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The application of different methods for microbial development assessment in Pilot Scale Drinking Water Biofilters

Title (in Polish)

Zastosowanie różnych metod do oceny rozwoju mikroorganizmów w filtrach biologicznie aktywnych oczyszczających wodę - skala pilotowa

Type

Original paper

Keywords (in English)

biological activated carbon filters (BAC), biofilm, drinking water treatment, microbiological activity, identification of microorganisms, pilot scale

Abstract (in English)

Objectives
Effective removal of water contaminants is one of the priority goals for water treatment plants. The one of the advanced method of water treatment is the process of biofiltration in the biologically activated carbon filters (BAC). It enables efficient elimination of dissolved organic matter and some inorganic pollutants. The production of high-quality drinking water requires the appropriate method of filter work control based on biofilm growth assessment. The first aim of the study was to assess the microbial development in beds of two BAC filters with the use of various methods. The second aim was to compare the obtained results and indicate the method which could support filter operators during routine control of biofiltration process.

Material and methods
The study was carried out in a pilot scale on models of rapid BAC filters during two filter runs. Analyzes of microorganisms was performed with the use of culture methods (HPC), metabolic activity assay (with the FDA), epifluorescence microscopy – total cell count method (TCC) and biochemical method (system Vitek 2 Compact).

Results
No statistical correlation between HPC and metabolic activity assay was noted. Total bacteria number determined with the use of TCC was approx. 100-900 times higher than the number of colony forming units in the HPC method. The biochemical tests revealed the presence of several Gram-negative species.

Conclusions
The comparison of the applied methods shows that microbial activity assay is the most useful, fast, low-cost method which may be applied additionally to the HPC method at standard laboratory at water treatment plant.

Abstract (in Polish)

Cel pracy
Efektywne usuwanie zanieczyszczeń z wody przeznaczonej do spożycia jest jednym z głównych celów stacji uzdatniania wody. Jednym z zaawansowanych metod uzdatniania wody jest proces biofiltracji na biologicznie aktywnych filtrach węglowych (BAC). Umożliwia on skuteczną eliminację rozpuszczonej materii organicznej i niektórych zanieczyszczeń nieorganicznych. Produkcja wysokiej jakości wody pitnej wymaga użycia właściwej metody kontroli pracy filtra w oparciu o ocenę wzrostu biofilmu. Pierwszym celem badań była ocena rozwoju mikroorganizmów w dwóch filtrach węglowych (BAC) z wykorzystaniem różnych metod. Drugim celem było porównanie uzyskanych wyników i wskazanie metody, która mogłaby wspomóc rutynową kontrolę procesu biofiltracji.

Materiał i metody
Badania wykonano w skali pilotowej na modelach filtrów BAC podczas dwóch cykli pracy filtrów. Analizy mikroorganizmów wykonano metodą hodowlaną (HPC), poprzez oznaczenie aktywności metabolicznej (z FDA), przy pomocy mikroskopii epifluorescencyjnej (TCC) oraz identyfikacji biochemicznej (system Vitek 2 Compact).

Wyniki
Nie stwierdzono istotnej statystycznie korelacji pomiędzy metodą HPC a testem aktywności metabolicznej. Liczebność komórek bakterii określona za pomocą TCC była ok. 100-900 razy większa niż liczba jednostek tworzących kolonie uzyskana metodą HPC. Testy biochemiczne wykazały obecność kilku gatunków Gram-ujemnych bakterii.

Wnioski
Porównanie zastosowanych metod wykazało, że badanie aktywności metabolicznej bakterii jest najbardziej przydatną, szybką i niekosztowną metodą, którą można zastosować jako uzupełnienie metody hodowlanej w standardowym laboratorium na stacji uzdatniania wody.

1. Introduction

The production and supplying safety water by water treatment plant is based on effective processes of a removal of any microorganisms, parasites or substances in number or concentration harmful for human health. The requirements for drinking water constantly increases. The list of pollutants that should be reduced during water treatment grows as scientists discover their detrimental effect on human and the natural environment as well as develop the methods for their determination and elimination. In the result of the revised Drinking Water Directive formally adopted by the European Parliament at the end of 2020, the new parameters of water quality will have to be tested and lower to reach the parametric values recommended in ”the Directive” by the water supply entities in the European Union in the near future. These parameters include e.g. microplastic, per- and polyfluoroalkyl substances (PFASs), pharmaceuticals and endocrine-disrupting compounds (Directive (EU) 2020/2184).

The one of water treatment technology which can be involved in the elimination of many mentioned pollutants from the drinking water is a biofiltration. It is a process used at modern water treatment plants for decades. It enables the efficient removal of many organic chemical substances known as dissolved organic matter (DOM) (Pruss et al.2009, Kaarela et al. 2015, Papciak et al. 2016, Kaleta et al. 2017, Fu et al. 2017) as well as some inorganic chemical compounds e.g. ammonia (Hasan et al. 2020). This process also helps to ensure the biological stability of water supplied to the consumers by the reduction of biogenic substances required to the microorganisms growth (Szuster-Janiaczyk 2016, Chaukura et al. 2020).

Common used filtration medium in the biologically activated filters (BAC) process is a granular activated carbon (GAC), which properties such as adsorption capacity and large specific surface area (usually 600-1000 m^2) provide excellent conditions for the effective adsorption of chemical compounds as well as for the growth of microorganisms. The first process occurs after the filter start-up is adsorption. It leads to gradual filling the pores of carbon grains with DOM, what induces the microbial colonization. The phase of full acclimation of bacteria lasts 2-3 months. The biofilm is formed by the bacteria attached to the carbon grains surface as a results of the cell divisions. Bacteria living in biofilm secreted extracellular polymeric substances (EPSs) which act as a matrix stabilizing cells and protecting them against external factors. Biodegradation of DOC occurs as degradation of substances present in the treated water as well as the result of desorption of partially degraded compounds from the GAC pores. The microbial activity in the BAC extends the filtration time

and reduces the necessity of activated carbon bed regeneration (Seredyńska-Sobecka et al. 2006, Simpson 2008, Kołwzan 2011).

The filtration trough the BAC filter beds enables the great removal efficiency of contaminants of emerging concern e.g. pharmaceuticals, pesticides (Zang et al. 2017), endocrine disrupting chemicals, personal care products (Snyder et al. 2007), disinfection by-product precursors (Chaukura 2020), cyanotoxins, geosmin and 2-methylisoborneol, amines, aliphatic aldehydes, phenols/chlorinated phenols (Simpson 2008). It can be useful in per- and polyfluoroalkyl ether acids elimination (Hopkins et al. 2018).

The achievement of benefits of BAC application in water treatment technology depends on the appropriate methods of the filter work's control. The effects of biofiltration process is affected by many factors such as the quality of the treated water, water temperature, DO and pH level, flow velocity, the stage of biofilm growth, backwashing frequency (Korotta-Gamage and Sathasivan. 2017). The number, activity or biodiversity of microorganisms living in the bed can by studied with many methods, e.g. culture method – heterotrophic plate count (HPC), Eberhardt, Madsen and Sontheimer (EMS) test, the determination of microbial activity with the use of specific chemical compounds e.g. fluorescein diacetate (FDA) or 2,3,5-triphenyltetrazolium chloride (TTC), the quantification of adenosine triphosphate (ATP), the use of glucose-labeled ^{14}C, the observation with the use of microscopic techniques, the determination of chemical compounds of the biofilm (phospholipids, polysaccharides and proteins) and biochemical tests (Mądrecka et al. 2018). Nowadays, also the techniques of molecular biology are more frequently used to discover and describe the taxonomical diversity and functioning of microorganisms living in a biofilm of BAC e.g. polymerase chain reaction (PCR) based methods, 16S rRNA analysis, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and next generation sequencing (NGS) techniques used in a metagenomics (Douterelo et al. 2014). Most of these methods are still too expensive, demand special equipment or qualified laboratory personnel to use them in the routine analyses at water treatment plants. Studies on biofilm formation and functioning with a use of commonly known and old methods as well as comparing them with modern ones are still very useful.

The first aim of the study was to determine and compare the amount, activity and diversity of bacteria growing in beds of two BAC filter columns which differ with the method of bacterial inoculation. Research was conducted in a pilot scale and included two filter runs during the full microbiological activity of BAC. The evaluation of the changes of microbiological activity in the filters was made with the use of traditional culture methods

(HPC), the metabolic activity assay (with the FDA) and the epifluorescence microscopy techniques (TCC). Additionally taxonomical identification based on biochemical method (system Vitek 2 Compact) was used. The microbiological studies was carried out in water samples collected in the filter bed cross-section and were accompanied by chemical analysis of inflowing and outflowing water.

The second aim of the research was to compare the results obtained from chosen methods and indicate the most useful method, which could support filter operators during routine control of the biofiltration process.

2. Methods

2.1. Research installation

The research was conducted on a pilot scale with the use of physical models of rapid filters (Fig. 1). The research installation consisted of two filter columns - Filter 1 (F1) and Filter 2 (F2). Every filter column's height was 300 cm, inner diameter was 10 cm. and the total filter bed height was 210 cm. The filter medium was activated carbon (WG-12; Gryfskand Ltd. Poland) made of special, low-ash coal, connected by a binder and activated by water vapor characterized by the following parameters: iodine quantity 1,100 mg/g, methylene blue adsorption 30g/100g, specific surface area - BET 1,100 m^2/g, particle size 1.5-0.75 mm. In the vertical cross-section of each filter column five connectors for collecting bed samples (Z1-Z5) and five connectors for collecting water samples (W1-W5) were installed. The connectors for water samples were placed at following depths of the beds: 45 cm, 85 cm, 125 cm, 165 cm and 205 cm. The connectors for bed samples were located at beds' depth: 45 cm, 75 cm, 115 cm, 155 cm and 195 cm. The filter columns were surrounded by a water jacket. The continuous flow of water from the bottom to the top of the water jacket ensured an even temperature throughout the bed's depth. The filter beds were protected against the light and algae growth with the black geotextile cover placed on the filter columns. Both filtration columns were supplied with tap water from the municipal water supply network. The water supply network was supplied with infiltration water treated in a traditional iron and manganese removal system and disinfected with UV rays and sodium hypochlorite.

Figure 1.

2.2. Filters' operation

Main difference between both columns was the method of inoculation with bacteria. As BAC's start working F1 was immediately supplied with tap water and F2 for two weeks treat

in a closed circuit diluted with tap water backwashings from full scale water treatment plant. The backwashings were additionally supplied with nutrients such as ammonium chloride, potassium phosphate and natural organic compounds (onion extract) in order to accelerate the working of the bed (Holc et al. 2016a, Holc et al. 2016b). The filters were then used in scientific research and after several weeks an interval in the research started. During this interval, the filters were working with a water flow rate of 20 dm^3/h and were not backwashed for approximately 275 days (run 0), because there was no significant increase in hydraulic losses. After that time, the research described in this article began. They included two runes - both lasting 55 days for each filter. Filters worked in parallel with the weekly delay in backwashing. During two runs both filtration columns were supplied with municipal tap water and operated under the same hydraulic conditions: water flow rate 40 dm^3/h, the filtration velocity was 5.1 m/h and the bed contact time was 27.2 minutes.

Each filter were backwashed for 17 minutes with tap water at an intensity of 300 L/h, ensuring an expansion of approx. 30% (Komorowska-Kaufman et al. 2018).

2.3. Water sampling, laboratory analyses

Water samples for testing physical, chemical and microbiological parameters were taken once a week. The samples for physical and chemical parameters were taken from the filters' inflow and outflow, while the samples for microbiological analyses were collected form the five filters' depths.

Physical and chemical parameters included: temperature, pH, dissolved oxygen (DO), alkalinity, UV_{254} absorbance and total organic carbon (TOC). These parameters were analyzed in accordance to the Standard Methods guidelines (2017). For TOC measurements, a TOC/TN multi NC 310 (analitykjena, Swiss) was used. The determination of the carbon content was made by thermocatalytic decomposition of sample in the presence of an N/C catalyst at 800 °C with synthetic air as the carrier gas. Chemical oxygen demand (COD_{Mn}) was determined by acidic permanganate method according to Polish Standard PN-C-04578-02:1985.

The microbiological analyses involved: heterotrophic place count (HPC), microbial activity (FDA method), total cell count (TCC) and taxonomical identification based on biochemical methods. The heterotrophic plate count (HPC) allowed to determine the number of culturable bacteria. It was performed in accordance to the Polish standard PN-EN ISO 6222:2004. Pour plate technique on enriched agar and incubation at 22°C for 72 hours was applied. Microbial activity was measured by the determination of esterase activity with the use of FDA. Measurement of FDA hydrolysis rate (fluorescein luminescence intensity) was

made on the luminescence spectrometer (PerkinElmer Instruments LS55) and dedicated software (FLWinLab) at the excitation wavelength of 433 nm and the emission wavelength of 525 nm according to the methodology described by Mądrecka et al. (2018). The representative water samples of the first filter run were studied with the use of TCC method. The selected samples were taken from the depth in which comparable number of bacteria determined in HPC method were found in both filters. The water samples for microscopic analyses were preserved with buffered glutaraldehyde to a final concentration of 10% and stored at 4ºC in the dark. The samples were concentrated on black Nuclepore polycarbonate filters with the pore size of 0.2 µm (Whatman) and stained with fluorochrome DAPI (4',6-diamidino-2-phenylindole). The filters were next examined with cell secondary fluorescence (Porter and Feig 1980) under UV light excitation and a magnification of 1500× using the Olympus BX-60 epifluorescence microscope equipped with a Jenoptik Gryphax camera and a mercury light source (HBO 100W). The taxonomical biochemical identification of microbial cultures obtained for both filters was performed with the automatic Vitek 2 Compact (bioMérieux) and Gram-negative (GN) bacteria identification cards according to the producent's protocol (bioMérieux, Marcy l'Etoile, France).

3. Results

3.1. Physical and chemical parameters of water

Filter 1 (F1) and filter 2 (F2) inflow and outflow water quality during the tests is shown in Table 1 and Table 2, respectively. Generally water inflow to both filtration columns differed slightly due to the weekly delay of the start time of the filtration cycle in F2. Larger differences in water quality were found between the two runs. In the run 2, all analysed inflow water parameters were slightly lower than in run 1, except for COD_{Mn} and TOC, which were slightly higher and temperature that was approx. 2.5ºC higher than in run 1.

Table 1.

Table 2.

As a result of filtration through a biologically active bed, the content of organic compounds in water expressed by COD_{Mn}, TOC concentration and UV_{254} absorbance decreased in both filters. The dissolved oxygen concentration also decreased after flowing through both filters.

Amount of organic matter removed by filtration (ΔCOD_{Mn}) of water through F1 at the end of the run preceding the test (run 0) was equal 0.49 mg O_2/dm^3 and dissolved oxygen consumption (ΔDO) was 1.59 mg O_2/dm^3. In run 1, the filter efficiency decreased and only 0.26±0.18 mg O_2/dm^3 of COD and 1.24±0.59 mg O_2/dm^3 of DO were removed, while in run 2

it increased again to ΔCOD_{Mn} equal 0.54±0.38 mg O_2/dm^3 and ΔDO equal 1.05±0.27 mg O_2/dm^3.

In F2, at the end of run 0, the leaching of the biofilm from the bed had already occurred, the increase in the concentration of COD_{Mn} in outlet from filter was found, while oxygen was still used for the processes taking place in the bed, ΔDO was similar to that during the entire study and amounted to 1.33 mg O_2/dm^3. In both research runs, the efficiency of F2 was similar. It removed in run 1 and run 2, respectively, 0.49±0.21 mg O_2/dm^3 and 0.50±0.14 mg O_2/dm^3 of COD_{Mn}, and 1.54±0.36 mg O_2/dm^3 and 1.31±0.23 mg O_2/dm^3 of DO. During the run 2 the obtained filtration effects were more stable than in run 1.

The average efficiency of organic compounds removal during whole research period (run 1 and 2) was as follows: COD_{Mn} 10.25±5.71%, TOC 7.17±10.59% (max 16.00%) and UV_{254} absorbance 31.27±18.82% for F1 filter and COD_{Mn} 14.45±7.23%, TOC 5.57±10.44% (max 20.18%) and UV_{254} absorbance 29.71±10.77% for F2 filter. The removal efficiency of organic pollutants in the filters was very variable during the cycle. The obtained effects indicate that despite the same hydraulic conditions and quality of inflow water, the F2 filter worked better, which should be justified by the presence of microorganisms from the inoculated backwashings.

3.2. Results of HPC method and microbial activity assay

3.2.1. Development of microorganisms in the filter during filtration run

Changes in the number of bacteria and microbiological activity in the section of the bed depending on the duration of the filtration run are shown in Fig. 2. Average values obtained from the analysis of water samples taken on a given day in the whole bed profile (connectors 1-5) are presented. It is clearly visible that the number of HPC bacteria in water samples taken at the end of run 0 from both filters and in the whole run 1 of F2, was varied at different depths, which is indicated by significant standard deviation. The differences in microbial activity measured at different depths were much smaller. Run 1 in both filters showed a higher number of bacteria in the water samples taken from the bed than in run 2, which was a consequence of the very intensive development of biofilm in the previous ran, which was carried out for a long time with half the filtration velocity. The filtration velocity affects the structure of the biofilm, which becomes more compact at higher velocities (Pruss 2007). With time, all the scattered fragments break away from it and the amount of slowly flowing bacteria decreases (Fig.2b). Also in F1, during run 1 the number of slow-flowing bacteria in water samples has decreased. Probably in F1 there was less biofilm volume (biomass

concentration removed during rinsing was lower - results not included) than in F2 and the decreasing effect which we recorded in F2 from the 27th day of the cycle started earlier (Fig.2a). In run 2 in both filters the number of HPC bacteria in water samples increased with time as the biofilm was developing. It was noticed that the number of bacteria in F2 was always higher than in F1.

Figure 2.

The difference in microbial activity in both filter runs in both filters was much greater than the difference in HPC bacteria (Fig.2.c-d). The highest activity was found in run 0 samples. During run 1 in F2 the microbial activity decreased slightly, while in F1 it definitely decreased during the first 40 days and later it almost doubled. During run 2 an increase in the measured microbial activity was recorded in both filters and it was definitely higher for F2.
In order to determine the capacity of bacteria living in the filter to decompose organic compounds, the microbial activity/HPC bacteria indicator was used. It informs about decomposition yield of FDA by one bacteria in water samples from the filter and was called unit activity (A/min)/CFU). The change of this indicator during the filtration run is similar to the activity (Fig.2.e-f). However, a high variability of this parameter was observed at different depths of the bed.

3.2.2. Development of microorganisms in the filter bed profile

Figure 3 shows changes in the number of HPC bacteria and microbial activity in the profile of the studied filter beds. The averaged values obtained from the analysis of water samples taken from the depth of 45, 85, 125, 165 and 205 cm, for the entire cycle are presented. The greatest differences in the values of the measured parameters were recorded on the last day of run 0 that preceded the research period. In the tested runs, the average number of bacteria grown from the water samples did not change unambiguously with depth (Fig.3.a-b).

Detailed analysis showed that it is difficult to determine a clear trend, because in the following days of the filtration run, the number of bacteria in the samples from the bed profile both increased and decreased, or changed irregularly. In run 1, however, it is possible to determine the depth at which the greatest number of bacteria was usually determined, i.e. 165 cm in F1 and 125 cm in F2. In run 2 in the F1 filter bed, the number of bacteria in water samples decreased slightly with depth. In the F2 filter bed, it decreased at a depth of 85 cm and increased slightly later.

In F1, the highest microbiological activity in the samples of water collected in the bed

profile was recorded at the end of run 0 (Fig. 3.c-d). In subsequent runs, it was approximately constant throughout the depth of the bed, although there were significant fluctuations in activity in the samples from different bed depths in the consecutive days of the run, similar to the amount of HPC bacteria. In run 1 it was slightly higher than in run 2. In the F2 bed, in run 1 the microbiological activity was comparable to that in F1 bed, while in run 2 it was much higher and slightly decreased with depth.

Figure 3.

The microbial activity/HPC bacteria indicator reached its highest value in F1 in run 0 at a depth of 85 cm. In the remaining runs, up to a depth of 125 cm, an increase in the unit activity of bacteria was found. While below this depth in run 1 a decrease in unit activity was observed, and in run 2 its further increase. The F2 also showed the highest unit activity in run 0 at depths 85 and 125 cm. Also in the remaining two runs, up to a depth of 85 cm, the unit activity increased, later it started to decrease and stabilized at a depth below 165 cm. In the entire profile of the F2 bed, higher unit activity was found in run 2 than in run 1, which confirms that proper backwashing of excess biofilm allows for the development of appropriate bacteria and better diffusion of nutrients.

3.3. Relationship between HPC and microbial activity in BAC

No correlation was found between the number of cultured bacteria and microbial activity determined by the FDA method in all analyzed water samples taken from different heights of the deposit on different days of the cycle (Pearson correlation coefficient $R < 0.3$) (Fig. 4). The spread of both HPC bacteria and microbial activity was greater in F2. Much more bacteria that did not show proportionally high activity were found in filter F2 in run 1. This may confirm the assumptions that these bacteria come from fragments of “old biofilm” left over from backwashing.

Figure 4.

3.4. Total cell count method

The total bacteria number and biomass determined with the use of an epifluorescence microscope in the water samples collected from filter bed were changing during the filters’ run (Table 3, Fig.5). Before the first filter backwashing, at the end of run 0, after about 275 days of continuous filtration, the total number and biomass of bacteria were several times

higher in F1 than in F2 (about 6 times in case of bacteria number and about 8 times in case of the biomass). The first backwashing led to biofilm separation and washing it out of the beds. In the result of backwashing, in 13th day of filters run 1 the bacterial number and biomass decreased. The total cell number were similar in both filters, but the biomass was lower in F1 than in F2. In 34th day of the run 1 the bacterial number and biomass increased what indicated the biofilm growth. The bacterial number in F1 was only slightly higher than in F2, while the biomass was two times higher than in F2. The higher bacterial biomass was the result of higher number of morphological forms characterized by the large size of cells e.g. rods. After the second backwashing, in 6th day of the run 2 the total cell number and biomass was higher than before the second backwashing. In that day the total cell number was similar in both filters but the total biomass in F1 was about 25% smaller than in F2.

Table 3.

The analysis made with the use of TCC method has shown the differences between F1 and F2 in the structure of morphological forms (Table 3, Fig.5, Fig.6). The number of *Spirochaetes*, filamentous forms and vibrios were higher in F1 than in F2. The number of cocci, diplococci and rods was changing in both filters, but at the end of the investigated period it was of higher in F2. The differences in the morphological structure of bacterial cells indicated that the taxonomical structure of bacterial community developed in both filters was also different. The biomass of free-flowing bacteria derived from the biofilm was changing during filter runs in the range of 0.012-1.092 mg/dm^3. The total bacteria number determined with the use of an epifluorescence microscope was approximately 100-900 times higher than the one obtained using HPC method.

Figure 5.

Figure 6.

3.5. Taxonomical identification based on biochemical method – Vitek 2 Compact

The biochemical identification performed with the use of Vitek 2 Compact resulted in recognition of several bacterial species. The most common species noted in both filter columns during two filter runs was *Stenotrophomonas maltophilia*. Other identified taxa were: *Brevundimonas diminuta/vesicularis, Shewanella putrafaciens, Sphingomonas paucimobilis and Pseudomonas putida*. The last mentioned species was detected only in F2

during the run 2.

Identified species are Gram-negative bacteria, preferring mesophilic environment and usually noted as rods. Most of them are aerobic, exception *S. putrafaciens*, which is facultative anaerobe. They are mainly noted in natural environment such as water, soil or rhizosphere, but some of them are also isolated from food or are part of animals' microflora. They can be also opportunistic human pathogens, mainly related with the nosocomial infections and observed in immunocompetent patients. They are able to metabolise various inorganic or organic compounds. Strains of the most often noted species *S. maltophilia* are chemoorganoheterotrophic and use limited range of organic compounds as main carbon and energy source e.g.: glucose, mannose, maltose, cellobiose, acetate, propionate, fumarate, lactate, citrate, l-alanine, d-alanine, l-glutamate, l-histidine, and l-proline. *Brevundimonas diminuta/vesicularis* is chemoorganotrophic and oligotrophic taxon. Its nutritional spectrum is also restricted. It utilizes e.g. pyruvate and organic acids. *Shewanella putrafaciens* strains can form hydrogen sulfide from various sulfur compounds. It is also known, that this bacterium can reduce ferric, manganese and other metals. *Sphingomonas paucimobilis* degrades aromatic hydrocarbons, e.g. it can assimilate betaine, D(+)galactose, l-aspartate, succinate, p-hydroxybenzoate, cis-aconitate. D(+)trehalose, trans-aconitate, L(-)malate and l-serine. A distinctive feature of *Pseudomonas putida* is ability to production siderophores called pyoverdines. It is a fluorescent species, which is noted mainly in soil and water. It can metabolize a widely spectrum of organic compounds as a source of carbon (Garrity 2005a, 2005b). A diverse metabolism is a reason ,why *P. put*ida is used or considered to be useful in bioremediation of various pollutants (Zamule et al. 2021)

4. Discussion

The presented research concerns the BAC filters, which have been operated for more than 9 months since the start-up and microbial inoculation. The chemical analysis of inflowing and outflowing water revealed average efficiency of organic compounds removal during the two analyzed filter runs - max 16.00% of TOC in case of F1 and max 20.18% in case of F2. These data indicate that probably the adsorption capacity of GAC was exhausted and the DOM removal was based mainly by biological degradation (Simpson 2008). However, it is worth noting that TOC removal efficiency of F2 was greater, what can be explain by the colonization with the microorganisms from the backwashings derived from existing water treatment plant. The results of previous studies carried out in a pilot scale have proved that inoculation the BAC filter with backwashings accelerates the carbon filter activation (Holc et al. 2016a, 2016b). The results of TCC method showed the differences in the composition of

morphological structure of bacterial population living in two analyzed BAC filters. Biochemical identification carried out with the use of Vitek 2 Compact also indicates some taxonomical differences of microorganism growing in the filters biofilms. These differences may results in more effective working of F2. Removal of organic matter from water is a result of oxidation in the respiratory processes of microorganisms and the increase of their biomass. Decrease in oxygen concentration and following increase in carbon dioxide concentration in the treated water indicates the development of microorganisms in the filter bed (Pruss et al., 2009, Liao et al., 2012, Elhadidy et al., 2017, Kołaski et al.2019). These findings were confirmed in the presented studies. The removal of oxygen in F1 (0.49±0.21 mg O_2/dm^3 in run 1 and 0.50±0.14 mg O_2/dm in run 2) was evidently smaller than in F2 (1.54±0.36 mg O_2/dm^3 in run 1 and 1.31±0.23 mg O_2/dm^3 in run 2).

The comparison of HPC method and microbial activity assay reveals no correlation between number of cultivated bacteria and microbial activity in both BAC filters. Moreover, the results of third analyses method – TCC show that total bacteria number determined with the use of an epifluorescence microscope was approximately 100-900 times higher than the number of colony forming units obtained from the HPC method. It means that only about 0.001-0.01% of bacterial cells counted with the use of microscopic techniques was able to grow on culture media. These values might be overestimated, because one colony is the results of binary division of one cell or cell arrangement. However the results confirm poor detectability of microorganisms with the use of culture methods.

All methods used in the research have advantages and disadvantages. The HPC is an old and easy method of determining the number of culturable heterotrophic microorganisms that can be carried out in a standard microbiological laboratory. This method enables the calculation of the number of various taxonomical or ecological groups of microorganisms. Its main disadvantages can be quite long time of bacterial incubation in some cases or difficulties in taxonomical identification of microorganisms. Its main disadvantage is that only a small percentage of bacteria living in various environments is able to grow on culture media in the laboratory conditions - only 0.01% of waterborne bacteria (WHO 2003), up to 15% of bacteria of activated sludge (Wagner et al., 1993) and 0.05-8.3% of bacteria occurred in drinking water (Burtscher et al. 2009) The methods of microbial cultivation have to deal with the impossibility to recreate the wide range of ecological factors affecting microorganisms in their natural environment. Many bacteria is also able to reduce their metabolism and enter the "viable but nonculturable state" (VBNC), e.g. as a result of environmental stress during

samples collection. Bacteria in VBNC state are still alive but not detectable with the use of culture methods (Oliver 2010). Despite these deficiencies, the HPC method is still recommended by the low regulation related to the drinking water quality in the European Union (Drinking Water Directive), United States, Canada or Australia (Van Nevel et al 2017).

The second analyzed method - the measurement of microbial activity using the FDA is based on the determination of esterase activity (Olszewska and Łaniewska-Trokenheim 2013, Lis et al. 2016). Esterase is an enzyme, which converts fluorescein diacetate to fluorescein - a green fluorescent chemical compound. Only live cells are capable of showing fluorescence. The advantages of this method are simplicity, low cost and shorter time of performance than HPC method. This method is commonly used to measure the activity of bacteria originating from the natural ecosystems such as soil (Adam and Duncan, 2001) and freshwater (Battin, 1997) or developing in the man-made environment e.g. activated sludge (Kijowska et al., 2001; Ziglio et al., 2002). However, some studies have shown that the ability to absorb fluorescein diacetate depends on the taxonomical features and stage of cell growth (Chrzanowski et al. 1984).

The next method applied in the research - total cell count method (TCC) is more sophisticated but can be used to accurately determine the number of microorganisms. It involves epifluorescence microscopy techniques and fluorochromes - the fluorescent dyes that stain cell compounds. The method allows to count bacterial cells, describe their morphology, measure the cell size, and in some cases also recognize their physiological state. The main dyes used for bacteria observation are e.g. acridine orange, 4',6-diamidino-2-phenylindole (DAPI), propidium iodide, ethidium bromide, PO-PRO-3 and SYTOX Green or the a set of SYTO®9 and PI dyes known as LIVE/DEAD® BacLightTM Bacterial Viability Kit which enable to distinguish the live and dead bacterial cells (McFeters et al. 1995, Boulos et al. 1999, Sadowska and Grajek 2009). Although the determination of bacteria cell number with this method may be very accurate, it is susceptible to errors, if bacteria cells are formed in clusters or are attached to microscopic fragments of carbon grains (Van der Kooij et al., 2014). It is also more time-consuming and difficult in performance than the two above mentioned methods.

Biochemical tests are usually based on the determination of immunological or biochemical properties of microorganisms which allow the taxonomical identification, e.g. analytical profile index (API) tests or system Vitek 2 Compact (bioMérieux). The use of biochemical tests is an expensive method, requires special laboratory equipment and commercial test, but is easy to perform. However, it should be noted, that the number of taxa that can be identified

with this method is limited. Such tests are mainly design to use e.g. in medical analysis to detect pathogenic and opportunistic microorganisms. Less common and non-human taxa may remain unidentified or misidentified (Pincus 2013).

The results of the presented study and above mentioned benefits and deficiencies of biofilm growth assessment indicate that despite the HPC method is still required to perform by the low regulation in many countries in the world, the application of additional method of biofilm growth control gives valuable information. One of these methods is the measuring the microbial activity. It is easy to carry out in the standard laboratory, no time-consuming and low-cost method.

5. Conclusions

Microbiological analysis of water samples using HCP method, epifluorescence microscope and the microbiological activity test (FDA) showed microbiological activity of both carbon filters. The results of the HPC and FDA test in water samples taken from the vertical profile of the filtration columns confirm that the filter bed was biologically active over its entire depth. Comparing the results for both columns, higher activity values were observed in the F2 column, which indicates a more developed biofilm, possibly as a result of earlier inoculation of the bed. The activity of the filtration column is very unevenly distributed in the bed depth. The analysis of the results showed that the backwashing of the filter beds of both columns cause only a slight decrease in microbial activity in the filters, which does not adversely affect the entire cycle. Backwashing, on the other hand, plays an important role in controlling the amount of free-flowing bacteria and the thickness of the biofilm, which has an impact on the results of water treatment.

The study did not show any correlation between the number of HPC bacteria and microbiological activity measured with FDA method. Similarly, no correlation was observed between these parameters in samples of deposits of biologically active filters operated on a technical scale (Kołaski et al. 2019).

Studies have shown that the use of an epifluorescence microscope to determine the counts of heterotrophic bacteria allows to determine the actual counts of bacteria, including those incapable of growing on culture media. The total bacteria number determined with the use of an epifluorescence microscope was approximately 100-900 times higher than the one obtained using the HPC method.

The bacteria identified in the water samples belong to Gram-negative strains that use carbon from the decomposition of organic compounds.

Despite its imperfection continuous testing the microbial community of BAC filter with

HPC method allows to assess the stage of biofilm formation and it microbial activity. It helps to biofilm growth control and effective removal of contaminants in BAC filters. Although the TCC methods may determine the bacterial cell number very precisely it is time-consuming, requires special laboratory equipment and trained personnel to perform. The biochemical method may give the additional information about the taxonomical structure or changes of the microorganisms in the biofilms but they contain a limited list of microorganisms that can by identified by the design tests.

The comparison of the various methods shows that microbial activity assay with the use of FDA is the most useful, no time-consuming and low-cost method which can be applied at standard laboratory at water treatment plant. It provides valuable information which can support BAC filters operators and be used as a routine control of the biofiltration process additionally to the HPC method required by the law regulations.

Acknowledgement

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Table 1. The filter 1 (F1) inflow and outflow water quality during experimental period (average value±standard deviation)

		Run 1		Run 2	
Parameter	Unit	Inflow	Outflow	Inflow	Outflow
Temperature	°C	14.46 ± 0.67	17.14±1.53	16.80±0.87	18.80±1.38
pH	-	7.82±0.18	7.64±0.15	7.71±0.15	7.49±0.22
Total alkalinity	mg $CaCO_3/dm^3$	3.86±0.12	3.74±0.14	3.78±0.07	3.62±0.05
Dissolved oxygen	mg O_2/dm^3	4.83±0.43	3.59±0.39	4.30±0.32	3.25±0.40
COD_{Mn}	mg O_2/dm^3	3.37±0.24	3.11±0.18	3.68±0.29	3.19±0.13
TOC	mg C/dm^3	4.63±0.26	4.20±0.02	4.64±0.32	4.39±0.54
UV_{254}	m^{-1}	0.846±0.580	0.494±0.006	0.760±0.195	0.514±0.113

Table 2. The filter 2 (F2) inflow and outflow water quality during experimental period (average value±standard deviation)

		Run 1		Run 2	
Parameter	Unit	Inflow	Outflow	Inflow	Outflow
Temperature	°C	14.56±0.87	19.14±1.21	16.99±1.58	19.49±1.13
pH	-	7.85±0.14	7.55±0.06	7.70±0.12	7.48±0.12
Total alkalinity	mg $CaCO_3/dm^3$	3.82±0.09	3.72±0.15	3.77±0.09	3.60±0.07
Dissolved oxygen	mg O_2/dm^3	4.81±0.48	3.27±0.36	4.25±0.24	2.94±0.33
COD_{Mn}	mg O_2/dm^3	3.41±0.34	2.87±0.24	3.73±0.35	3.23±0.40
TOC	mg C/dm^3	4.58±0.25	4.36±0.30	4.74±0.31	4.42±0.71
UV_{254}	m^{-1}	0.645±0.013	0.468±0.058	0.770±0.200	0.507±0.073

Table 3. Cell number and biomass (expressed as wet weight) of bacteria and their morphological forms in selected water samples of F1 and F2.

Parameter	Filter no and filter depth	Run / operating time (day)					
		Run 0		Run 1			Run 2
		273	278	13	34	55	6
Cocci (10^3 cells/cm^3)	F1 – 125 cm	1475.7	NA	79.9	93.8	109.4	124.8
	F2 – 165 cm	NA	247.2	68.2	110.8	NA	159.8
Diplococci (10^3 cells/cm^3)	F1 – 125 cm	10.6	NA	2.1	0.0	2.8	0.0
	F2 – 165 cm	NA	4.3	0.0	4.3	NA	10.7
Coccobacilli (10^3 cells/cm^3)	F1 – 125 cm	314.3	NA	6.4	33.0	28.4	30.4
	F2 – 165 cm	NA	75.6	20.2	22.4	NA	25.6
Rods (10^3 cells/cm^3)	F1 – 125 cm	873.7	NA	66.1	65.0	32.7	91.3
	F2 – 165 cm	NA	184.3	38.3	27.7	NA	101.4
Diplobacilli (10^3 cells/cm^3)	F1 – 125 cm	21.3	NA	0.0	0.0	0.0	0.0
	F2 – 165 cm	NA	2.1	2.1	2.1	NA	8.5
Vibrios (10^3 cells/cm^3)	F1 – 125 cm	623.3	NA	27.7	34.1	35.5	39.6
	F2 – 165 cm	NA	109.7	27.7	32.2	NA	20.5
Spirilla (10^3 cells/cm^3)	F1 – 125 cm	42.6	NA	0.0	0.0	1.4	0.0
	F2 – 165 cm	NA	12.8	1.1	9.6	NA	5.3
Spirochetes (10^3 cells/cm^3)	F1 – 125 cm	218.4	NA	0.0	0.0	2.8	57.8
	F2 – 165 cm	NA	0.0	0.0	1.1	NA	12.8
Filamentous (10^3 cells/cm^3)	F1 – 125 cm	63.9	NA	0.0	0.0	5.7	3.0
	F2 – 165 cm	NA	5.3	0.0	0.0	NA	1.1
Trichome (10^3 cells/cm^3)	F1 – 125 cm	0.0	NA	0.0	0.0	0.0	0.0
	F2 – 165 cm	NA	1.1	0.0	0.0	NA	1.1
Total cell number (10^3 cells/cm^3)	F1 – 125 cm	3644.0	NA	182.2	225.9	218.8	347.0
	F2 – 165 cm	NA	642.5	157.7	209.9	NA	349.7
Total cell biomass (mg/dm^3)	F1 – 125 cm	1.092	NA	0.021	0.026	0.017	0.044
	F2 – 165 cm	NA	0.139	0.031	0.012	NA	0.060

NA – not analyzed

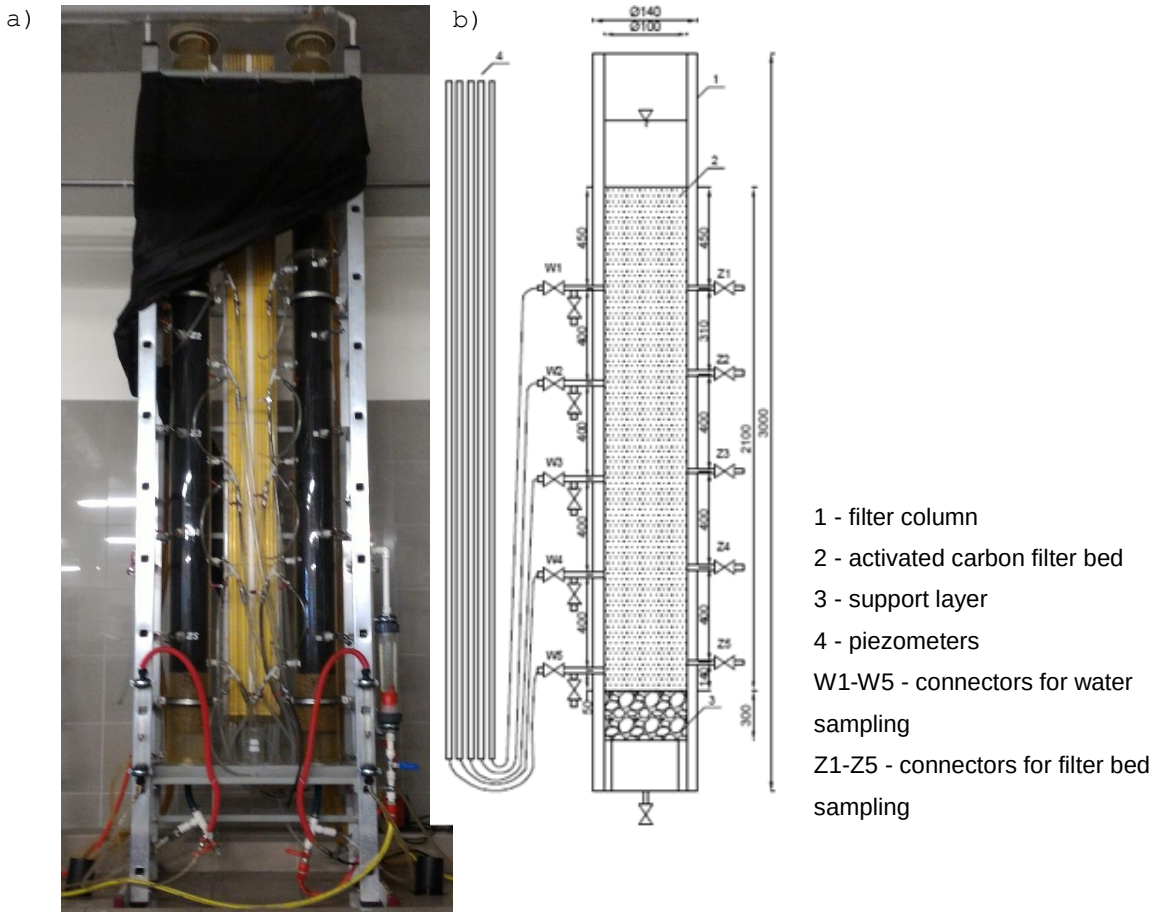

Figure 1. Research installation a) photography of two filter columns b) construction and dimensions of a single filter column.

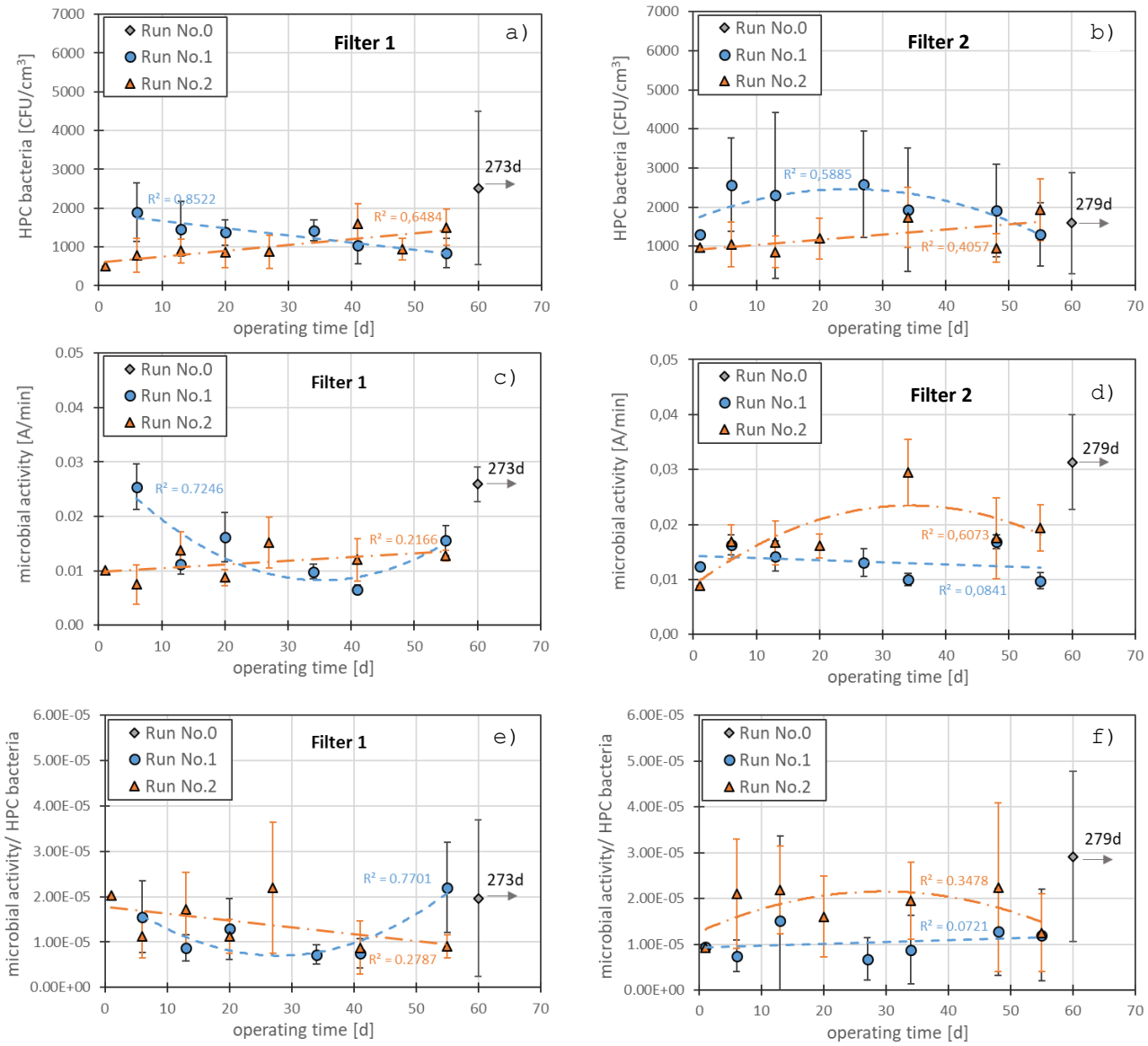

Figure 2. Change of average values of HPC bacteria (a,b), total microbial activity (c,d) and unit water activity (e,f) in water samples taken from the filter F1 (a,c,e) and filter F2 (b,d,f) bed during filter run

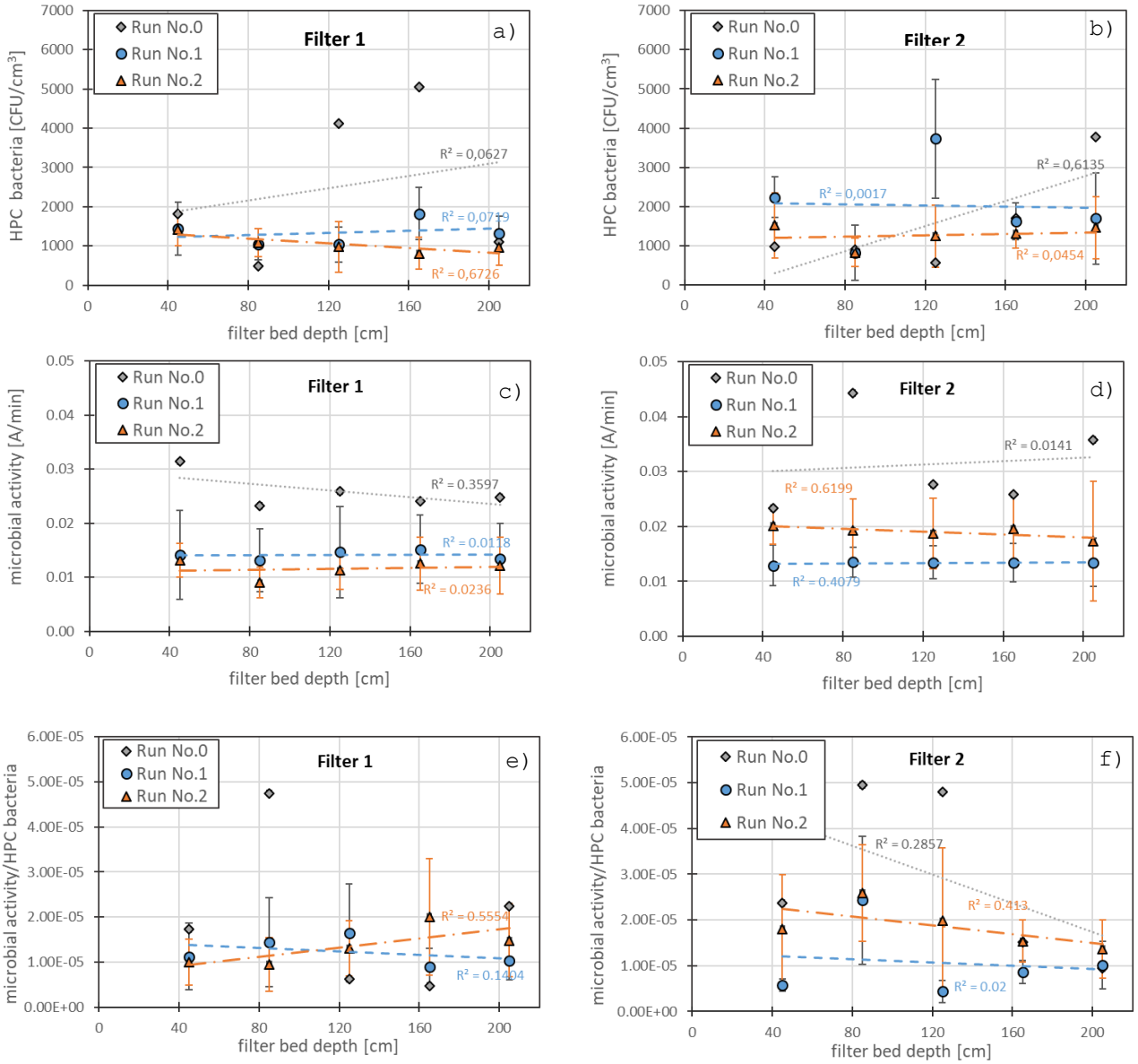

Figure 3. Change of average values of HPC bacteria (a,b), total microbial activity (c,d) and unit water activity (e,f) in water samples taken from different filter bed depths of filter F1 (a,c,e) and filter F2 (b,d,f) bed during filter run.

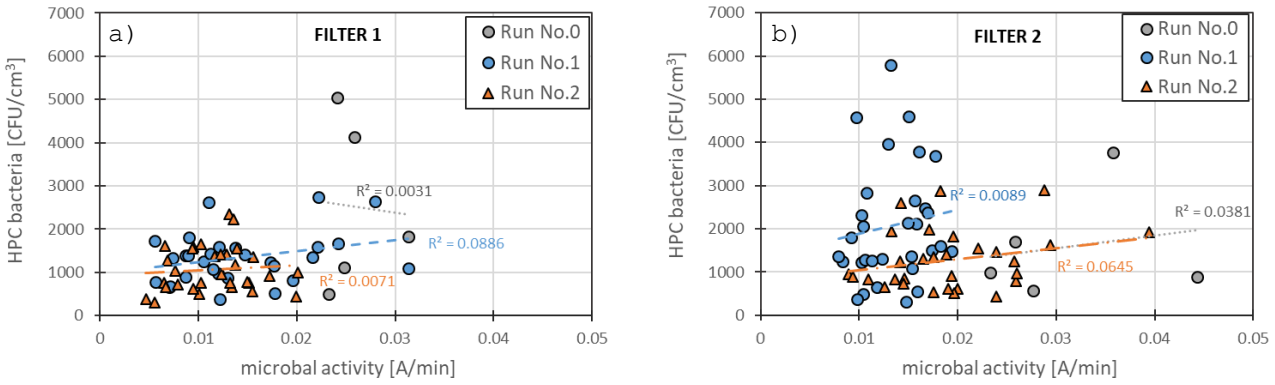

Figure 4. The relationship between microbial activity and the number of HPC bacteria for the entire filter (all layers) a) F1 b) F2.

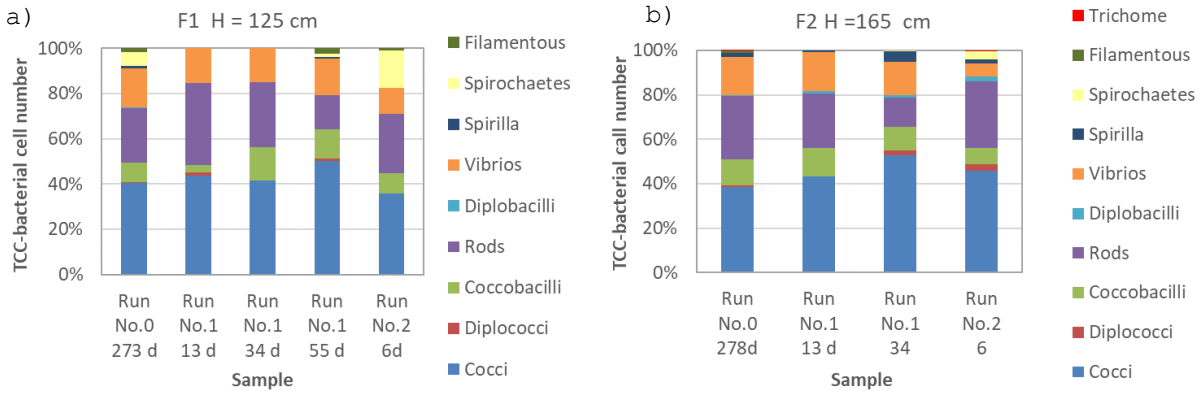

Figure 5. The percentage share of bacterial shapes and arrangements in the total cell number determined with TCC method in selected water samples

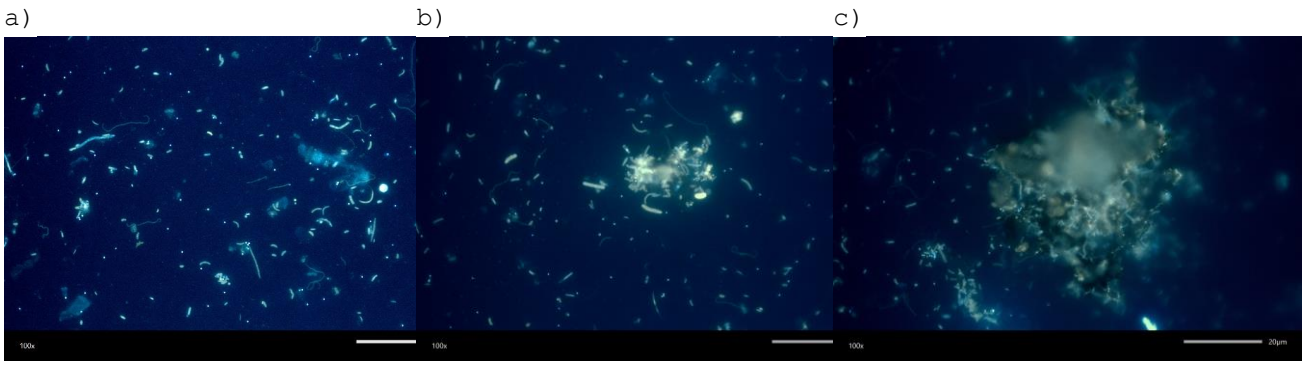

Figure 6. Bacteria observed under epifluorescence microscope (100x): a) morphological diversity of free-floating bacteria; b) biofilm fragments c) biofilm developed on the carbon grain

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