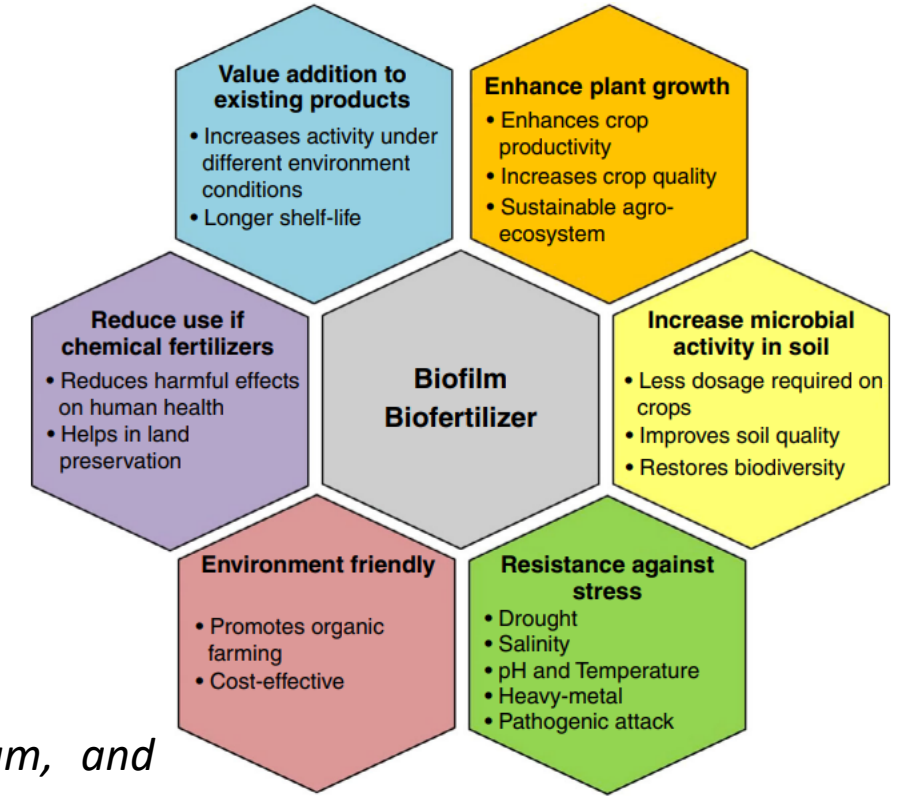
https://doi.org/10.1016/j.resmic.2023.104149

Agricultural applications

Biofilm as biofertilizer

A fertilizer that contains a healthy microbial community that has evolved in biofilm mode.

- **Substitute for chemical fertilizers**
- **Improved agronomic potential** (microbiological and plant growth promotion, biocontrol agents)
- Enhancement of **soil biological and chemical properties** (soil fertility)



The following are the types of biofertilizer:

- **Nitrogen-fixing biofertilizers** (e.g. *Rhizobium, Bradyrhizobium, Azospirillum, and Azotobacter*).
- **Phosphorus-solubilizing biofertilizers** (e.g. *Bacillus, Xanthomonas, and Aspergillus*).
- **Phosphate-mobilizing biofertilizer** (e.g. *Arbuscular Mycorrhiza, Ectomycorrhiza)*
- **Plant growth promoting biofertilizer** (e.g. *Rhizobium, Azotobacter, Azospirillum*).

https://doi.org/10.1111/jam.14609

Techniques for biofilm research

Direct characterization of biofilms in soil is challenging due to:

- **Spatial heterogeneity**
- **Opacity**

Ex-situ processes are advantageous to further our understanding of the functional capacity of a sampled soil community.

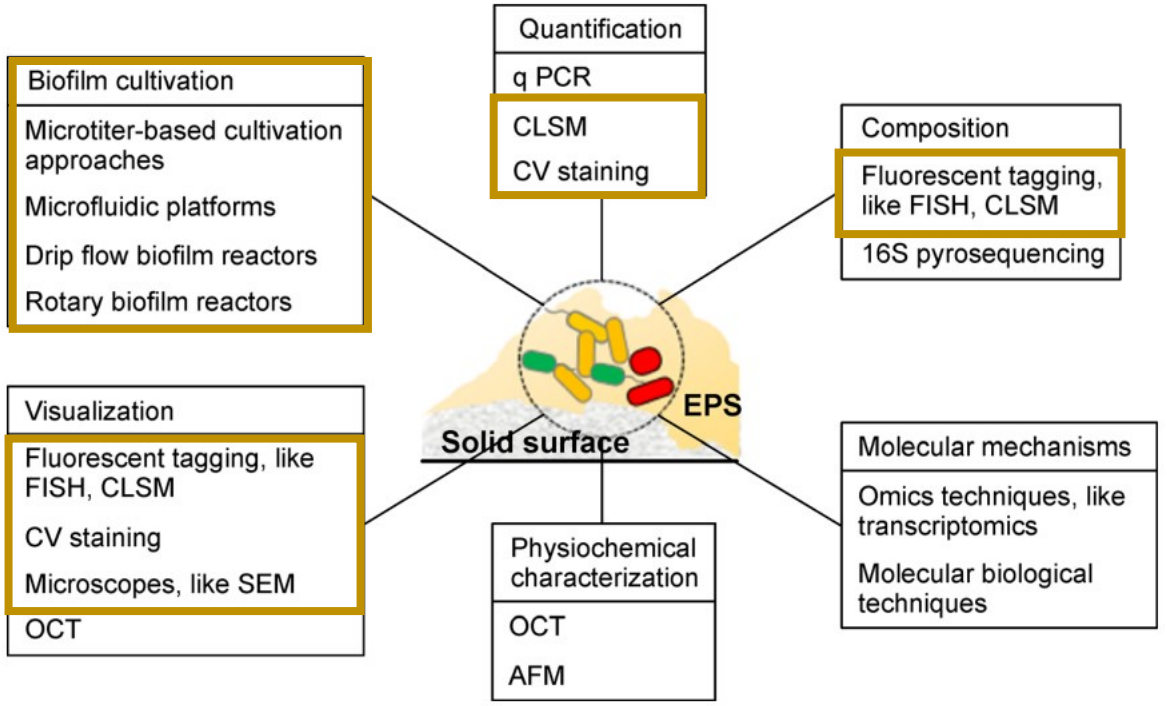
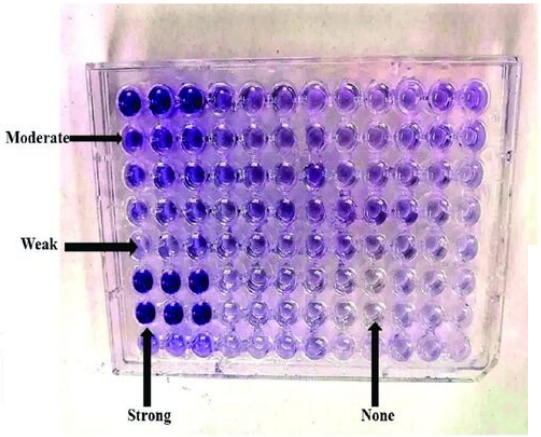


Fig. 2 Cultivation systems and analytical techniques for *ex-situ* biofilm research. Rods with different colors represent bacteria in biofilm. FISH, fluorescence in situ hybridization; CLSM, confocal laser scanning microscopy; SEM, scanning electron microscope; CV staining, crystal violet staining; OCT, optical coherence tomography; AFM, atomic force microscope.

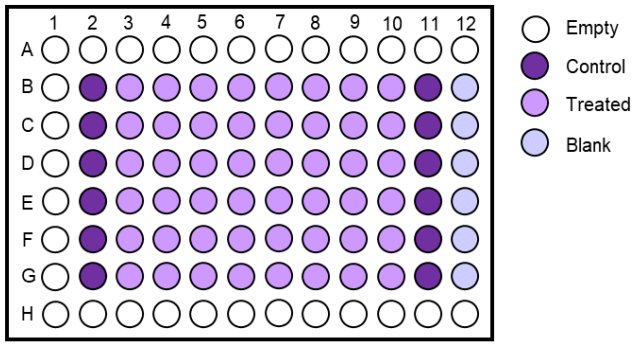
https://doi.org/10.1007/s42832-019-0017-7

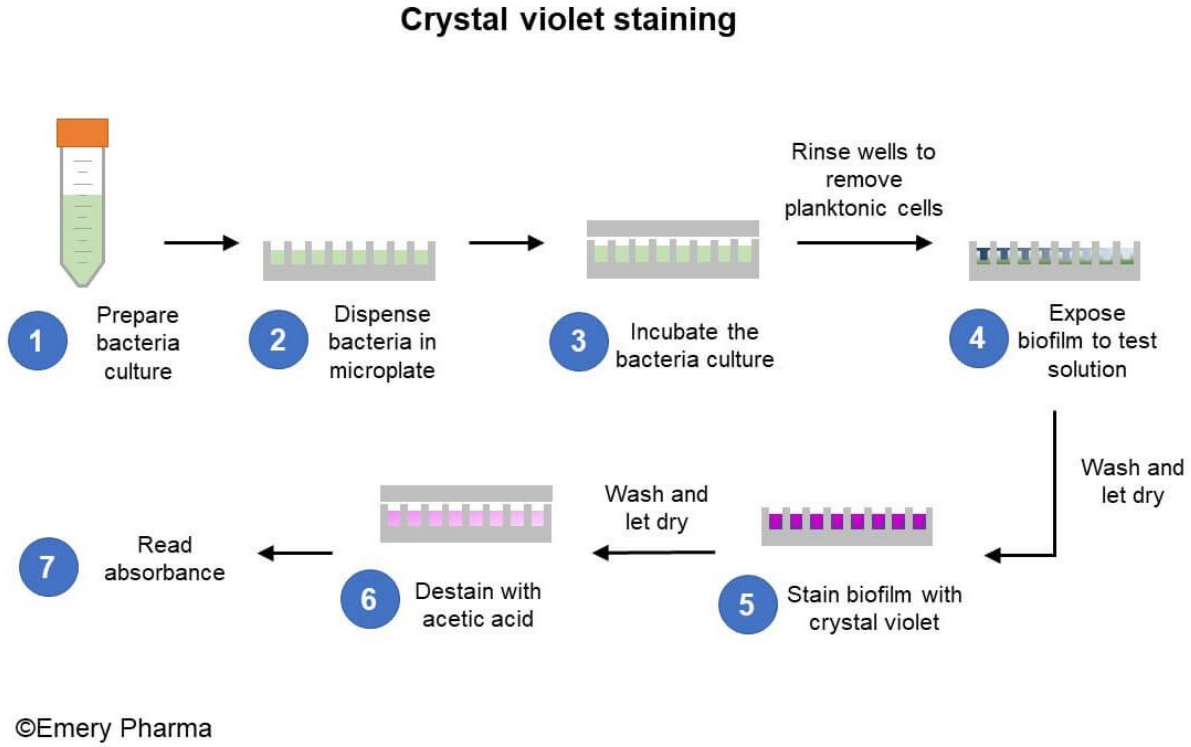
Biofilm cultivation: Microtiter-based cultivation approaches

- Growth of static biofilms
- Indirect quantification of the biomass
- Test of anti-biofilm compounds



O'Toole e Kolter, 1998





https://doi.org/10.3390/microorganisms11092244
https://doi.org/10.1186/s12951-020-00724-0

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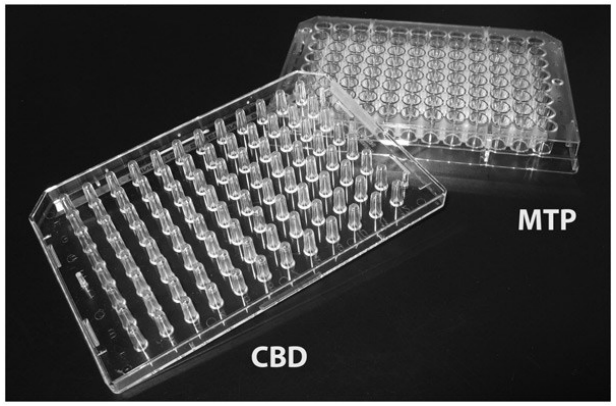
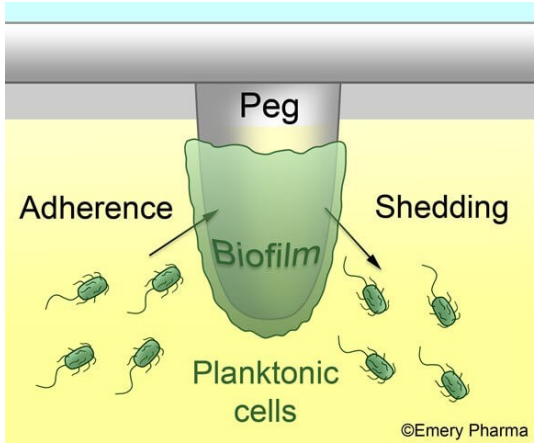
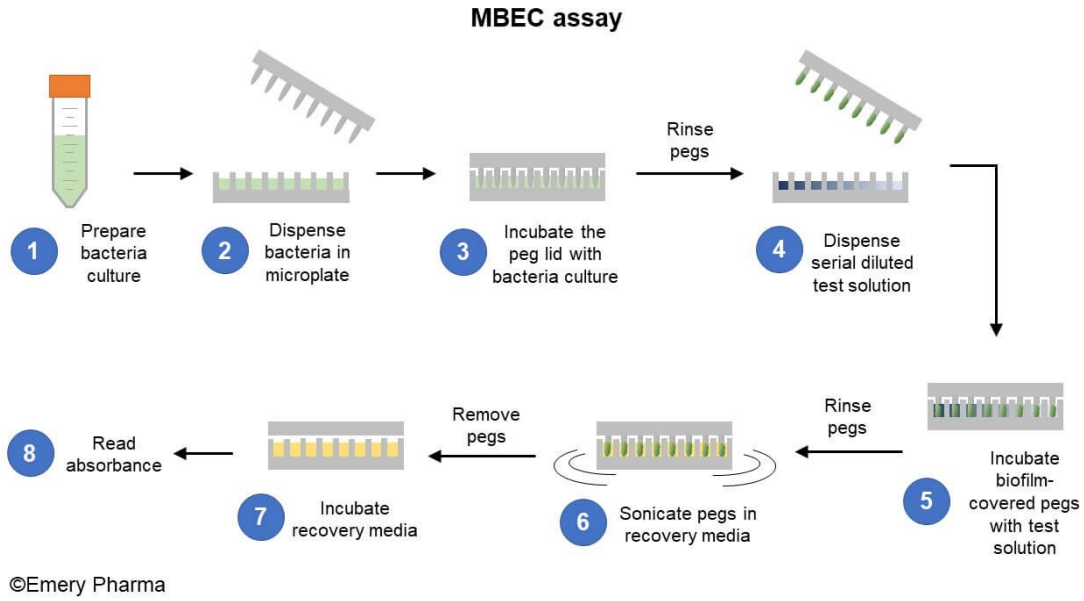


Figure 2. The microtiter plate (MTP) system and the calgary biofilm device (CBD). © Claus Sternberg.





Minimal biofilm eradication concentration

https://doi.org/10.3390/microorganisms11092244
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Biofilm cultivation: Microfluidic Platforms

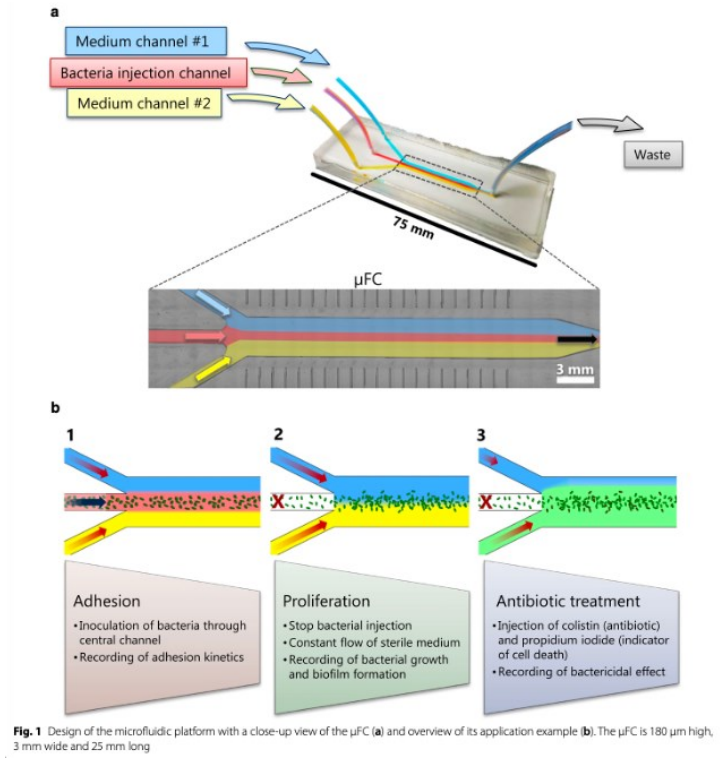
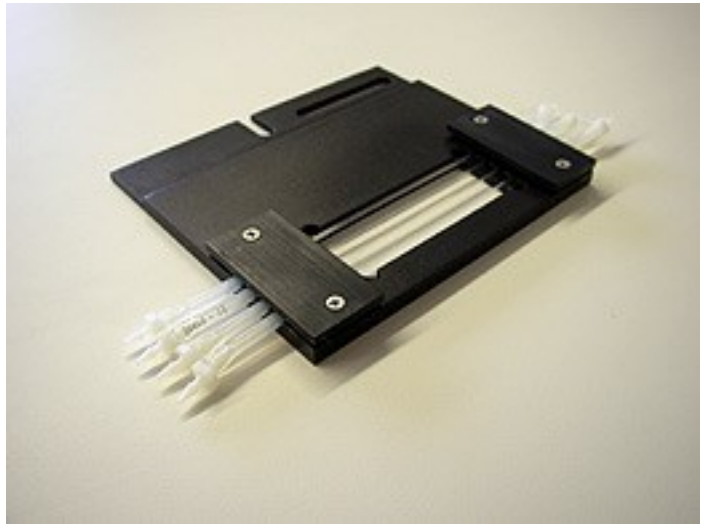
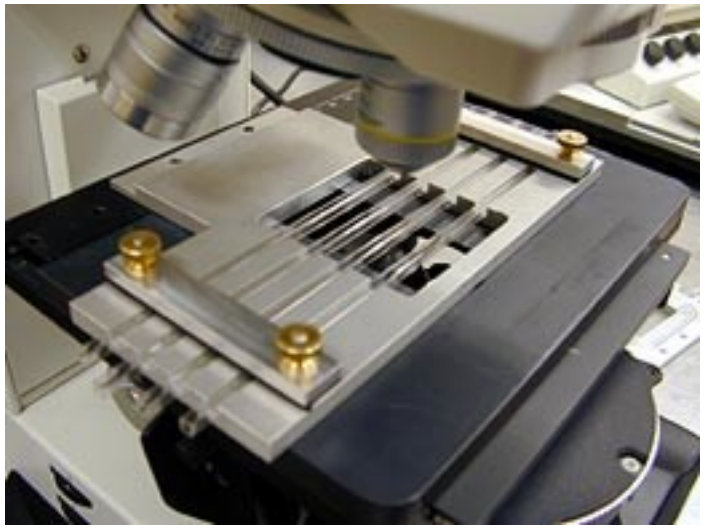


Fig. 1 Design of the microfluidic platform with a close-up view of the μFC (**a**) and overview of its application example (**b**). The μFC is 180 μm high, 3 mm wide and 25 mm long





Flow Cell Functionality: Enables biofilm growth in varying flow conditions. Mimics real environments with continuous nutrient replenishment.

Applications: Studies mature biofilms and assesses impacts on structure, detachment, gene expression, and distribution of EPS

https://doi.org/10.3390/microorganisms11092244
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Biofilm cultivation: Drip flow and Rotary biofilm reactors

Drip-flow BR

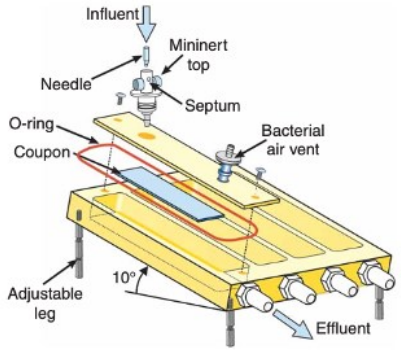


Figure 1 | Schematic diagram of a drip flow reactor showing its various components. Reprinted with permission from ref. 15.

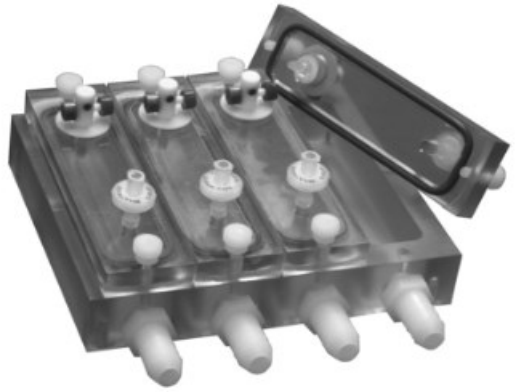


Figure 5. The Drip Flow Biofilm Reactor. Commercial version of the drip flow biofilm reactor, with four chambers each accommodating a microscope slide. © Bryan Warwood. Reuse not permitted.

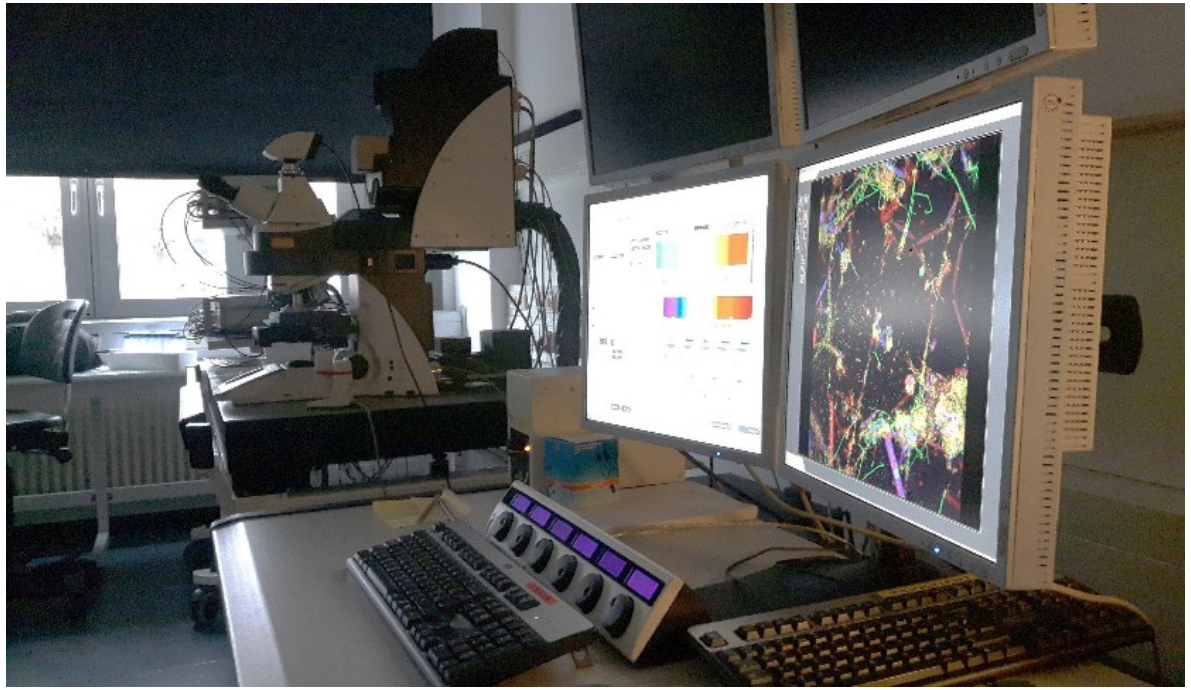
Drip flow biofilm reactor: controlled growth of biofilms by allowing a nutrient-rich liquid to drip or flow over a substrate.
Valuable for studying the dynamics of biofilm formation in a controlled environment (e.g. temperature, flow rate, and nutrient composition); Compatible with coupons of various geometry.

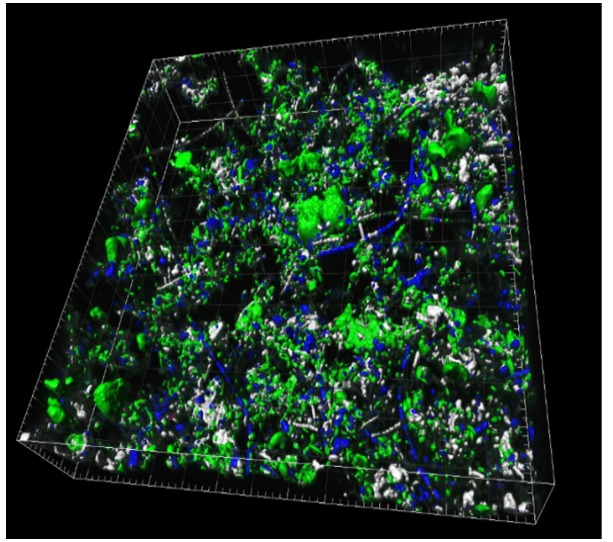
Rotary BR

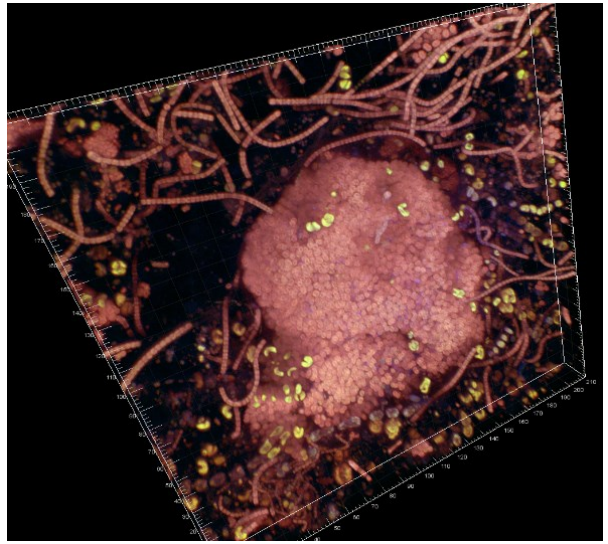
A **rotary biofilm reactor:** rotating structure with the continuous movement of the structure that helps maintain optimal biofilm thickness and distribution, while a steady nutrient supply supports microbial development, making it a useful tool for controlled biofilm studies.

https://doi.org/10.1080/1040841X.2016.1208146

Biofilm visualization and quantification: Confocal scanning laser microscopy (CLSM)







- Utilizes a focused **laser beam** to illuminate a specimen.
- Incorporates a **pinhole** aperture to selectively capture in-focus light.
- Enables three-dimensional imaging and visualization of fluorescent signals.

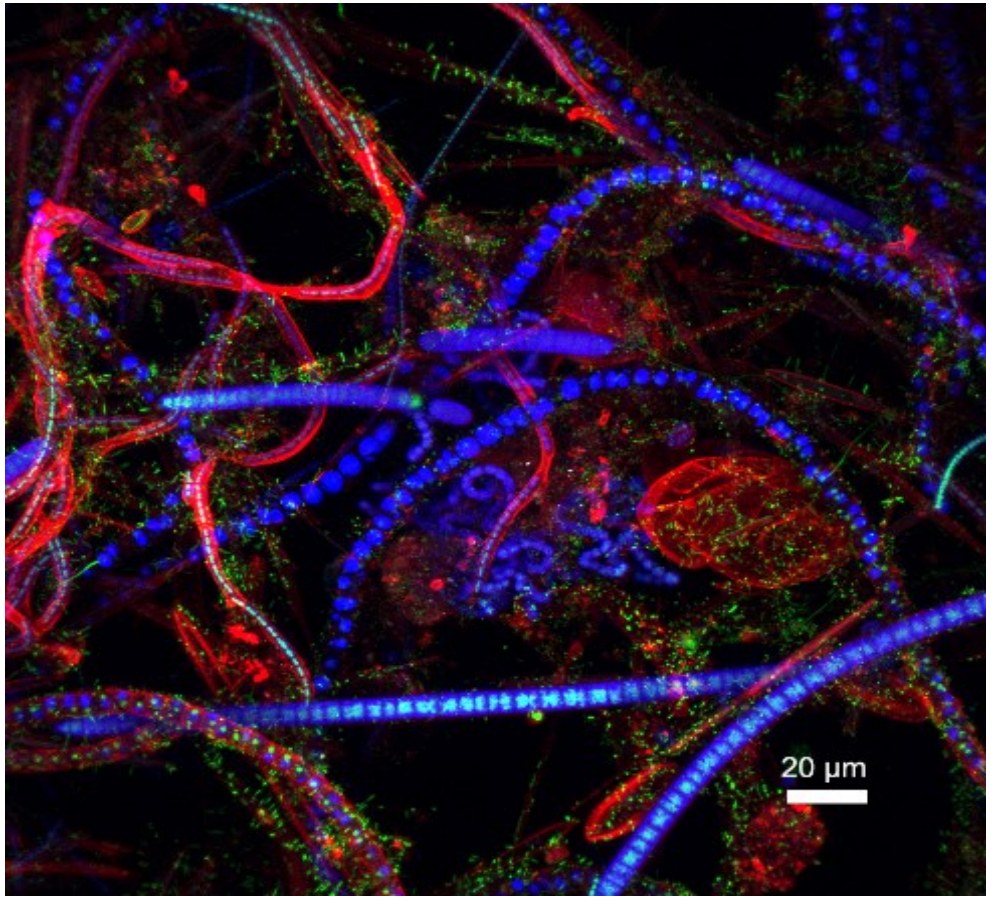
Confocal laser scanning microscopy (CLSM) is one of the tools most widely used at present to study **biofilm structure** because it enables the direct ***in situ*** **and non-destructive** investigation of native multicellular structures using specific fluorescent markers.

Biofilm visualization and quantification: CARD-FISH Coupled with CLSM

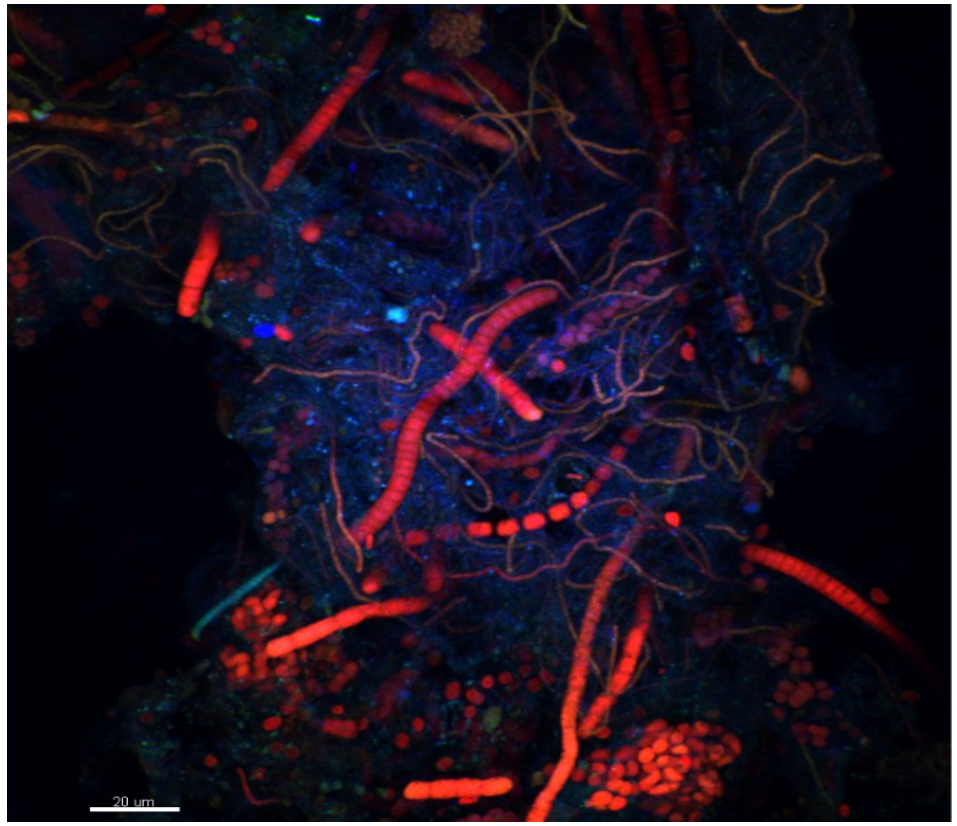
Microbial ecology in complex environments:
spatial organization and composition of microbial communities.

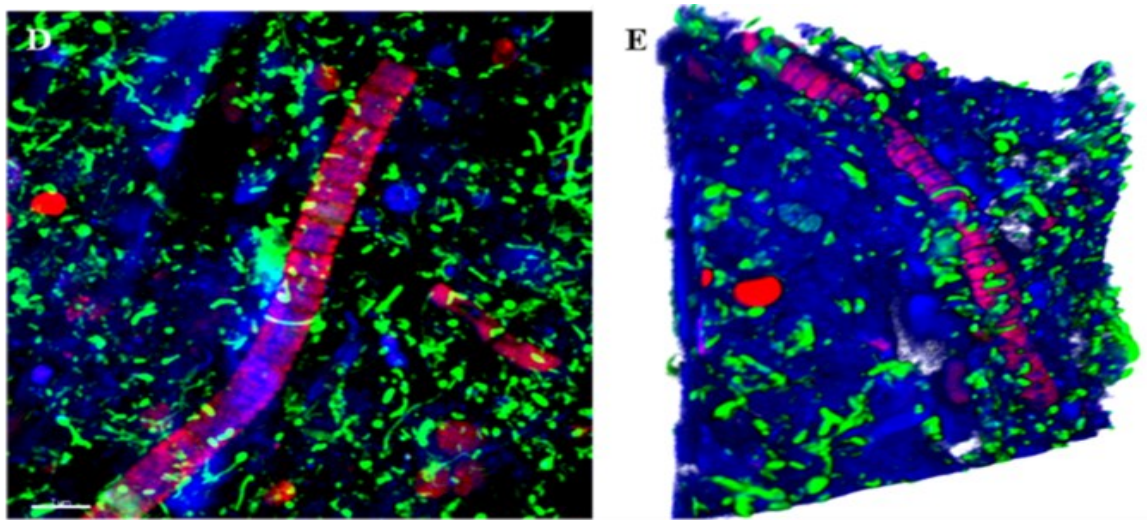
CARD-FISH (Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization): Targeting, identification and quantitative assessment of microbial populations in environmental samples. **Fluorescence Detection**: Probes sequences **complementary segments of 16S or 23S rRNA (15-30 base pairs)**.

CARD-FISH Coupled with CLSM enables to quantitatively analyze the abundance, distribution, and interactions of specific microbial groups within the biofilm.



Biofilm visualization and quantification: CARD-FISH Coupled with CLSM





CARD-FISH/CLSM signals

- **Bacteria** (EUB338 probe): 488 nm (emission 519 nm, green channel); Ar/HeNe Green
- **DAPI**: Blue 405 nm (emission 461 nm, blue channel);
- **Chlorophyll a**: red/pink 543 nm (red channel) scale bar 20 µm (left) / 7 µm

Biofilm visualization and quantification: biovolume (µm³)

CLSM micrographs: bacteria targeted with SybrGreen (green signal; ex. 490 nm; em. range 505-560 nm), matrix glycoconjugates with Glycoconjugate Aleuria aurantia AAL-A568 (red signal, ex. 578; em. range 590-650); autofluorescence of chlorophyll a signal for algae and cyanobacteria detection (blue signal) ex. 630; em. Range 650-720 nm)

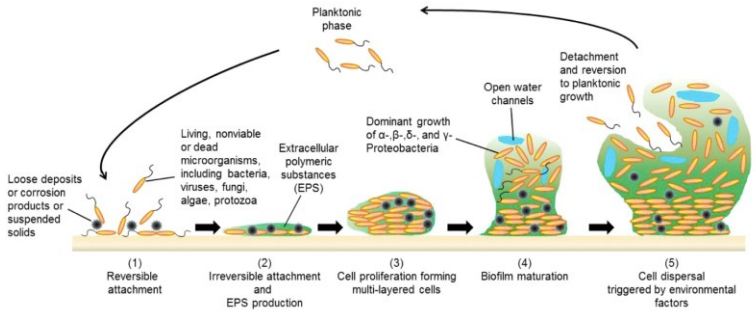
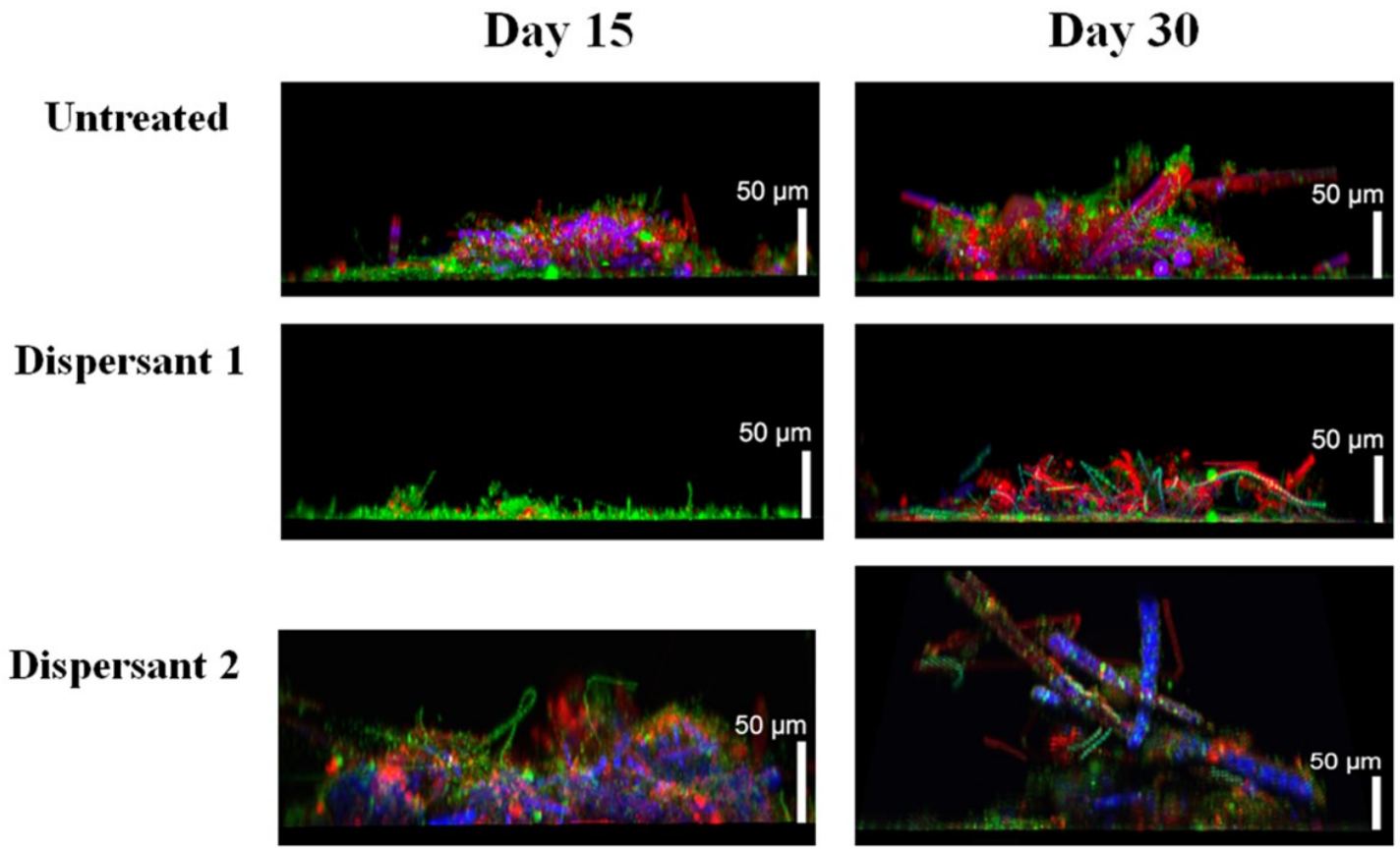
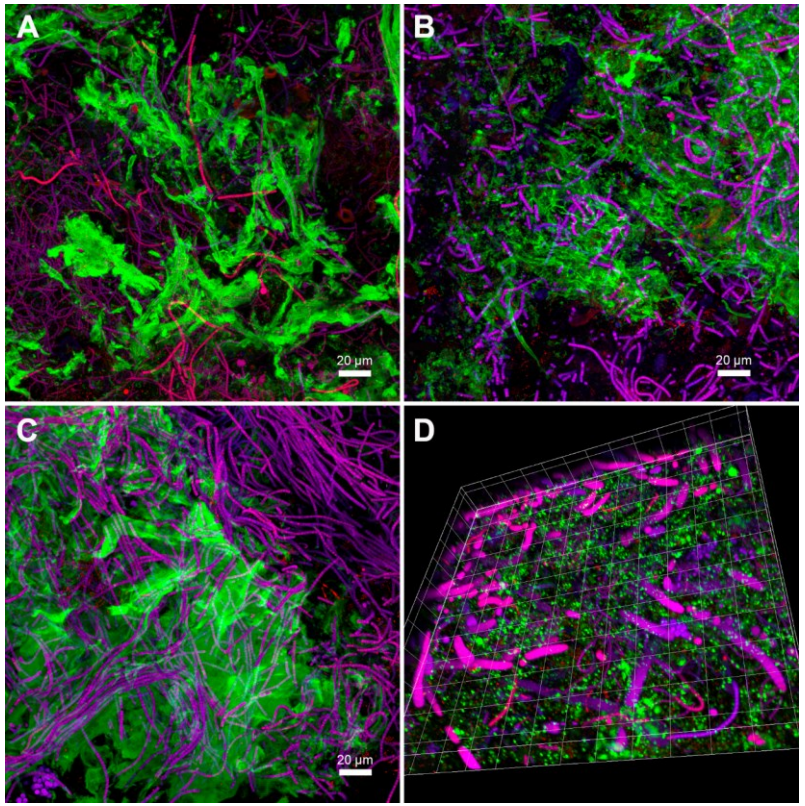


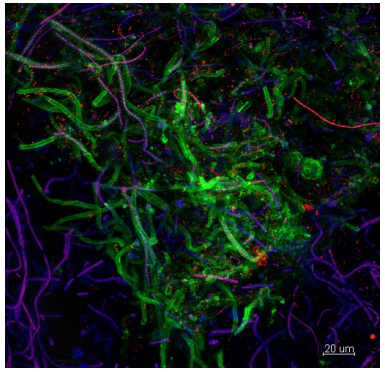
Figure 1. (a) Biofilm growth on different pipe materials. Reprinted with permission from Ren et al.[43] Copyright 2015, Springer. (b) Biofilm life cycle in DWDS.

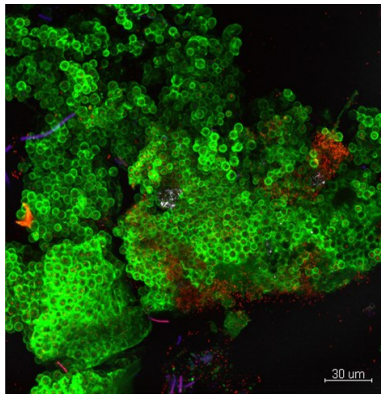


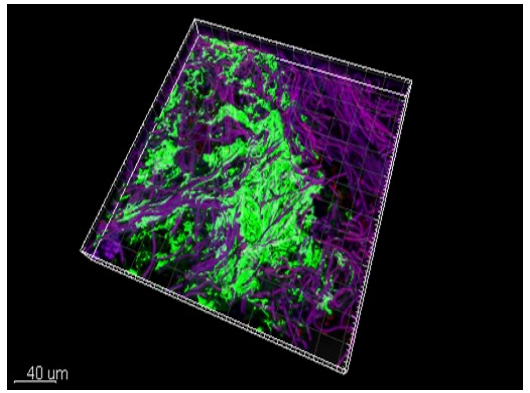
EPS: Fluorescence lectin-binding analysis (FLBA) analysis

Fluorescence lectin-binding analysis (FLBA) represents the only option for non-destructive and in situ glycoconjugate analysis and, therefore, is widely used in glycoconjugate/biofilm analysis in combination with other fluorochromes, e.g. specific for nucleic acids. The most frequently used lectin is Concanavalin A (Con A) from the jack-bean, Canavalia ensiformis, binding to mannose and glucose residues.









- **Lectins** (ex 490 nm em 515-560 nm)
- **Algae-Cyanobacteria** (Autofl. signal; ex 630 nm em 650-720 nm)
- **Nucleic Acids** (Sytox Orange, ex 543 nm, em 575-620 nm)
- **Reflection signal** (em 495-505 nm)

doi:10.1080/08927014.2018.1541454
https://link.springer.com/protocol/10.1007/978-1-4939-0467-9_4

Biofilm qualitative approach: Scanning Electron Microscopy (SEM)

SEM uses a focused beam of electrons for high-resolution imaging of specimen surfaces. Captures three-dimensional-like images by detecting emitted secondary electrons. Provides high-resolution images revealing biofilm surface topography. Enables detailed visualization of biofilm architecture and microbial cells

Applications:

- Quantitative analysis of biofilm characteristics (thickness, roughness, and the density of microbial cells).
- Study of microbial attachment, detachment, and interactions.
- Elemental composition analysis through coupling with Energy-Dispersive X-ray Spectroscopy EDS.

Advantages:

- Detailed imaging at micro and nanoscale.
- Useful for studying biofilm development and dynamics.

Limitations:

- Requires sample dehydration, affecting native biofilm structure.
- Provides surface information, not internal structure.

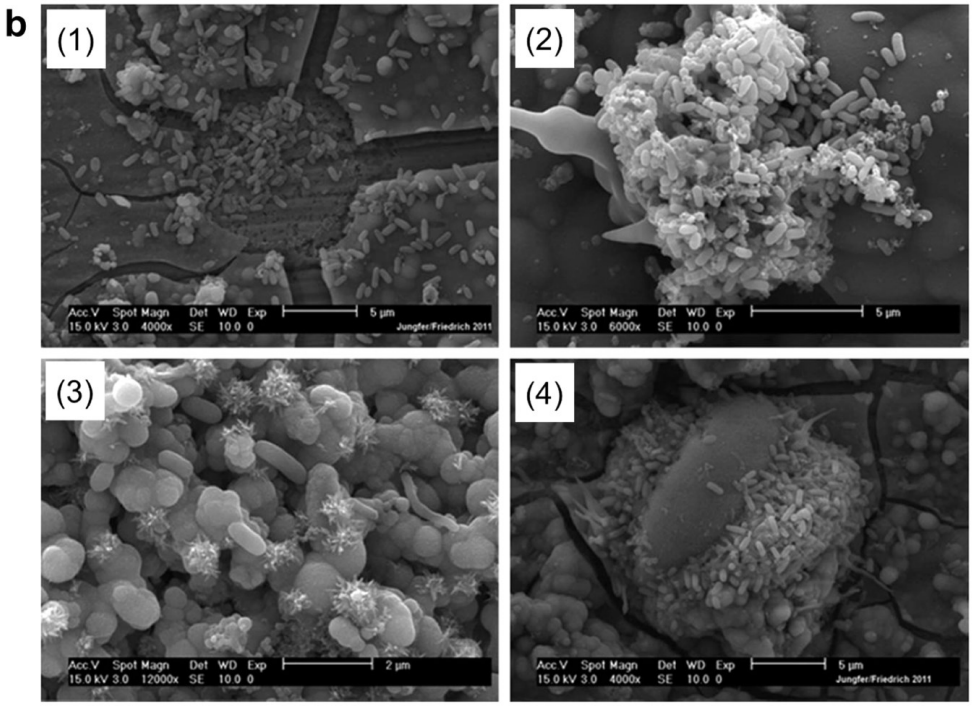


Figure 4. (a) Epifluorescence images of biofilms on copper pipes with (1) aggregating bacteria and (2) homogeneously distributed bacteria stained with the BacLight viability reagents.[222] Green: bacteria with intact membranes. Red: bacteria with damaged membranes. Scale bars =10 μm. (b) Environmental scanning electron micrographs of biofilms on copper surfaces.[222] Images (1)−(4) show the presence of multilayered bacterial aggregates with different morphologies on Cu surfaces. Note the multispecies microbial communities in (3). Reprinted with permission from Jungfer et al.[222] Copyright 2013, Taylor & Francis.

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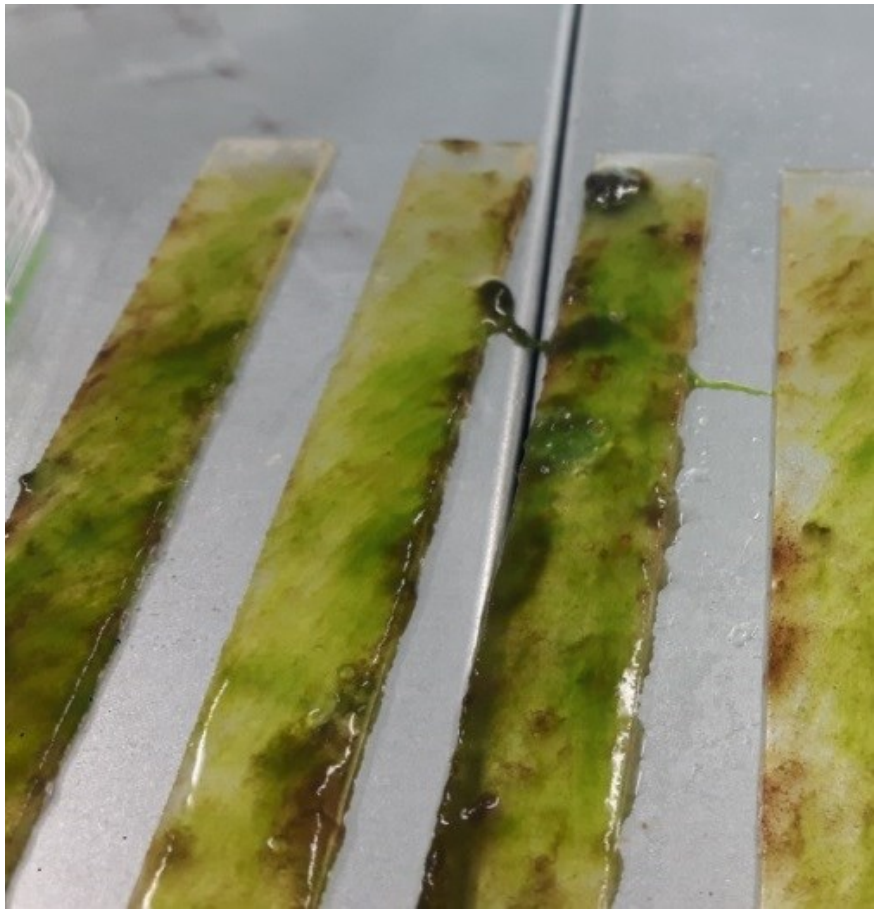
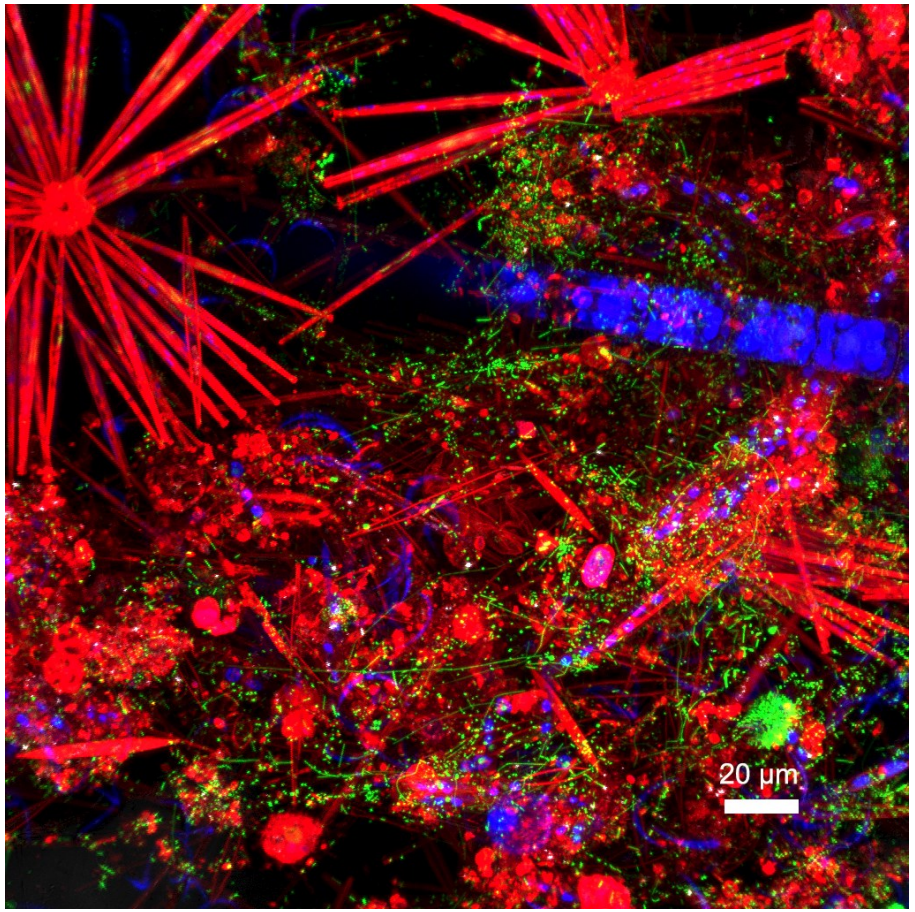
Multispecies biofilm challenges: macroscale and microscale heterogeneity





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Thanks for your attention

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