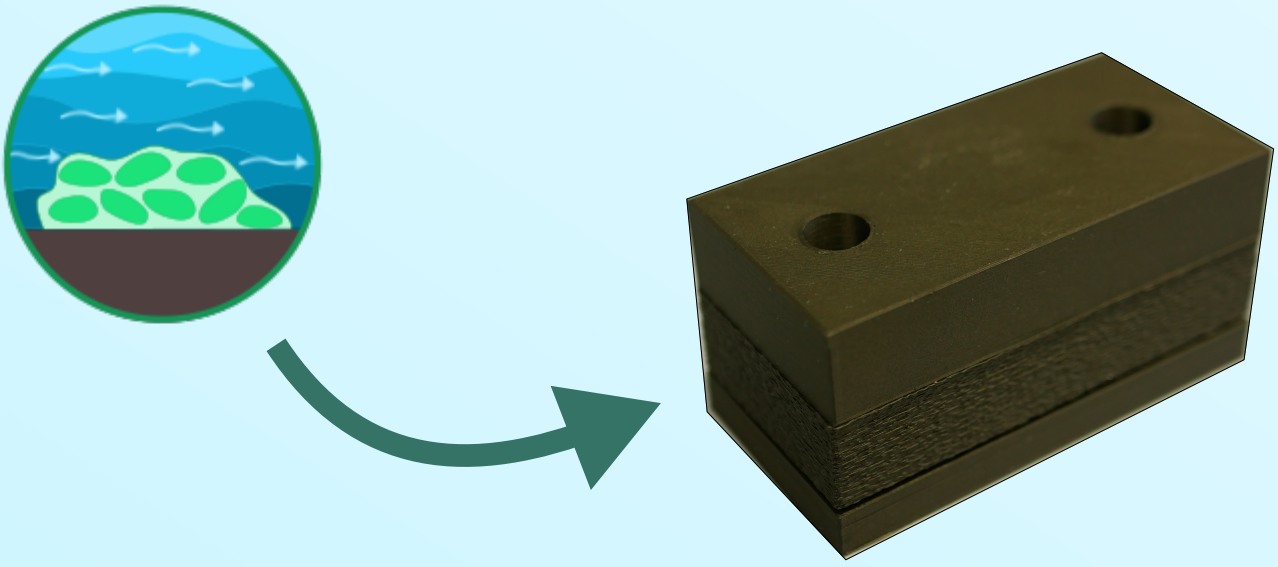
B-TOX

reduction of wastewater toxicity using a *B. subtilis* biofilm

Manual:
How to build and use our flow chamber



☑ Material:

Material for performing flow chamber test:

- ☐ Template for bottom part
- ☐ Template for middle part
- ☐ Template for top part
- ☐ (Template of Platelet if you want grow a biofilm)
- ☐ Polylactic acid (PLA) but we strongly discourage it´s use, for further information see recommendations below
- ☐ 3D-printer
- ☐ Sample
- ☐ 2 short hoses
- ☐ Peristaltic pump
- ☐ Vessel filled with phosphate-buffered saline
- ☐ Screw clamp
- ☐ Parafilm
- ☐ Scalpel

Material for our assay:

- ☐ Microtiter plate
- ☐ Lysogeny broth (LB) media
- ☐ Plate reader

Step 1.
Printing the flow chamber

1. You can download the flow chamber by scanning the QR-code or clicking here:
https://2020.igem.org/wiki/images/5/51/T--TU_Darmstadt--templates_flow_chamber.zip
They are saved as .stl-files which can be directly used for 3D-printing.

2. Before printing the templates, a few parameters have to be adjusted for successful printing. They depend on the printer type but also on the surrounding temperature and the used filament. Ideally the chosen filament is autoclavable. Parameters for our printer (Creality Cr10V2 3D printer) were set as described in the Gcode-files which can also be found in the zip file. Try out printing with small pieces first if you are unsure about setting parameters. If all the parameters are set correctly, printing of the pieces can be started. We recommend watching the printing process until you can see that the first layers are printed successfully. This prevents the loss of time and resources in case the settings were incorrect.

3. 3D-printing takes time. It varies with every printer type. Nonetheless, it can take up to several hours (from 2 up to 8 hours) of printing until a piece of the flow chamber is printed. Enough time has to be considered between printing and using the parts in the lab. This will prevent you from starting your experiment with missing a piece.

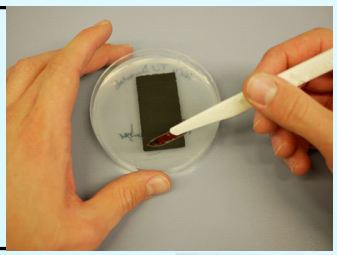
Step 2.
Application in the laboratory

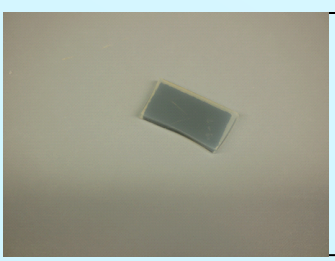
Ensure that the flow chamber is leakproof before running any experiments. See our recommendations at the end of the document. There we give tips on what can be changed compared to our setup in order to better seal the flow chamber.

0. Sterilize flow chamber by leaving it for at least 2 days in disinfectants. If you use PLA ethanol and isopropanol will be fine. If you use other filaments please check if they are chemically stable in the used disinfectants. You can also check if it is autoclavable which can then be an alternative.

1. Biofilms can be either grown within the bottom part of the flow chamber or in a petri dish. We recommend the latter since it is much easier to handle.

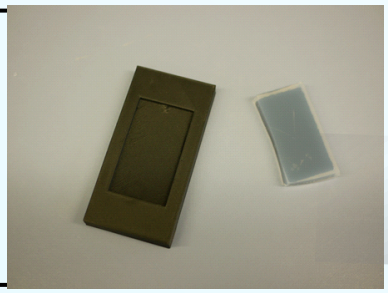
2. Subsequently for incubation a piece of the size of a platelet gets cut out.

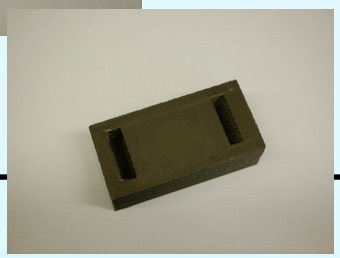




3. Platelet with your sample can be easliy transferred into the bottom piece of the flow chamber.

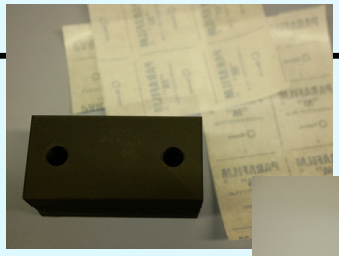
4. The bottom part of the flow chamber needs to be put down with the notch facing up. Insert the sample either directly or on the platelet. The biofilm should face up.

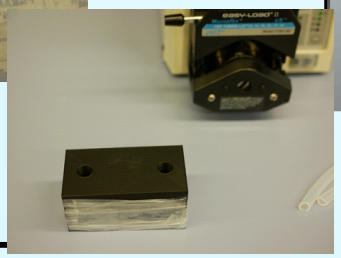




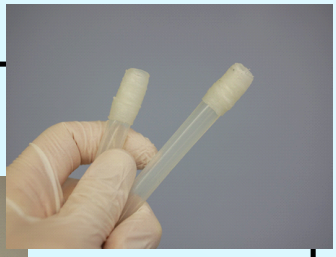
5. Then the middle part is put on top of the bottom piece. The lowering allows the liquid to flow over the biofilm in a thin layer. The side with two holes for the hoses should face up.

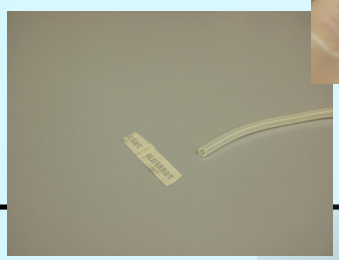
6. The upper part is put on top of the other two parts and closes the flow chamber. Seal flow chamber on the sides with parafilm to prevent leakage.



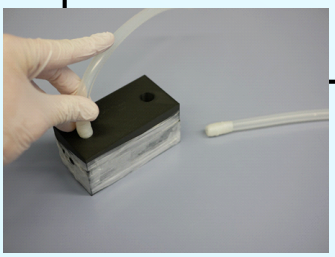


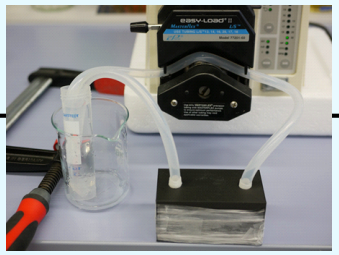
7. Lastly, the ends of the water hoses are taped with parafilm to make the cycle leakproof.





8. Subsequently, they are inserted into the flow chamber by pushing them into the holes. The other end of each hose is put in a vessel with the chosen liquid (e.g. PBS). One hose is attached to the peristaltic pump. It is simply inserted in the pump head.



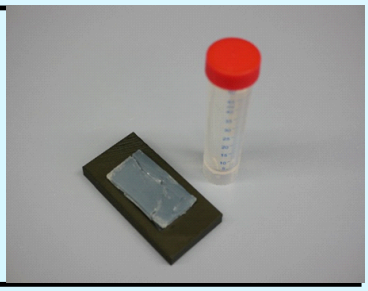


9. Using a screw clamp improves the waterproofness. Close the pump head by switching the smaller lever and make sure the vessel is filles with enough liquid.

10. Turn the peristaltic pump on and adjust the desired flow rate.

11. After pressing "start" the experiment has begun. The liquid is now pumped through the chamber.

12. After performing the experiment, collect all the liquid in the vessel and disassemble the flow chamber. Wash and sterilize the parts before next usage.

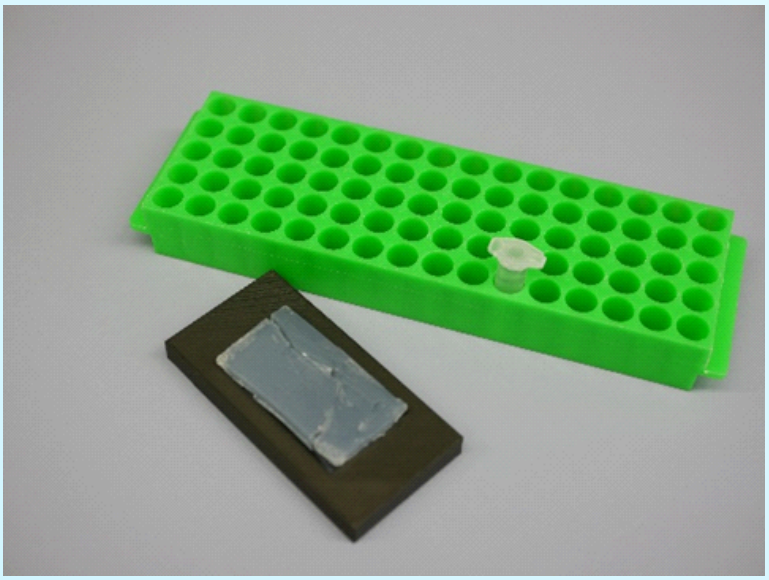


Step 3.
Analysis based on concentration of living cells

We had a short time frame of two weeks to establish biofilm growth and the assay. Unfortunately, we were not able to grow a *B. subtilis* biofilm following a method based on MSgg media[1]. For illustration of the assay setup, we used red dye to prepare serial dilutions in microtiter plates.

1. a) Centrifuge the flow sample at 6000 xg for at least 6 min and resuspend the cells in 2 mL LB media. This is the first sample which corresponds to the number of cells that have been washed off.

b) Additonally, mutiple areas of defined size are scrapped off the biofilm and each sample is resuspended seperately in 2 mL LB media. These second samples are the remaining cells per mm^2 to which we can compare the washed-out amount of living cells. As controls are essential, conduct the assay without a biofilm.



2. Both samples are transferred into a microtiter plate and serial diluted with LB media. After a few hours of initial growth, a growth curve of the cells in the plate is recorded by measuring the optical density at 600 nm (OD_{600}) of each well. We also thought about combining this assay with the Sensor Brick system from last years' TU Darmstadt iGEM Team.

Comparing optical densities of the serial diluted samples of the biofilm and the flow samples, estimation of the ratio of cells that have been washed off compared to all cells in the biofilm is possible. The dilution series of red dye on microtiter plates below represents samples after a few hours of incubation at perfect conditions. Blue rectangle marks the resuspended cells of the biofilm in 2 mL LB media. Each dilution step in our example was performed 1:1.5 for the sake of a visible serial over multiple steps. Assuming that the Xth (*e.g.* 7th) dilution of the biofilm sample corresponds to the optical density of the flow sample, we can calculate the ratio of cells washed out by using the following formulas.

Formula 1: $x = \frac{1}{dilution\ factor^{number\ \ of\ dilutions}}$

Formula 2: $Ratio\ \frac{Flow}{Biofilm} = \frac{x}{1+x}$

In case of our example with a dilution factor of 1.5 and 7 dilution steps, 5.53 % of all living cells were washed out. The corresponding dilutions are outlined with a yellow rectangle

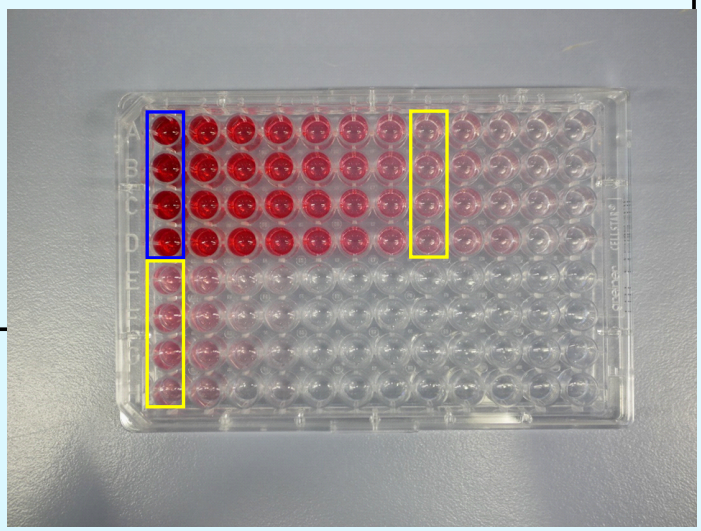


Figure 1. Exemplary layout of the microtiter plate assay for estimating the amount of washed-out living cells using serial dilution of red dye instead of Bacillus subtilis cultures. In practice, rows A-D would contain samples of the resuspended biofilm in LB media. Rows E-H contain flow samples. Serial dilutions in LB media are horizontally prepared from the samples in column 1. The blue rectangle outlines undiluted cells of a certain area of the biofilm. Yellow rectangles show samples of equal optical intensity and thus roughly similar numbers of cells. Measurements can be conducted in common plate readers with absorbance function.

Our recommendations for further development

We were only able to use PLA filaments for printing of our flow chamber. However, this polyester is not waterproof which is why we had to wrap the whole flow chamber in parafilm. We highly recommend to seek for an alternative as raw material for your own prints. Additionally, we recommend designing threads into the holes of the upper flow chamber part leading to less leakage by using hose connectors. Sealing of the parts on top of each other could be improved using a plug-in principle to assemble the parts.

Lastly, we recommend designing a lid for the bottom part if you want to grow a biofilm directly in the notch. Otherwise, the agar or your liquid culture might dry out.

References

[1] Kerstens et al., A flow cytometric approach to quantify biofilms, Folia Microbiologica, 2015, DOI 10.1007/s12223-015-0400-4