EXPERIMENTAL BIOFILMS WITHIN DRINKING WATER TREATMENT PLANT ORIGIN; EVALUATION OF NUTRIENT CONCENTRATION AND TEMPERATURE INFLUENCES UPON THEIR DEVELOPMENT

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Abstract. From the planktonic free-floating state, microorganisms pass to the solid state, the biofilm, cells being strongly attached to each other and usually to the interface. This changing in cells' behavior induces surface colonization and complex interactions development within the biofilm. If the biofilm's role into the natural aquatic habitats is, undoubtedly, a positive one, consisting in water self-purification, drinking water pipe networks biofouling can be responsible for a wide range of water quality and operational problems.

This exploratory experiment was performed in order to investigate, in a time interval of 7 days, the influence of certain environmental factors such as nutrient concentration and temperature upon in vitro biofilm's development, origin in the biofilm of water treatment plant. The method used for in vitro biofilm growth monitoring is the colorimetric measurement of the biomass.

Descriptive analyses, including the mean value, variability, trends, correlations and graphic displays were performed. The correlation analysis shown that the biofilm development in the discussed experiment was influenced as by the origin source as by the temperature, time and nutrients concentration. The biomass increment was significantly different for the biofilms with clarifier and sand filter sites origin, grown at 22˚C, while at 8˚C, the differences were not significant from a statistical point of view.

For all the dilutions, moments and temperatures considered, the biofilm's development with clarifier origin registered was significantly higher than the biofilm with sand filter origin.

Keywords: biofilm, drinking water treatment, biofouling, colorimetric measurement of biofilm density

INTRODUCTION

Microorganisms mainly display two types of behavior: the free floating state of planktonic cells, form in which single-celled organisms freely float or swim independently into the liquid medium, and the attached state, the biofilm, which is characterized by strongly attached cells, both to each other and usually to an interface, forming a solid surface. The biofilm represents a structural community of autotrophic and heterotrophic microorganisms, encapsulated into the polymeric matrix, adherent to a living or inert surface. Biofilms are also often characterized by surface attachment, structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances. Microorganisms in biofilms include different types of bacteria, fungi, and higher organisms like protozoa, nematodes, larvae, crustacea. Recently, researchers have shown that viruses and parasites like *Cryptosporidium, Legionella pneumophila* or *Helicobacter pylori* can be trapped in biofilms associated to the drinking water distribution system. Although viruses and such parasites do not grow into a biofilm, they can attach to it after a contamination event [7, 10].

Planktonic microorganisms' research yielded relevant data, applied in diverse area, and lies at the basis of standard methods elaboration. Examination of several environmental habitats, extreme or otherwise, such as drinking water pipelines, has revealed only relatively small numbers of planktonic cells. In aquatic systems the biofilm bacterial count per square centimeter of surface has been estimated to be approximate 1000-fold higher than the corresponding planktonic count per cubic centimeter [13].

If the biofilm's role into the natural aquatic habitats is, undoubtedly, a positive one, consisting in water self-purification [15], in drinking water pipe networks can be responsible for a wide range of water quality and operational problems, such as: loss of distribution system disinfectant residuals, increased bacterial levels, reduction of dissolved oxygen, taste and odor changes, red or black water problems due to iron or sulfate-reducing bacteria, microbial-influenced corrosion, hydraulic roughness, and reduced materials life [2].

This exploratory experiment was performed in order to investigate, in a time interval of 7 days, the influence of certain environmental factors such as nutrient concentration and temperature upon modeled in laboratory biofilm's development, origin in the biofilm of water treatment plant.

MATERIALS AND METHODS

Biofilm samples were taken from two distinct points of a drinking water treatment plant: site A – a clarifier's walls and site B – a sand filter bed (sand grains). Also water temperature and pH were measured by the moment of sampling.

The sequence of water treatment steps follows:

- Passing through micro strainers to remove coarse suspensions;
- Reagents administration (aluminum sulfate for coagulation, lime for pH correction, polyelectrolyte as a flocculent agent) and primary disinfection by pre-chlorination;
- Sedimentation by settling in clarifiers (flocculation and coagulation take place here, under the reagents activity);
- Filtering through sand filters with dual-current;
- Disinfection by chlorination;

- Storage in tanks, wherefrom water enters the distribution system, the flow being adjusted by the users' needs [4, 21].

From the site A sampling was done by scraping the biofilm deposit from the inner walls of the clarifier with a sterile spatula, followed by biofilm concentration by centrifugation for 10 min. at 5000 rpm. After the supernatant exude, an aliquot of 0.2g of sediment was homogenized in 100ml peptone water, thereby achieving the suspension used for in vitro biofilm modeling. At sampling moment, the water into the clarifier registered 13.5˚C and 6.78 pH.

From the site B the sand was sampled into a sterile container and the biofilm suspension had been prepared by washing 25g of sand in 100ml peptone water in pursuing the biofilm detach. The sand filter was working for 23h (every 36h an automatic bubbling washing is performed). The water registered 13˚C and 6.66 pH at sampling moment.

Of these biofilm suspensions, series of glass tubes containing 2ml Tryptic Soy Broth culture medium in 1/10, 1/20, 1/40, 1/60 and 1/100 dilutions had been inoculated, with 1ml aliquots. Subsequently, batches and blanks were shaken for 10minutes and incubated at 8˚C and 22˚C, for 24h, 48h, 72h and 7 days. At selected intervals was performed the evaluation of biofilm's density, developed upon the inner walls of the test tubes using the colorimetric method: discharging the culture broth; washing the tubes three times with bidistilled water; dyeing the biofilm with 3ml Merck crystal-violet solution; washing the staining solution after 10 minutes; re-eluting the crystal-violet dye using 3ml 95% ethanol solution; measuring the absorbance of the ethanol/crystal-violet solution at two wavelengths (588nm and 594nm) using a Lambda 40Bio Perkin Elmer spectrophotometer. The operations' sequence had been iterated for the blanks, except the biofilm inoculation step. Reflective color measurement provided a rapid, non-destructive and quantitative measure of biofilm accumulation [1, 9, 12].

These incubation temperatures had been chosen for certain reasons: 8˚C represents the average temperature for the year 2008, of the raw water entered the plant, and 22˚C is considered the optimum growing temperature for the mesophilic bacteria, being indicated by the drinking water regulations [5, 6].

Tryptic Soy Broth, being a universal culture medium, free of inhibitors and indicators, was considered ideal for growing a wide range of microorganisms. Its composition in g/l is: Casein enzymic hydroslate 17g, Papaic digest of soybean meal 3g, Dextrose 2.5g, Sodium chloride 5g, Dipotassium phosphate 2.5g.

Descriptive analysis, including the mean value, variability, trends, correlations and graphic displays were performed. The significant differences between the biofouling ability of A and B biofilm were evaluated using the t-test (paired two sample for means), and the test is considered significant for p-value <0.05 [14, 20].

RESULTS

The batches' absorbance values measured at both wavelengths (588nm and 594nm) were similar, so the reading at λ=594nm has been taken into the future considerations.

The charts of the absorbance tendency depending on the nutrients concentration reveal as follows:

The biofilm suspension with clarifier origin, incubated at 8˚C yielded a biomass which grows directly proportional to the nourishing concentration and to the time range considered, except for the 1/10 dilution of the broth. The same suspension, cultured at 22˚C registered a growing peak, corresponding to 1/60 dilution of the broth (Fig. 1).

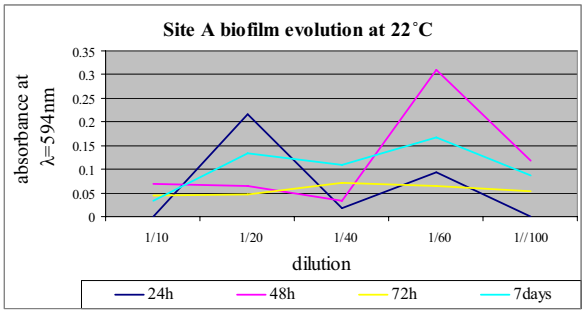

Figure 1. The biofilm's from the clarifier evolution on different dilutions at 22˚C.

The biofilm suspension with the origin in site B, the sand filter, incubated at 8˚C yielded a low biomass production in the first two days, subsequently distinguishing two growing peaks, corresponding to the 1/20 and 1/100 broth dilutions. The similar batch cultured at 22˚C registered a maximum of growth for the 1/60 dilution.

The statistical analysis revealed results as shown in Table 1 & 2.

Regarding the time evolution of biofilms, in the period of 7 days considered, the generally increasing trend has slightly decreased for the A samples: the batches cultured at 8˚C and measured at 48h and for the biofilms grown in high dilutions broth and measured at 72h (Fig. 2). A representative chart from Fig. 3 shows the trend of biofilm evolution in time, dilution 1/100, origin in site A and B.

Table 1. Results of the time correlation analyses for the biofilm growth.

CLARIFIER		Dilution	SAND FILTER	
8ºC	**22ºC**		**8ºC**	**22ºC**
- 0.5438	0.3433	**1/10**	0.5158	0.9622
0.9178	- 0.4403	**1/20**	0.8973	- 0.0734
0.4687	0.9885	**1/40**	0.7926	0.7664
0.9035	- 0.0316	**1/60**	0.7469	0.857
0.7864	0.4963	**1/100**	0.8012	0.0608

Table 2. Statistical results for the clarifier and filter biofilm growth.

Incubation temperature	Origin of biofilm	Mean	Standard deviation	P-value
8˚C or 22˚C	A	0.07238	0.076492	0.003028101
	B	0.03708	0.041134	
8˚C	A	0.05786	0.076485	0.057749846
	B	0.02969	0.044407	
22˚C	A	0.08690	0.075604	0.01313507
	B	0.04447	0.037216	

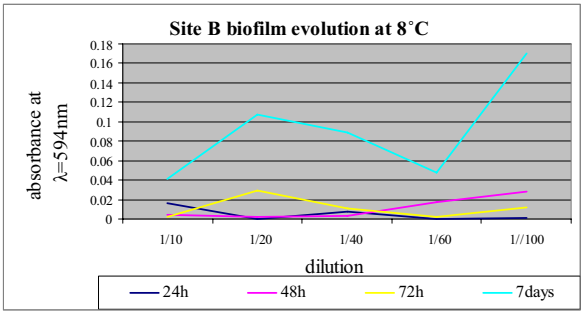

Figure 2. The biofilm's from the sand filter evolution on different dilutions at 8˚C.

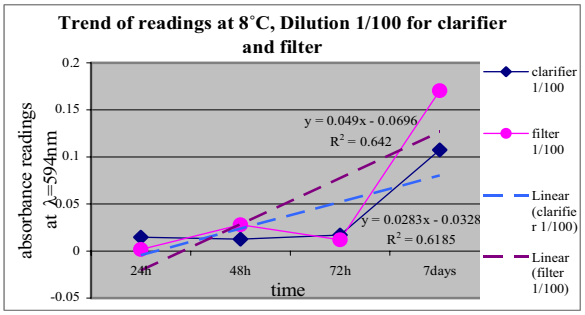

Figure 3. The trend of biofilm evolution in time, dilution 1/100, origin in clarifier and filter.

DISCUSSIONS

Chemical and microbiological parameters of the raw water and filtered water are weekly monitored, the last ones being considered as indicative value for the planktonic microbial density. They subject for another future study.

Table 3 reveals the average parameters for water quality for the year 2008, the environment conditions and microbial load for biofilms development. Parameters were determined according to standard regulations [5,6, 16-19].

Table 3. Parameters of the raw and filtered water, average values for 2008.

Type of water	CFU at 22˚C/1ml	CFU at 37˚C/1ml	T (˚C)	pH
Raw water	1572	534	8	7,12
Filtered water	596	113	-	6,89

Note: CFU=Colony Forming Units, T=temperature.

The differential increment of the biofilm mass developed by the sample origin in site B, the sand filter, cultured at 8˚C consisting in two growing peaks, for the 1/20 and 1/100 dilutions of the liquid medium might have two possible explanations:

-the initial biofilm suspension sampled from the site A contains at least two populations characterized by colonization abilities, but with different trophic necessities, which have been the promoters of the biofilm development;

-a diauxic growth of the population initializing the biofilm; while dextrose depletion, the microbes expressed the ability to use other available nutrients of TSB, contained by the casein enzymic hydroslate or soybean meal.

Achieving of the maximum development at low nourish concentrations (dilution series 1/60 at 22˚C and 1/100 at 8˚C) might be due to the presence of oligotrophic populations. The water into the sand filters is usually scarcer in nutrients, compared with the water within the clarifiers, which contributes to a microbial population's selection in order to become able to induce biofouling [8].

It must identify the real causes which led to record growth of two peaks when incubating the biofilm suspension origin in the clarifier, by the species of the biofilm consortium determination and their metabolic activity analyzing.

Nutrients concentration into the culture media represents a substantial influence factor of selection for the microbial populations which are able of different niche colonization and biofouling. Hereby, the water within the clarifiers containing higher amounts of organic and inorganic matter, bacteria are more sensitive to nourish deprive, evolving in dense biofilms directly correlated to the nutrients concentration. While, the microorganisms adapted to the oligotrophic medium into the sand filters had developed a maximum ability to induce biofilms in low feed conditions.

Statistical interpretations revealed an important correlation of the biofilm development with the incubation period, at 8˚C, for the following situations: clarifier 1/20 (r=0.9178) and 1/60 (r=0.9035), filter 1/20 (r=0.8973), while, at 22˚C a similar trend is not registered (Table 1).

Depending on the sampling sites, the evolution for the biofilm within the sand filter is more stable meaning that, both the 8˚C and 22˚C biomasses registered an ascending trend, with a singular exception (22˚C, dilution 1/20). Instead, the biofilm origin in the clarifier evolved differently: the dilutions 1/40 and 1/100 shown an ascending trend, but the rest registered a different tendency.

The early stage of 2-3 days may be insufficient for in vitro biofilm development, as for in vivo, planktonic bacteria being not able to change their behavior in order to surface colonization and complex interactions development within the biofilm. The change in behavior is triggered by many factors, including quorum sensing, as well as other mechanisms that vary between species. When a cell switches modes, it

undergoes a phenotypic shift in behavior in which large suites of genes are up- and down- regulated [3].

The correlation analysis shown that the biofilm development in the discussed experiment was influenced as by the origin source as by the temperature, time and nutrients concentration.

A whole, biomass increment was significantly different for the biofilms with A (clarifier) and B (sand filter) sites origin, grown at 22˚C (p-value 0.013), while at 8˚C, the differences were not significant from a statistical point of view (p-value 0.057).

Overall, as shown in Table 2, for all the dilutions, moments and temperatures considered, the biofilm's development with clarifier origin registered was significantly higher than the biofilm with sand filter origin.

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