METHODOLOGIES FOR EVALUATING BIOCLOGGING EFFECT IN POROUS MEDIA

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Abstract

This study aims to establish two new approaches for visualization and quantification of biofilm growth in porous media. Biofilm growth, stated as bioclogging, is one of the components of the clogging effect on subsurface-flow treatment wetlands and one of the causes of their significant life time reduction. On the other hand, biofilm growth is also responsible for soil improvement when using ureolytic bacteria which, in presence of a calcium source (feeding solution), precipitate calcium carbonate (biocement) due the hydrolysis of urea biologically catalyzed. The biocement clogs the soil pores, bonding the soil grains and leading to an increase of overall strength and stiffness and a reduction of medium permeability. So far, the measurement and prediction of the clogging effect on porous media, and specially the bioclogging effect, are not well-known. The methods presented here, which one of them takes advantage of a microfluidic device, allowed computing average biofilm growth rates and intend to be a helpful tool for calibration of numerical prediction models.

Key-words: Constructed wetlands, bioclogging, biocementation, microfluidics, biofilm growth, visualization tool.

1. General introduction

This paper is divided in two main parts. The first presents a methodology for evaluation of the bioclogging effect mainly focused on treatment wetlands. The second presents a different methodology for evaluation of the bioclogging effect regardind the biocementation of soils and the ground improvement by MICP (Microbially Induced Calcite Precipitation) technique. Finally, the paper ends with a brief general conclusion about the two methodologies presented. The experimental work was developed at IST, in the laboratory of environment and Geotechnics (Decivil) and Bioengineering (DBE), and also at INESC-MN.

2. A methodology for evaluation of the bioclogging effect in porous media

2.1 Introduction

Clogging phenomenon is one of the main problems affecting the life time of constructed wetlands (CW). It consists in the generalized occupation of the medium pores by sediments, chemical precipitates and biofilms, obstructing the normal wastewater subsurface flow and leading to shorter retention times and potential contaminant overflows. Bioclogging, in particular, is the clogging effect induced by the presence of bacteria and their extra polymeric substances, both attached to the grains of the porous medium. According to Nivala et al. (2012), predicting and modelling the clogging effects is an obstacle that has not yet been properly solved.

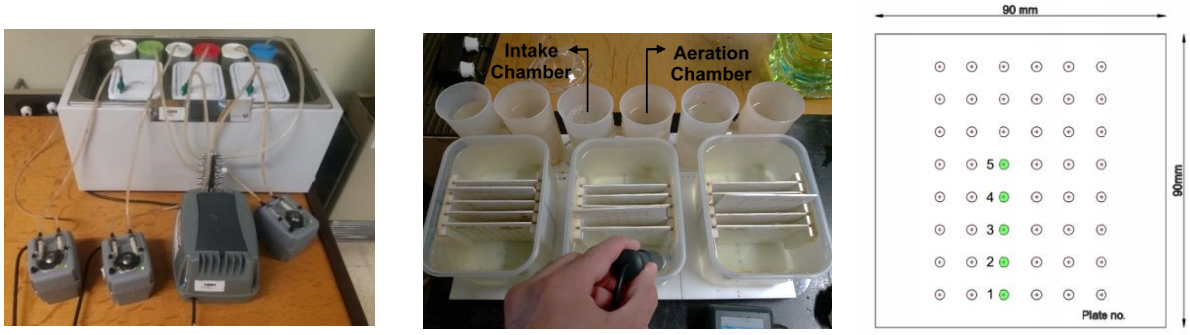
Samsó et al. (2016); Samsó and Garcia (2013) refer the application of an average biofilm thickness on their models although without experimental validation. Recently, Martinez-Carvajal et al. (2018) proposed a methodology for pore analysis within the filling media of constructed wetlands using X-ray tomography.

To the author's acknowledge, there are no other studies pretending to measure biofilm growth inside the filling media pores of constructed wetlands. The method presented here intends to be a helpful tool for the calibration of numerical prediction models. It consists in defining a visualization tool for biofilm growth inside the pores of the filling media. This tool is tested on the analysis of laboratorial data, also presented, where bioclogging was monitored for approximately 3 months. Available data allowed the calculation of average biofilm growth rates.

2.2 Material and Methods

2.2.1 Description of experimental installation

The experimental installation (Figure 1) consists in three independent reactors partially submerged on a warm bath equipment to prevent temperatures to decrease below 20ºC. Each reactor (Figure 2) is composed by a box where 5 PVC plates are vertically disposed, perpendicularly to the recirculating flow direction. Each box is coupled to an aeration chamber where a compressor is permanently injecting air to oxygenate the recirculating solution. A second chamber acts as the intake of the pumping system, composed by a peristaltic pump which maintains a constant recirculating flow from the intake chamber to the entrance of the PVC plates' box, closing the recirculating circuit (figure 1 a) and b)). Each PVC plate has 48 drilled holes with the same diameter (1, 2 or 3 mm) (figure 1 c)). The holes simulate the pores of a filling medium. Each reactor has 5 plates, all with the same diameter. The experimental installation was placed in a room with low light conditions to prevent the proliferation of algae within the system. The holes monitored in each plate are identified in Figure 1c).



a) b) c)

Figure 1 - a) Global view of the experimental installation, b) inside view of the reactors and c) PVC plate.

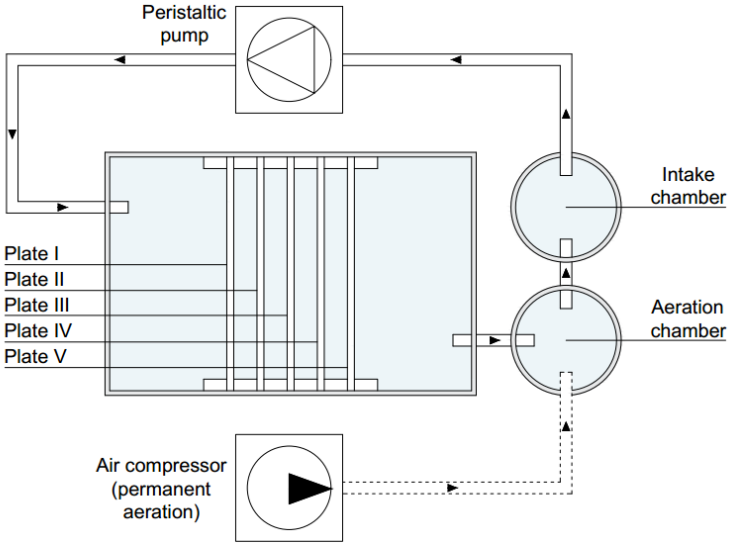


Figure 2 – Operating scheme of one of the reactors

2.2.2 Inoculation and system operation

The system started operating on 8th May 2018. Initially, the recirculating solutions were renewed once per week. During the first two weeks of operation the system was inoculated with a solution composed by 50% in volume of detached biofilm solution from an experimental constructed wetland bed and 50% synthetic sewage solution. Synthetic sewage solution was composed by sodium acetate trihydrate dissolved in distilled water and liquid plant fertilizer NPK 6-3-6. The mass of sodium acetate trihydrate was defined to obtain a chemical oxygen demand (COD) of 800 mg/l. After the first two weeks of inoculation, ending at 25th May 2018, the recirculating solution started to be weekly renewed only with 100% synthetic sewage solution. On 18th June 2018, a respirometry analysis indicated that substrate was being consumed in less than two days which could be limiting biofilm growth and the renewal of the recirculating solution was increased to three times per week, on Mondays, Wednesdays and Fridays.

2.2.3 Biofilm growth data acquisition

All the PVC plates were periodically observed by a NIKKON stereoscope with a 10 Mp MOTIC incorporated digital camera. In each PVC plate photographs were taken to five defined holes. The photographs are automatically scaled by the stereoscope software.

2.2.4 Data processing

The scaled photographs, where is visible the biofilm growth inside the holes, were analysed using MATLAB and its image processing toolbox. Biofilm growth inside the holes was calculated using an algorithm to count the evolution of pure black RGB pixels (R=0; G=0; B=0) in images taken at different times. The algorithm converts the pixels count to a mm^2 area. A sample of 75 different holes, totalizing 1320 photographs, were processed with this algorithm.

The area of the hole that is not occupied by biofilm or any other substance is pure RGB black. Once a thin layer of biofilm grows, the RGB code of those pixels will be no longer pure black. For each picture the pure black area was determined and used to calculate a corresponding equivalent diameter, which is the diameter of a circle with the same area. The difference of the equivalent diameter of pure black areas of two different pictures from a specific hole taken at different times was used to determine an average biofilm thickness (Eq. 1). The biofilm thickness growth rate can then be calculated through Eq. 2.

$$K = (D_t - D_{t-\Delta t})/2 \quad \text{Eq.1}$$

$$k = \frac{K}{\Delta t} \quad \text{Eq.2}$$

Where:

- D_t is the equivalent diameter of the pure RGB black area of a hole in time t [mm];
- K is the average biofilm thickness [mm];
- k is the average biofilm thickness increase rate [mm/day];
- Δt is the time interval between the two pictures [days];

2.3 Results and Conclusions

An example of the biofilm growth inside a 2 mm hole is presented in Figure 3, showing that there was clearly biofilm growth inside the holes during the experiment. However, a substantial biofilm evolution was only observed after the renewal of the recirculating synthetic sewage solution was increased to three times per week.

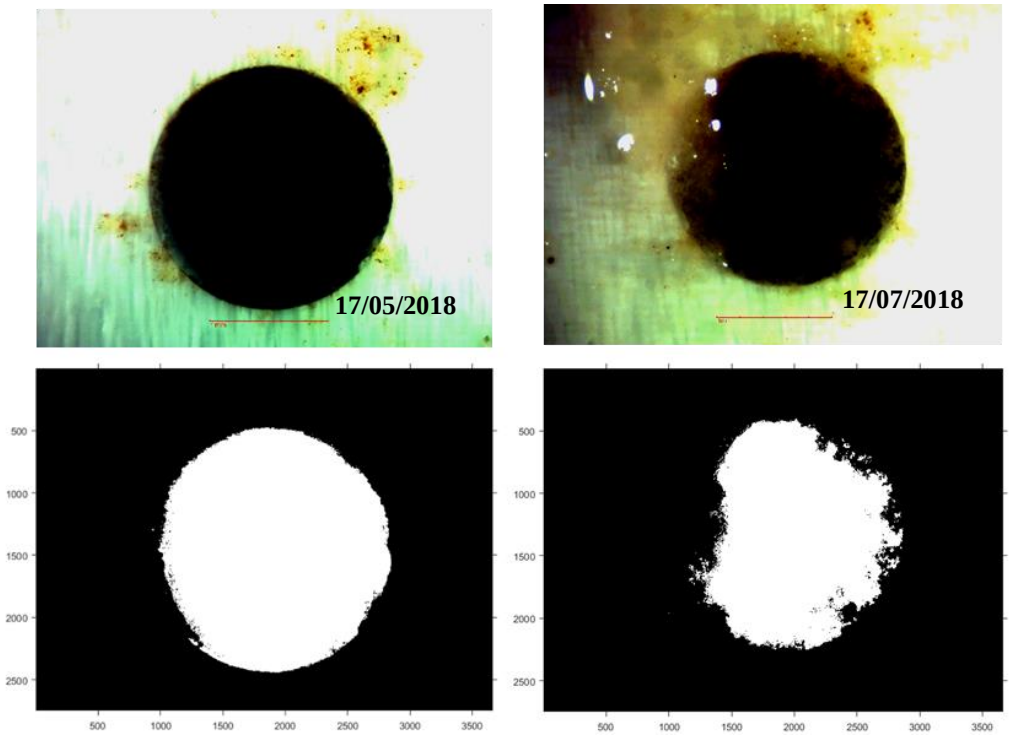


Figure 3 – Evolution of the biofilm growth inside a 2 mm hole (top) and the corresponding MATLAB negative images (bottom)

Results of average biofilm thickness and biofilm thickness increase rates obtained in a sample of 75 holes are shown in figures 4 and 5. It can be observed that biofilm thickness depends on hole diameter. Higher diameters have higher biofilm thickness and, as consequence, higher biofilm growth rates. This fact is against the assumptions made by Samsó and Garcia (2016). In their prevision model, they have considered that biofilm thickness grows independently of the pore diameter.

In the second footage campaign there are some negative values present. This interference is because, in the first observation campaign, PVC plates were photographed in dry and clean conditions, making the lateral limits of the holes visible and reducing the count of pure black RGB pixels. In the second observation, plates have been inoculated and these limits were no longer visible, increasing the pure RGB black area and resulting in a negative evolution of biofilm thickness.

The method developed proved to be adequate to observe and measure biofilm growth. Future studies with different operation conditions will allow to better understand the dynamics of biofilm, as well as determine increase rates that can further be used in modelling studies.

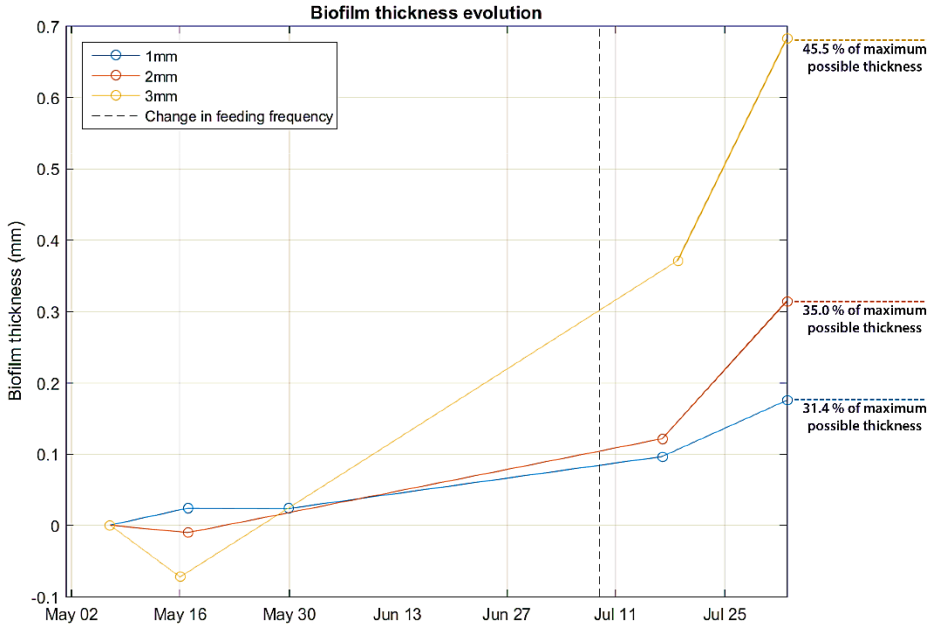


Figure 4 – Biofilm thickness evolution

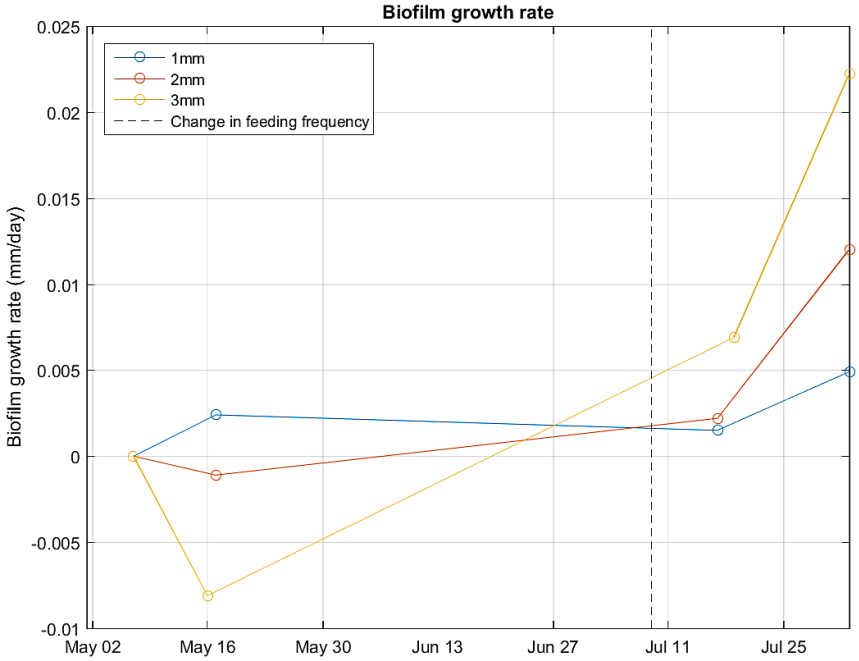


Figure 5 – Biofilm growth rate

3. Preliminary tests on a microfluidic device

3.1 Introduction

Soil improvement using ureolytic bacteria or other biological agents (Ivanov and Chu 2008; Mitchell and Santamarina 2005; Whiffin 2004) is a promising technique currently under investigation. It is based on the fact that calcium carbonate (biocement) is precipitated due to the hydrolysis of urea in the presence of a calcium source (feeding solution). The biocement clogs the soil pores, bonds the soil grains, increases soil overall

strength and stiffness and reduces the medium permeability (Gomez et al. 2015; Ng et al. 2012; Van Paassen et al. 2010; 2009). The study presented here is focused mainly on pore clogging effects, which is important because: (i) the treatment requires accessible pores; (ii) enough quantity of feeding solution (calcium source) must be provided to precipitate the intended quantity of biocement; (iii) fluid must circulate for treatment progress (add more biological agent and/or feeding solution); (iv) fluid velocity must be small enough to avoid washing away the precipitate and biological agents; (v) pore geometry depends on grading size distribution and void ratio; (vi) permeability depends on pore geometry and reduces with the progression of the treatment; and (vii) bonds geometry, and consequently the soil improvement achieved, depends on the amount of biocement and pore geometry.

In this study, the dosage definition of enzyme and feeding solution was done in small test tubes to find the maximum amount of precipitate found when changing the concentrations of both. Based on it, selected concentrations of enzyme and feeding solution were tested in a microfluidic device conceived to mimic a two-dimensional uniform porous size media. To the author's acknowledge, this is the first study taking advantage of microfluidic devices to study biocementation using this technique.

3.2 Materials and Methods

3.2.1 Description of the microfluidic device

The microfluidic device is composed by a top acrylic plate of 2 mm thickness and a bottom acrylic plate with 3 mm thickness. Figure 6 presents the two plates and their respective elements and dimensions. In the bottom plate, a channel with 1 mm height (where the flow occurs) and several obstacles in form of columns with 1 mm diameter were printed using micromilling techniques (Silvério and Freitas 2018). In the top plate, two inlets/outlets with 1 mm diameter were drilled. This plate has also a square fit where a high-quality glass is inserted for better visualization of the obstacles below. Both plates are joined together with 4 M3 screws and a uniform layer of silicone on the areas where there are no printed elements. The device is totally symmetrical, enabling flow direction to occur both ways. A picture of the final device presented on figure 7. The total volume of solution that the device can accommodate is inferior to 0,5 mL.

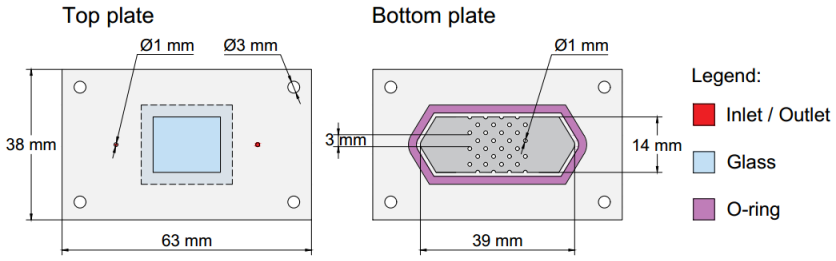


Figure 6 – 2D scheme of the microfluidic device

Figure 7 – Finished microfluidic device

3.2.2 Tests to define enzyme and feeding solutions dosages

Two laboratory tests were conducted to: (i) find the optimal concentration of urease enzyme and (ii) study the behavior of a constant urease solution in presence of different feeding dosages.

The powder of urease enzyme jack bean was dissolved in PBS (phosphate buffered saline). The feeding solution used in the tests was the same of previous studies on biocementation. Their components were defined by Pedreira (2014).

Both analysis consisted in measure the dry mass after a period of 24h, after letting the chemical reactions to occur in a 30ºC laboratory stove. The solutions were prepared in microcentrifuge tubes (eppendorf type). Each microcentrifuge tube was filled with a 1 mL volume of the different solutions. At the end of the 24h reaction period, the tubes were centrifugated to accumulate the precipitates at their bottom and to eliminate most of the liquid phase. After this procedure, the tubes were left 24h in a 60ºC laboratory stove to completely dry the remaining precipitated mass.

It was found out that urease concentrations superior to 15 mg/mL do not increase significantly the precipitated mass. The behavior of the measured dry mass is very similar to the stoichiometric production of calcium carbonate, indicating that it should be the major component of the precipitates. As shown in figure 8, it can be observed that higher feeding dosages resulted in higher amount of precipitates. However, it can also be seen that the tendency of higher feeding dosages is to decrease the precipitated mass when compared to the stoichiometric production of calcium carbonate. That may indicate an inhibition mechanism of urease enzyme.

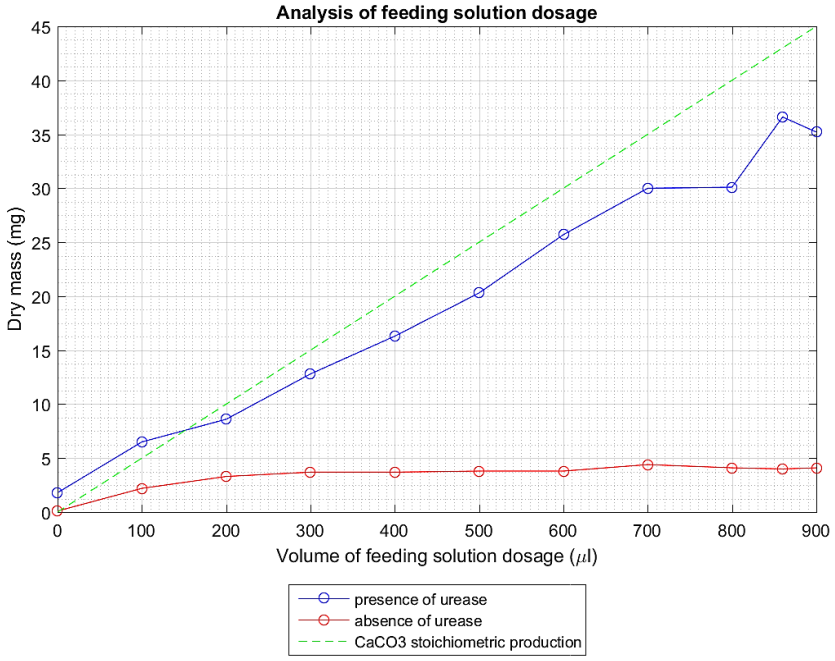


Figure 8 – Analysis of feeding solution dosage fixing urease concentrations to 15 mg/mL

3.2.3 Experiments on the microfluidic device

Five solutions were injected on the microfluidic device (Table 1). The objective was to reproduce the behavior of laboratorial results previously presented. The injection of the different solutions in the microfluidic device was carefully done using a 5 mL syringe. A special equipment visible in Figure 9 was used to fix both microfluidic device and a digital camera, making possible the observation of the device from a stationary position. During the injection and a reaction period of 24h, several photographs were periodically taken with a counter-light effect produced by a LED plate.

Table 1 – Solutions injected in the microfluidic device

Urease concentration (mg/mL)	**% (in volume) of feeding solution**
15	0
15	10
15	50
15	86
0	100

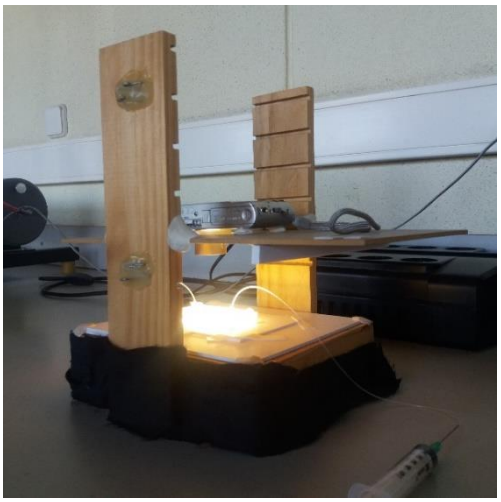


Figure 9 – Microfluidic device during an experiment

3.2.4 Data processing

The photographs taken in the different experiments were analyzed with the MATLAB image processing toolbox. It was built a program to count and identify the pixels between a range of blue RGB values (Table 2). The channel blue was identified as the most contrasting color channel between the precipitates color and the background. The range of blue values were determined by visual inspection of the RGB code of sediments in original photographs.

Table 2 – Blue RGB values range used to process the different photographs

Experiment	**Blue RGB values range**
15 mg/mL urease	0-125
15 mg/mL urease + 10% feeding	100-135
15 mg/mL urease + 50% feeding	0-130
15 mg/mL urease + 86% feeding	0-130
100% feeding	0-115

3.3 Results and Conclusions

The original photographs of the 15 mg/mL + 86% feeding solution test and the respective processed images are sequentially presented in Figure 10. Figure 11 presents the pixels count results of the different experiments conducted on the microfluidic device. As it can be seen, only the test on 10% feeding solution and 15 mg/ mL urease concentration is divergent from the laboratorial results.

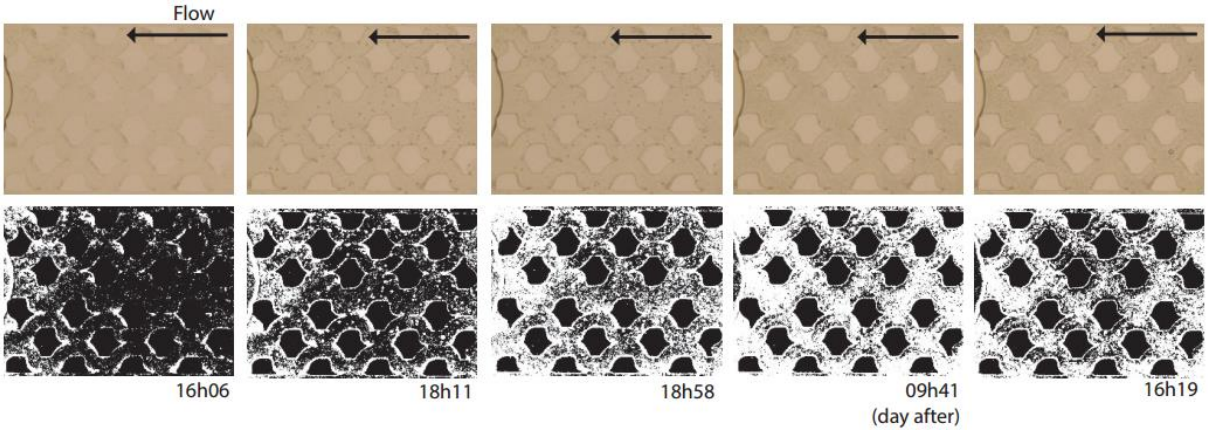


Figure 10 – Time lapse of unprocessed and respective processed images from 15 mg/mL urease + 86% feeding solution experiment

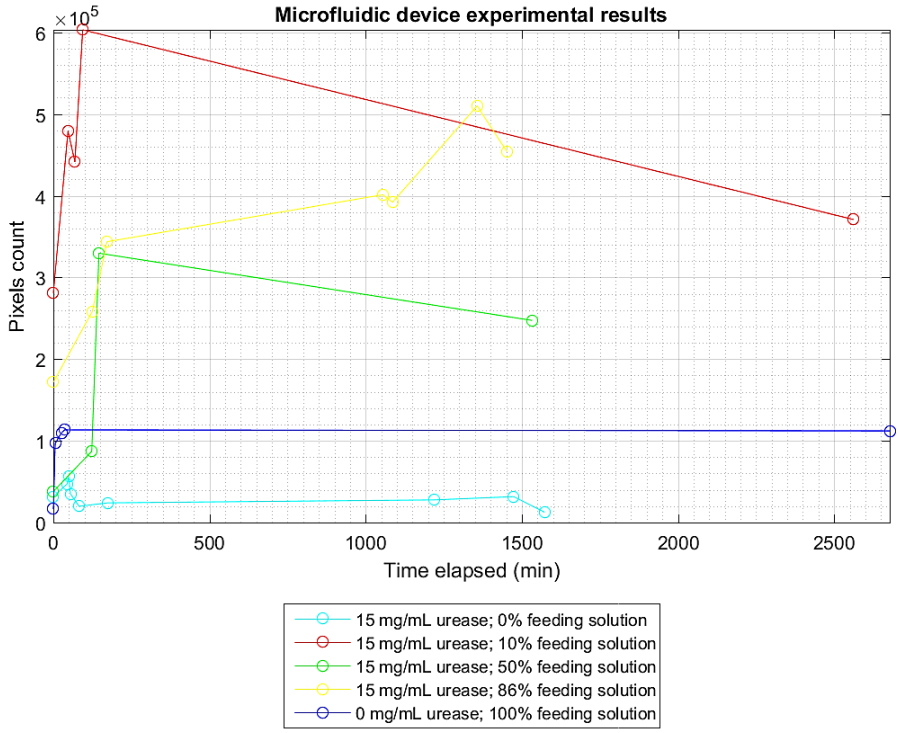


Figure 11 – Microfluidic device experimental results.

Qualitatively, the amount of precipitate was proportional to that of the concentrations used. The location of the precipitate was clearly related with the direction of fluid flow during inoculation. These preliminary results highlight the fact that the use of alternative testing devices such as the one developed may be a useful tool for studying clogging phenomena occurring during this treatment.

4. General conclusions

Both methodologies proved to be a useful tool for visualizing clogging effect in porous media. The methodology presented on section 2 has potential to be employed at large-scale in monitorization of constructed wetlands. For now, several tests changing the feeding and flow conditions may be conducted to increase the understanding of biofilm formation. The methodology presented in section 3 may immediately evolve to tests with bacteria in vivo. The microfluidic device enables the control of flow and environment conditions. The footage equipment also enables the real time monitoring of the microbial biofilm formation and calcium carbonate precipitation.

Although the two methodologies were developed for different proposes, they can be applied to both realities: the microfluidic device is a useful tool to study biofilm formation on wastewater environments and the PVC plates have the potential be used, with some limitations, to monitor the soil biocementation.

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