MONITORING BIOFILM PARAMETERS ON MBBR TECHNOLOGIES WITH LIGHT MICROSCOPE AND IMAGE PROCESSING

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***Abstract**: In the modern world, energetically optimized, decentralized, and environmentally friendly solutions are needed more and more. The trend of urbanization leads to ever growing suburban towns and stagnating or decreasing cities, due to the spreading practice of home office and the need of home-grown foods. These areas around big cities present great opportunities for industrial companies and storages to settle, as more space costs less money, however well-educated workforce is available. This means that the small wastewater treatment plants of suburban towns can't handle the increasing amount of wastewater in terms of hydraulic capacity and organic matter. In the R&D project MICROBI we developed a special MBBR material, which has relatively high surface area compared to other materials. In MBBR technologies it is hard to monitor the growth of the biofilm in terms of mass, surface area used by bacteria, and thickness of biofilm, which are important parameters that determine the efficiency and capacity of the carrier material. To measure these parameters TTC paint was applied to the biofilm samples, then I took light microscope pictures, calibrated the photos' pixels to mm, and with Image Pro processing program I separated the biofilm from unsettled material parts. With this new combination of methods, I can improve the monitoring of these systems and provide useful data for the challenges of modelling MBBR systems. With this data it is possible to determine more accurate kinetics for this special biofilm material, which makes this new technology even more useful, and applicable in the wastewater cleaning process. In the future this knowledge can be a part of a small (50-100 m3/day), autonomous wastewater cleaning technology that can provide smart, local solution for biological wastewater treatment.*

***Keywords**: Biotechnology, MBBR, Wastewater, biofilm monitoring,*

INTRODUCTION

In MBBR systems it is difficult to measure the biofilm thickness, which is essential to understand better the kinetics of this water cleaning method. Traditional carrier size ranges from 2,2 mm to 50 mm in length and 9-64 mm in width [Aygun and Berktay (2008), Das and Naga (2011), Kermani et al. (2008), FLOCOR (2013), BIOSPHERE-BR (2013), Fxsino (2013), Barwal and Chaudhary (2014)]. They are usually made of HDPE, PE, and PP [Barwal and Chaudhary, 2014]. Our special carrier differs in size and material from these, so it is crucial to determine its exact size range, and the ability to carry biofilm microorganisms on its surface. The aim of this study is to determine the diameter, area utilization (settle rate) by microorganisms and the difference in growth rate in four types of new carriers.

To measure the biofilm thickness on carriers Hoang et al. (2014) used Variable pressure electron scanning microscope (VPSEM) without pre-treatment and Atlas image processing software (Tescan USA Inc.,

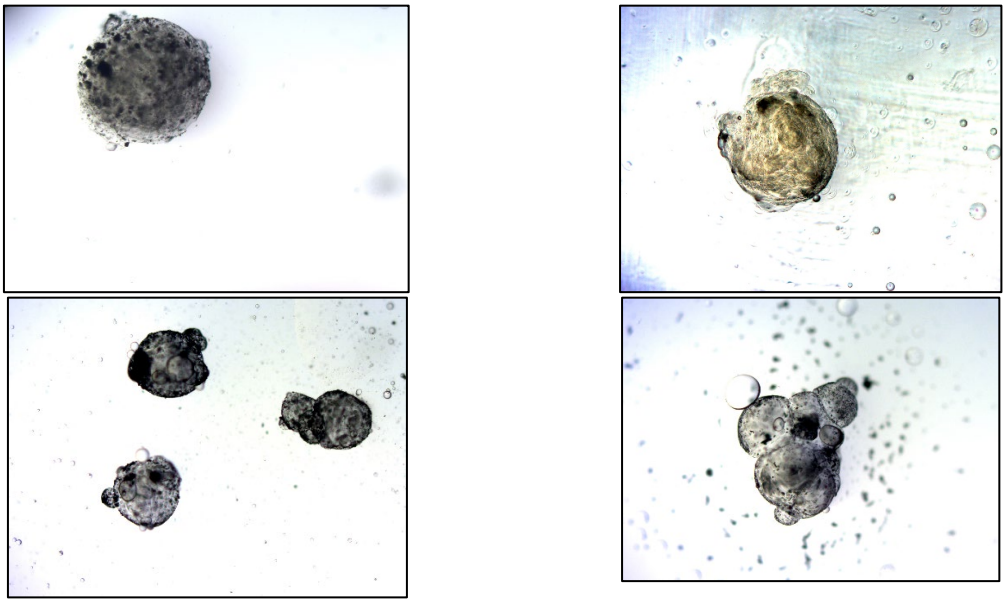
Cranberry, PA) to analyse the pictures. Arabgol et al. (2022) also used VPSEM but used Fiji (Schindelin et al., 2012) software, which is developed for biological microscope image processing. Bjornberg et al. (2009) also used microscopy to determine the biofilm thickness. In their study Spot Advanced software developed by Diagnostic Instruments Incorporated (Sterling Heights, MI) was used to take pictures, and ImageJ, a software program developed by the National Institute of Health (Bethesda, MD) used for processing the images and separating the biomass, background and carrier area.

Tetrazolium salts are widely used colouring materials in biochemical applications for more than fifty years now. One of its compounds, the trifenil tetrazolium chlorid has been first synthetised more than a century ago (Berridge et al., 2005). Szilágyi (2008) used TTC paint to colour the mitochondrial dehydrogenase enzymes in rat brain samples and used UTHSCA ImageTool software to analyze the parts of the brain cells. Hegyi (2014) also showed that TTC paint can detect living microorganisms and their biological activity.

Studies by Barwal (2014) showed that in MBBR systems only around 70 percent of the carriers' surface area is utilized by microorganisms, due to the geometry of the carriers. The studies also showed that a biofilm thickness less than 100 µm is required for full substrate penetration through the biofilm. Torresi (2016) suggests that a biofilm thickness over 200 µm does not significantly increase the functionality of nitrification activity. In studies of Bjornberg et al. (2009) there is a carrier surface area utilization rate on the outer parts of 30 to 50 percent, and a 100 percent in the inner areas.

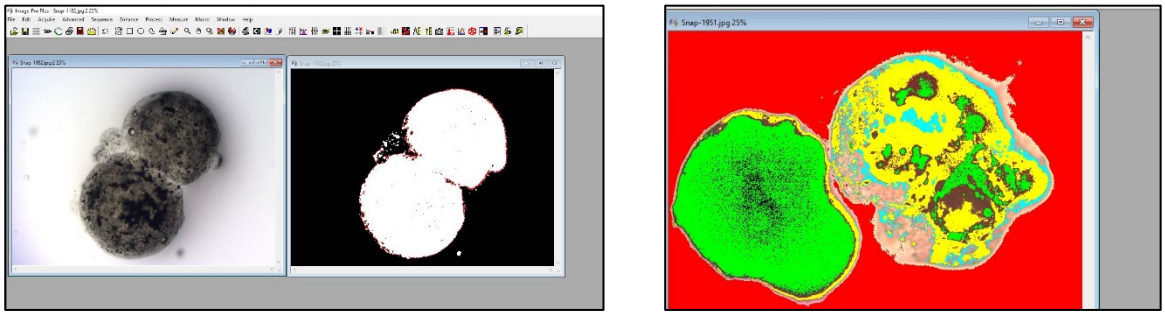
MATERIAL AND METHOD

The experiments have taken place at laboratory scale on four identical SBR (sequence batch reactor) glass reactors between 27.05.2023. and 14.07.2023. Each of the reactors contained 10 percent of the total volume carriers such as the original carrier, carrier with activated carbon adsorbent and two new carriers which were produced by cold polymerization with different types of oils (***Picture 1***). The two new types have iron adsorbent inside them.



Picture 1. – Different types of carriers used in experiment, Top left has added activated carbon, top right is the original, bottom left is made with rapeseed oil and bottom right is made with sunflower oil

The four reactors were fed with treated wastewater, to which we added substrates such as KH_2PO_4, NH_4Cl, Na-acetate, $NaHCO_3$, and calcium carbonate for puffer capacity. I set up the influent water to be optimal for autotrophic organisms so the experiments would last until nitrate appeared in the effluent. The cycle of the reactors was 6 hours of aerated cycle, 10 minutes of deposition, two minutes of water outtake by peristaltic pumps and two minutes of water intake also by peristaltic pumps. I took water samples directly from the effluent three times a week and took one sample from each barrel of influent water. Microscopic analyses were taken simultaneously with water chemical measurements. In the water phase I measured the following parameters: Nitrate-nitrogen, Nitrite-nitrogen, Ammonium-nitrogen, Phosphate-phosphorous, and Chemical oxygen demand. I also measured in the reactors the following parameters: pH values, Oxygen demand and temperature values. From the biomass I sampled the fully aerated reactors and measured the organic, inorganic and dry matter. I took samples from the original, undried sample and TTC painted it for light microscope measurements.
In measuring chemical parameters, I used the following standards: MSZ ISO 6060 (COD), MSZ 1484/13-09 (nitrate-nitrogen nitrite-nitrogen), MSZ ISO 7150-1:1992 (Ammonium-nitrogen), MSZ 448/18-77. For the local parameters (pH, Oxygen demand and temperature) I used Hach HQ40 portable multimeter.
For the microscope measurement I used a Zeiss Lab A1 light microscope with 5X zoom and dark contrast. For microscope camera I used Zeiss AxioCam ERc 5s. For the pictures I used Zen Blue (3.1 version) software. I took 50 pictures of each sample, at each sampling time during the experiment period. Pictures were taken to Image Pro software where I first separated the whole carrier from the background and measured their diameter (mean., max., min.), and the polygons area. The next step is to separate the TTC coloured biomass area from the background and the unsettled parts of the carrier. Pictures taken through the process can be seen in ***Picture 2***.



Picture 2. – Separating background from carrier (left) and separating biomass from unsettled parts (right)

For the data analysis I used Microsoft Excels Pivot function to handle the amount of data I had. For statistical analysis I used Past program.

RESULTS AND DISCSSION

First, I analysed the influent's chemical and local parameters to determine if the difference between the reactor results is caused by external parameters. In ***Figure 1.*** I show the pH, OD, and temperature values for each reactor over the experiment period.

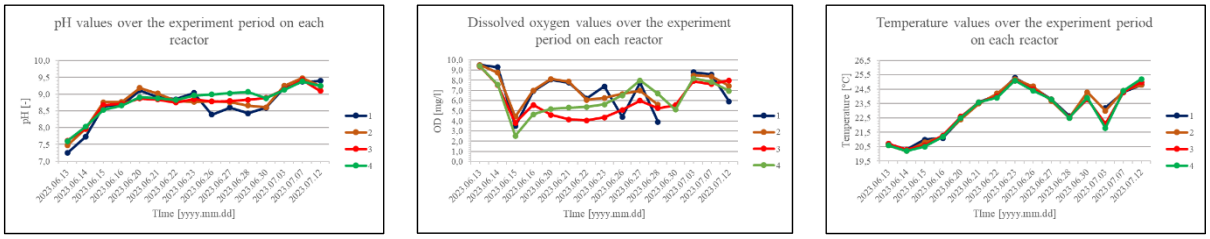


Figure 1. – Results of local parameters measured in the reactors (left pH values, middle: Oxygen demand values and right: temperature values)

As shown in ***Figure 1.*** no major difference occurred in the reactors' parameters. There was a slight difference in oxygen values, but almost all the time it was above 4,0 mg/l, so it was way above the WWTP average. In the third sample date it was around 2,0 mg/l due to a malfunction of aeration system, but I managed to repair it. You can see a slight increase in pH, that is due to the nitrification process. Temperature also increased similarly in every reactor, which was caused by the summer weather, that can affect even the air-conditioned laboratories.

As it is shown in ***Figure 2***., the concentrations were identical to each reactor. However, we started to increase the phosphate-phosphorous concentrations due to the phosphate absorbing abilities of new cold polymerized reactors. The nitrite-nitrogen and nitrate nitrogen amount in the influent were insignificant due to the lack of microbial activities.

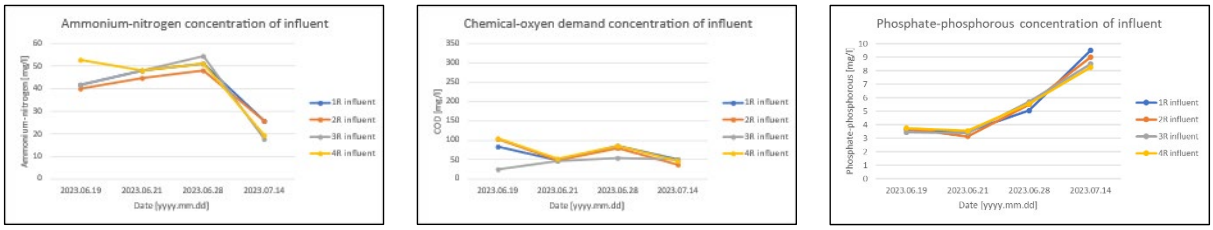


Figure 2. – Results of chemical parameter measurements (left: Ammonium-nitrogen, middle: Chemical oxygen demand and right: Phosphate phosphorous)

I measured the chemical parameters to check the effluents of the reactors, that shows how the biomass is growing in each reactor. The results are shown in ***Figure 3***. In ***Figure 3.*** the first diagram shows how the ammonium-nitrogen decreases in reactors, as the biomass increases, and the first step of nitrification starts in each reactor. The slowest nitrifying reactor is the fourth. In the second diagram we can observe that nitrate began to appear in each reactor, first with the original carriers, while the fourth reactor was the slowest in this regard too. COD values should not decrease significantly as the biofilm is autotrophic. In the second reactor I measured a constant decrease during the experiment, but COD values never measured under 40 mg/l. Autotrophic cultures may need some organic matter, that can cause the decrease of chemical oxygen demand. Phosphate-phosphorous values decreased to zero in third and fourth reactors. It's probably because of the phosphorous adsorbent ability of the new carriers, not the phosphate accumulation of microorganisms. As the experiment progressed, the phosphate begun to appear in those reactors, as the carriers begun to „fill" with phosphorous. These values led me to the conclusion that the third and fourth reactor had a phosphate limited culture, which explains the difference in microscope measurements. The organic matter is higher in the third and fourth reactor, due to the different, filamentous organisms.

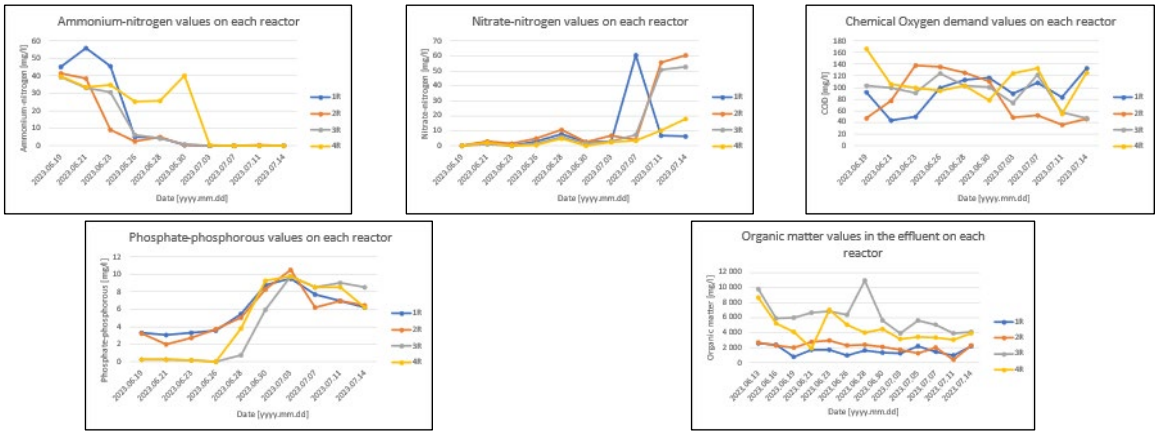


Figure 3. – Chemical parameters in effluent such as ammonium-nitrogen (top left), nitrate nitrogen (top middle), chemical oxygen demand (top right), phosphate-phosphorous (bottom left) and organic matter (bottom right)

After I examined the chemical and local parameters of each reactor, the next step was to deal with the microscopic data. For the third and fourth reactor I have 5 measured dates, and for the first two reactors I measured the carriers under microscope 12 times - control samples included. That means around 1700 pictures in total.

In ***Figure 3.***, the diagrams show the growth rate of each reactor during the experiment period. It is calculated by subtracting the carriers' unsettled areas from the total area, divided by the full surface area. It is calculated for each picture, each carrier, then the average of each day can be seen in the graphs. The ***Figure 4.*** shows when the average settled area (%) reached the maximum of its capacity.

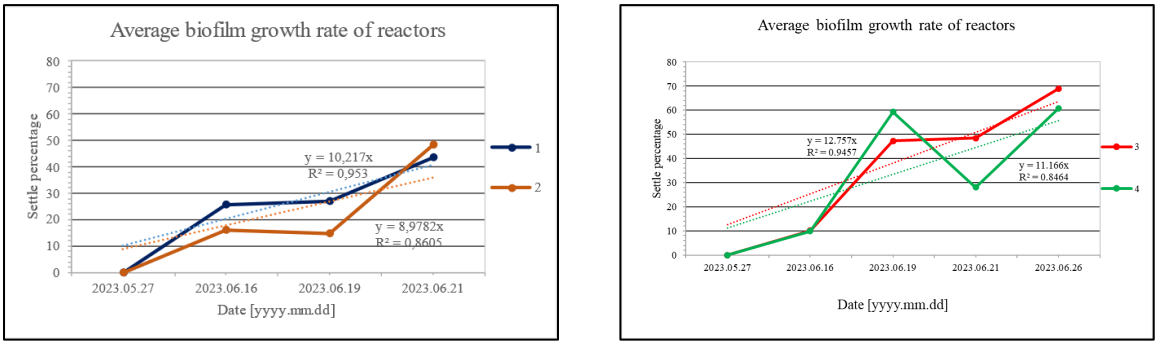
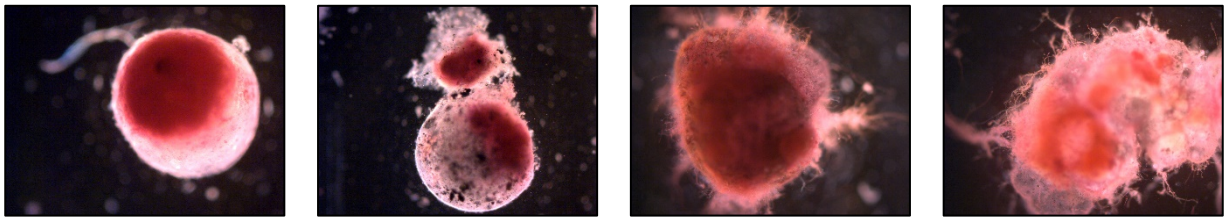


Figure 4. - Growth rate of each carrier types with autotrophic microorganisms

In the left you can observe the first and second reactors, where the carriers made by warm polymerization can be seen. The carrier without adsorbent reached 45,30% settle rate in the initial phase, while the second batch, that contained activated carbon adsorbent had 48,35% of all surfaces utilized. They reached this rate on the third sampling date (control time excluded), which is a faster rate than in the two other reactors, where phosphate was not available for the microorganisms. These reactors however reached a higher (69% in reactor 3, 60,82 in reactor 4) settle rate than the original carriers. This may be caused by the fact that on these carriers morphologically different organisms appeared. These values were smaller than the results of Barwal (2014), and in the range of Bjornberg et al. (2009)'s studies of the outer areas of carrier's surface, however literature data are about carriers marginally different in size and geometry.

In ***Picture 3.*** a picture of settled TCC coloured carriers in each of the four reactors is shown. The third and fourth reactor has filamentous organisms on the carriers' surface, which creates more surface area than on the two original carriers.



Picture 3. - The fully settled carriers of each reactor (first left reactor – 1, second left reactor - 2, first right reactor - 3, second right reactor - 4)

I measured the diameter (mean) values of each carrier at each measuring time and used Past software histograms to show how the carriers' diameter changes over time. Any change to the control samples can be via the growth of the biofilm, as it increases the diameter with its biofilm thickness. In the histogram analysis I used 6 bins each time (that means the number of columns) and set the axis to the same values. The first reactors data is shown in ***Figure 5***.

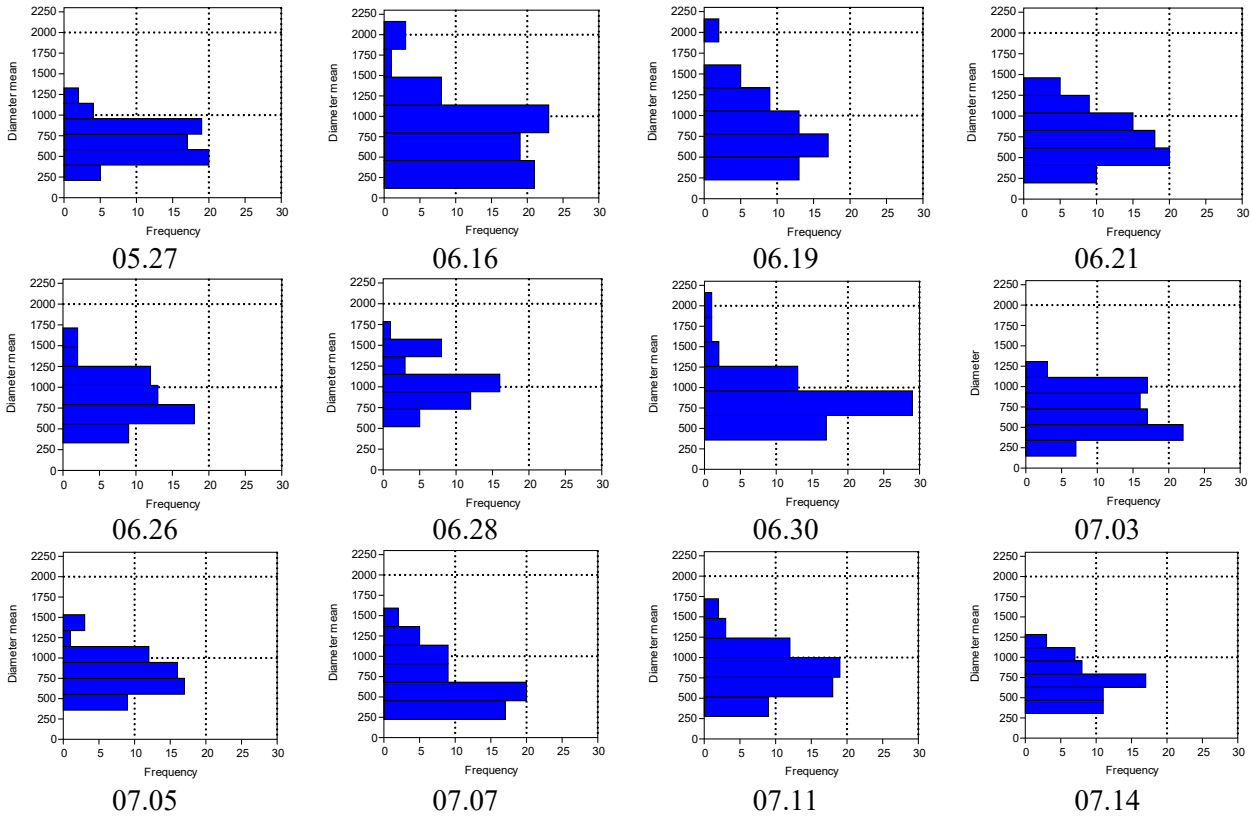


Figure 5. - The changes of carriers' diameter over time in reactor 1

In ***Figure 5***. the first chart shows the control sample diameter mean values distribution. The following charts show that higher diameter (above 125 0µm) values begin to appear, and the majority of carrier diameter moves

from 250-750 µm to 500-1250 µm. However, at 07.03. date there is a decrease, and after that it starts to grow again. Overall, the diagrams show a periodical growing-decreasing cycle of the biofilm.
In ***Figure 6.*** the diameter dispersion in each sample can be seen. In the first histogram you can see the control of the second type carrier. This type has slightly larger carriers than the first reactor, that has no absorbent in the carriers. The diameter size quickly rises, where the most common size is around 1000 µm until 06.26 sample. There is a decrease in diameter then, most carriers are around 750 µm, but there's still a significant number of carriers larger than 1250µm.

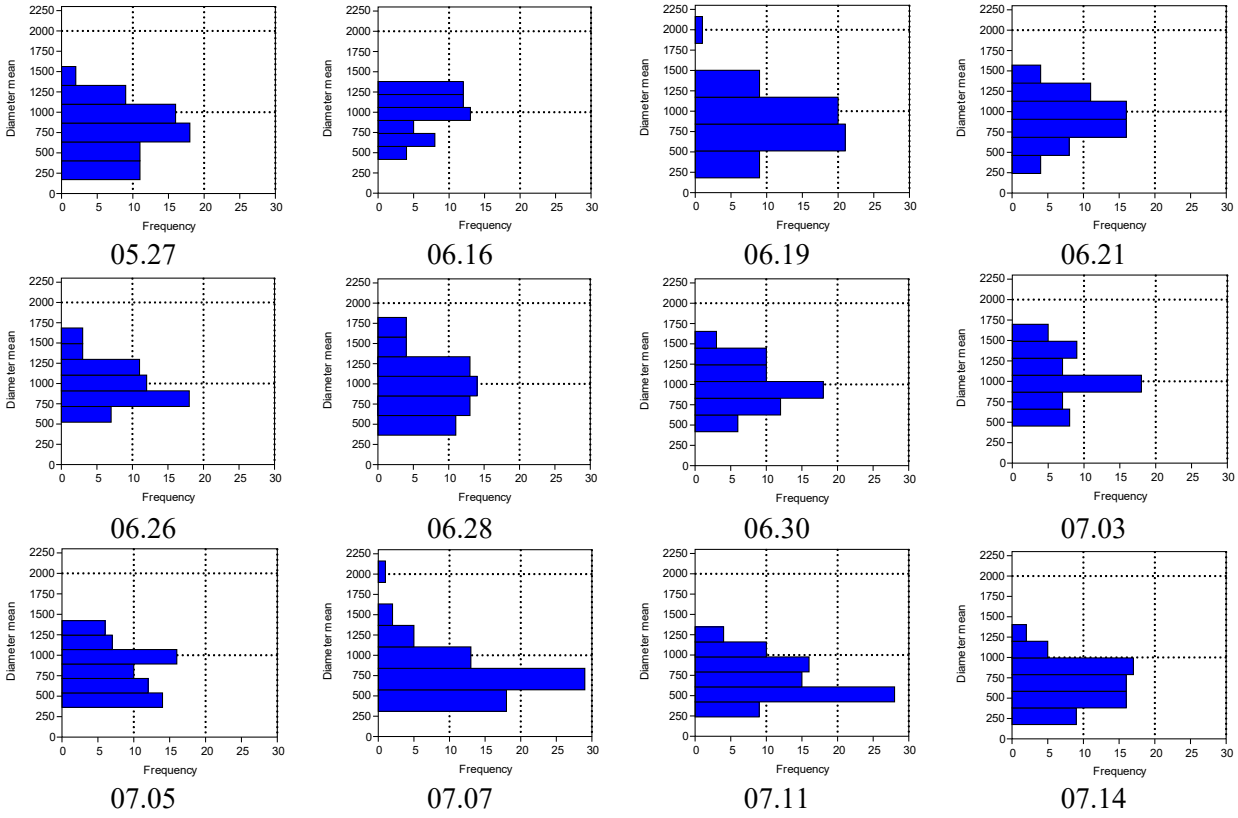


Figure 6. - The changes of carriers' diameter over time in reactor 2

After this there is a slow increase again, and at the last sample (07.14) most carriers are in the range of 1000µm. A similar trend is shown here, as in the first reactor.

In the next ***Figure 7***. we can see the diameter dispersion changes of reactor 3. The original carrier size (control, 05.27) significantly smaller than the traditional carriers. This is caused by the new synthesis method. Diameter mean range for most of the carriers are 300-600µm. The change in diameter however shows faster increase than the previous ones. In the first non-control sample the biggest bin has smaller share (33% vs. 12%) than the control, and the maximum is around 900µm.

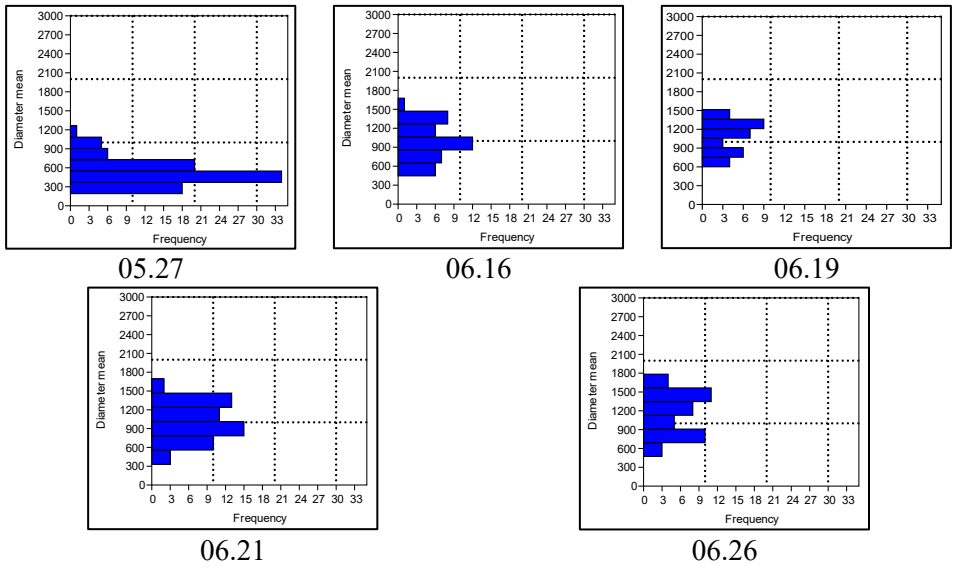


Figure 7. - The changes of carriers' diameter over time in reactor 3

The other samples are like this, with a decent increase in the 1500µm bin. In this reactor a slight increasing trend is visible. In ***Figure 8***. we can see the fourth reactors particle size changes over the experiment period. It shows that the control sizes are small, with most density in the 300-600µm range. Even at the first sampling time it changes dramatically, as seen at reactor 3. Fourth reactor grows biomass slower than reactor 3, as can be seen in every other parameter. Despite this, a growing trend, similar to reactor 3 can be observed.

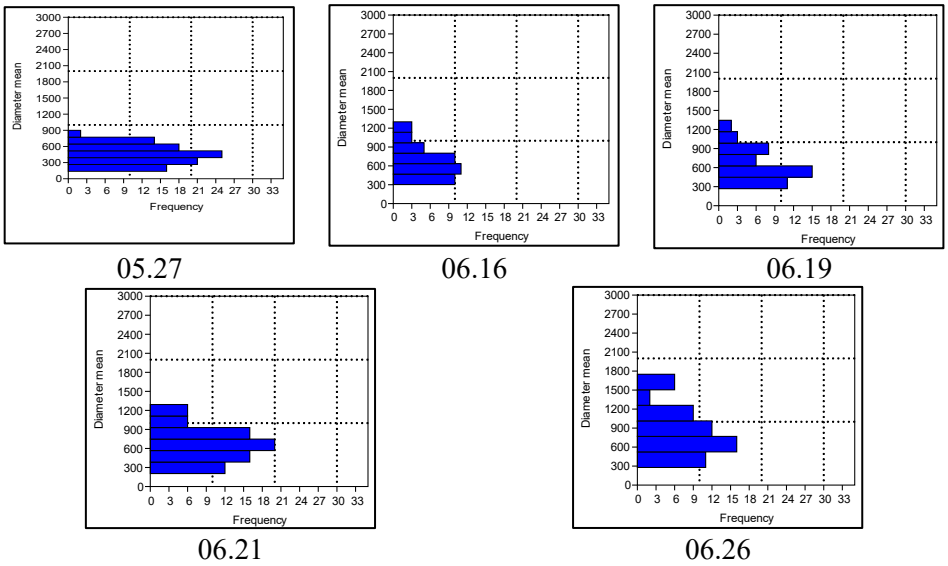


Figure 8. - The changes of carrier's diameter over time in reactor 4

In ***Figure 9.*** the average carrier thickness over the measuring period is shown. In the first chart it is clearly visible that as the biomass grows, then departs from the carrier, it changes its overall diameter. The values are slightly higher than the control, it suggests that a thin biofilm grows on the carriers. In the next chart we can also see a similar trend, but at the end of the experiment it goes a little lower than the original control values.

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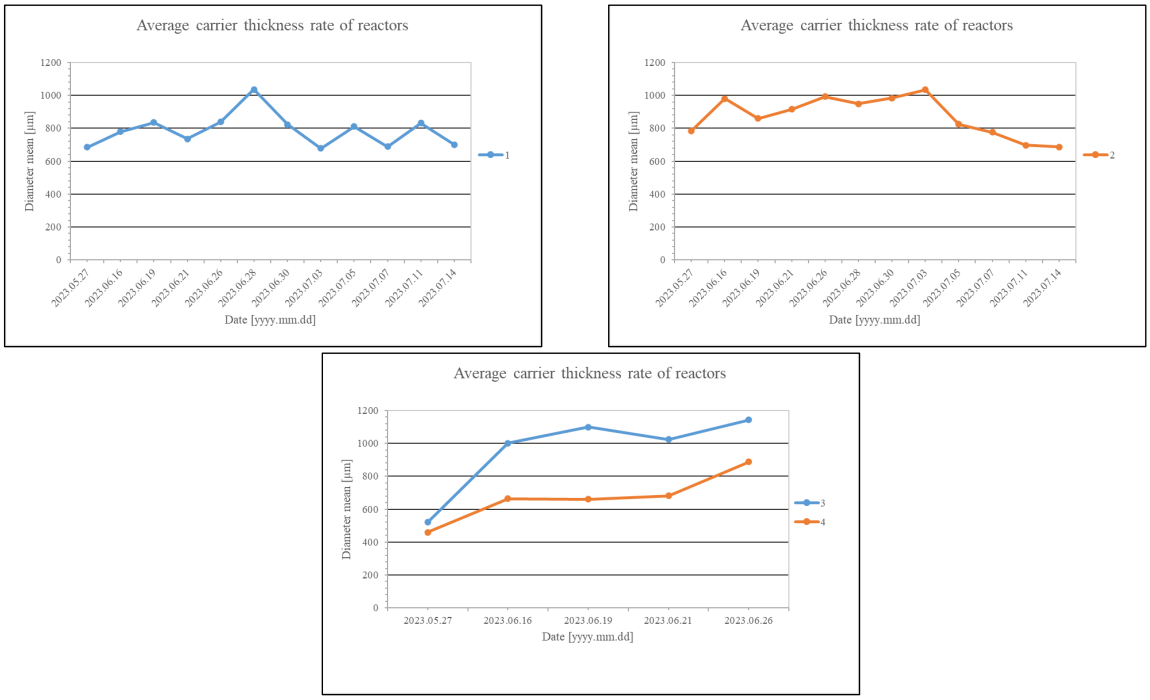

Figure 9. - Average carrier thickness in reactor 1 (top left), reactor 2 (top right), and reactor 3 and 4 (bottom)

The biofilm thickness is relatively small compared to the biomass's diameter, and in these calculations the carriers that has almost no biofilm on their surface are also included. In the reactor 3 and 4 there is a constant increase throughout the experiment period, however on those carriers, a different, phosphate limited, filamentous biomass grew, that shows less removal capacity but uses more surface area.

CONCLUSION & FURTHER PLANS

In this study I presented that TTC colouring with image processing and light microscopy is a suitable method for measuring and monitoring biofilm growth in terms of determining the carriers surface area, and also in monitoring the carrier's changing diameter, which is caused by the growing biomass. The results clearly show the methods ability to monitor the biofilm growing and detaching cycle, as can be seen in reactor 1 and 2. An increasing tendency can also be monitored, as in reactor 3 and 4. Further studies and calculations can be made based on the material shown in this article. To combine the settle rate with the calculations of overall diameter in further studies it is possible to determine the thickness of the biomass on these small carriers. Dividing the diameter calculations based on the settle rate make it possible to only measure those carriers that have actual biofilm on their surface. By isolating the carriers that have no biofilm on them, we can confirm if the new carrier materials' diameter changes over time.

The acquired data shows that this new type of carriers' surface is settleable for autotrophic microorganisms at a high rate. The second carrier was the most effective, and the fourth was the slowest. The original carriers have similar capabilities in terms of waste removal. They can settle quickly and remove ammonium from the influent water. These carriers don't reach the settling rate of the literatures values, however it's worth mentioning that these new carriers are about a hundred times smaller, even with slightly lower settle rate, their

biofilm concentration is even bigger than MBBR systems with traditional carriers. In this study only autotrophic microorganisms were used, for that is the most critical part of wastewater cleaning process. Another interesting result is that the third and fourth type of carrier were able to absorb a relatively high amount of phosphorous, which in this experiment caused a less effective microorganism culture on the reactors. In further studies it is important to determine their exact phosphate adsorbing capacity and after they overflow with phosphorous, how effectively organisms can grow on their surface. However, it's worth noting that even with phosphorous limited conditions, the biofilm on the carriers' surface were able to remove ammonium from the influent. This different filamentous biofilm has higher thickness and quicker growth rate than the original carrier's biomass.

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