Article

Optimization of Quantitative Analysis of Biofilm Cell from Pipe Materials

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Abstract: The quantitative analysis of biofilm can be used not only to assess the microbiological stability of tap water but also on its basis can assess: the degree of colonization of materials by bacterial cells, the rate of biofilm formation on the surfaces of pipes and determine their composition and number. The article presents the results of research on the development of an effective method of biofilm detachment from the surface of the galvanized steel. The number of biofilm cells was determined by methods: (1) luminometric ATP determination, (2) flow cytometry and (3) heterotrophic plate counts (HPC). The presence of the biofilm was confirmed by SEM and fractal analysis. The analysis of the obtained results showed that the most effective method of detaching the biofilm cells from the galvanized steel surface was the mechanical separation with a sterile cotton swab. The variant with the use of a sterile swab enables rapid collection of the biofilm from the surface of the ducts forming internal installations or water supply networks. Due to the simplicity and speed of obtaining results, the luminometric ATP measurement has been established as the best method for the quantification of biofilm cells. The results of this study were intended to provide reliable and useful data on the quantification of biofilm cells.

Keywords: pipe material; biofilm measurement; water supply system; environmental engineering

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1. Introduction

The purpose of the drinking water distribution system (DWDS) is to provide water of appropriate quality to the consumers, preferably of the same composition as when it left the treatment plant. Even with well-functioning drinking water treatment systems, there is concern that aging DWDS (exposed to more frequent network interruptions/repairs and associated pressure losses) may destabilize the pipeline material and stimulate the development of biofilms [1]. The release of sediments, chemicals and microorganisms from the surface of water pipes creates health and aesthetic problems related to discoloration and turbidity of drinking water [2–6]. One of the main reasons for changes in the quality of water during its transport is the lack of physicochemical and microbiological stability of the water leaving the water supply plant [7–10]. In order to obtain and maintain adequate quality of tap water, various water treatment technologies are used [11–13]. In turn, for the construction of distribution systems, materials are sought that show resistance to corrosion phenomena and do not support the development of biofilms on the internal surfaces of water pipes [14–16].

It is estimated that biofilm and loose sediment may contain over 98% of bacteria in the DWDS [17]. Due to its nutritional and protective properties, biofilm enables the development of various microorganisms such as: opportunistic pathogens (e.g., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Aeromonas hydrophila*, *Staphylococcus*

sp., *Mycobacteria*, and *Campylobacter jejuni*) [18–22], Pathogenic protozoa (e.g., *Cryptosporidium parvum*) [19,23], invertebrates (e.g., *Asellidae*) or worms (e.g., *Annelida*) [24]. The consumption of contaminated water with pathogenic biofilms poses a health risk to the consumers of tap water [6,25,26]. Biofilm microorganisms can cause the following diseases and infections, such as: pulmonary cystic fibrosis, typhoid fever, shigellosis, otitis media, periodontitis, endocarditis, urinary tract infection, and chronic osteomyelitis [22,27–31]. Biofilm present in drinking water distribution systems is a key factor necessary to understand and control the microbial risk of drinking water [12,32,33]. However, the current legal framework does not oblige to control the composition and stability of biofilm present in distribution systems [34].

Due to the fact that the study of biofilms in real DWDS is complicated, many reactors and laboratory simulated DWDS have been developed for this purpose [4,35–39]. These devices enable the formation of a biofilm under controlled physicochemical and microbiological conditions that ensure operational conditions similar to those prevailing in DWDS. The following methods can be used for the quantification of biofilm microorganisms: (1) standard heterotrophic plate count (HPC) bacteria using the spread plate technique on agar, (2) ATP bioluminescence assay (by measuring the level of adenosine triphosphate (ATP)) [10,34], (3) flow cytometry (by determining the concentration of bacteria in fluids) and (4) by direct counting of bacteria using fluorescent dyes (e.g., acridine orange) [14,16]. Detaching the biofilm from the surface of the tested installation material is the most complicated step in the entire process. For this purpose, the following physical methods can be used, i.e., sonication of materials with a formed biofilm [10,33,40], removing the biofilm with a sterile cotton swab, scraper or knife [6,34,39,41–43], shaking with the use of glass beads [16] or centrifuged [44]. However, there are still many doubts as to which of the mentioned methods most effectively separates biofilm from the porous structure of materials. The literature lacks information on the power of the sonicator and the duration of the ultrasound, which makes it impossible to repeat and compare the obtained results of the analyses.

The aim of the research was to develop an effective method of detaching the biofilm from the material in order to carry out a quantitative analysis of biofilm microorganisms. The paper presents the advantages and disadvantages of the applied microbiological methods for the quantitative determination of the number of microorganisms.

2. Materials and Methods

Subject of study: The galvanized steel coupons with dimensions: 1 cm × 1 cm × 0.1 cm were placed in 150 mL of untreated surface water. The coupons were incubated in the dark at 19–22 °C for 40 days to enable the formation of biofilm. It was assumed that biofilm was formed on both sides of the coupons, hence the colonized area was 2 cm² (sides omitted). The biofilm was detached from the coupons by: ultrasound (method 1) and sterile swab and ultrasound (method 2).

Detachment of the biofilm: After 40 days, the coupons were placed in a 30 mL of physiological fluid, i.e., (1) in Ringer's solution (8.5 g NaCl/L, 0.044 g Na₂HPO₄/L, 0.023 NaH₂PO₄/L) or (2) in PBS solution (8 g NaCl/L, 0.2 g Na₂HPO₄/L, 1.44 g NaH₂PO₄/L, 0.2 g KCl/L) and sonicated. A Vibra-cell Labo Plus 28 sonicator (ultrasonic power was 28 W) was used to transfer the cells from the coupons to the test solution, and the sonication time was: 30, 60 and 75 s. The biofilm was detached from the coupon also with a sterile cotton swab, which was placed in 30 mL of Ringer's solution and sonicated (60 s).

Quantitative determination of biofilm microorganisms:

- (1) HPC methods using A agar from BTL Sp. z o.o. Department of Enzymes and Peptons, catalog number P-0231 (composition: meat extract 2 g/L, yeast extract 2 g/L, glucose 10 g/L, peptone 5 g/L, sodium chloride 4 g/L, agar 15 g/L, and pH 7.2 ± 0.2), and R2A agar from Thermo Scientific with catalog number CM0906 (composition: yeast extract 0.5 g/L, starch 0.5 g/L, glucose 0.5 g/L, peptone 0.5 g/L, KH₂PO₄ 0.3 g/L, MgSO₄ 0.024 g/L, sodium pyruvate 0.3 g/L, agar 15 g/L, and pH 7.2 ± 0.2 at 25 °C).

- To determine the number of bacteria present on agar A, the samples were incubated at 37 °C for 48 h (mesophilic bacteria) and at 22 °C for 72 h (psychrophilic bacteria). When using R2A agar, the incubation time was extended to 7 days. The results converted to CFU/mL/cm² (taking into account the surface of the coupons of 2 cm²).
- (2) ATP value was analyzed using a luminometer (LuminUltra Photonmaster Luminometer, Fredericton, JCT, Canada), 100 uL of the sample was put in a sterile, polystyrene tube. For the determination of ATP(o)—the total number of microorganisms, 100 uL I changed it. of enzyme (BacTiter-Glo Microbial Cell Viability Assay) was injected into the sample by the automatic dispenser. For the determination of ATP(z)—the number of dead microorganisms, the test sample firstly was passed through a sterile MCE membrane filter and then the enzyme was delivered (this operation enabled the living organisms to remain on the surface of the filter so that it was possible to identify intracellular ATP(I) (ATP(I) = ATP(o)-ATP(z)). To enable proper dissolution and reaction of enzyme in the sample, the injection of enzyme was delayed by 30 s. A delay of 2 s was used between the injection of enzyme and the measuring time. The obtained values in RLU (Relative Light Units) were converted to RLU/mL/cm² (taking into account the surface of 2 cm²).
 - (3) Flow cytometry (FC) analysis was performed using a Cy Flow Cube 8 cytometer (Sysmex Partec GmbH, Görlitz, Germany), 2 mL of the sample was put into a sterile, polystyrene tube and then 20 µL of fluorescent dye was added. The dye SYBRGreen was used for measurements of the total number of microorganisms, while dye propidium iodide enabled the number of dead organisms to be determined. Before analysis, the samples were subjected to 10 s vortexing and incubated at 37 °C for 10 min. The obtained values were converted to number of particles/mL/cm² (taking into account the surface of 2 cm²).

The presence of the biofilm was further confirmed: (1) by observation, using an electron scanning microscope JOEL SEM 5500 LV (JEOL Ltd., Tokyo, Japan) and (2) by estimate of fractal dimension, using a Taylor Hobson laser profilometer Talysurf CLI 1000 (Taylor Hobson Ltd., Leicester, UK) with TalyMap and FRAKTAL_Dimension2D software (J. Konkol, FRACTAL_Dimension2D, version 1.0, a program, 2000, Poland). The principle of operation of this method consists in enclosing each section of the profile by a box of width ε and calculating the area $N(\varepsilon)$ of all of the boxes enclosing the whole profile. The total height of the roughness profile (P_t) was also determined for each profile. The profiled lines with a length of 5 mm separated from the surface of the coupons were determined with a discretization step of 0.5 µm. This procedure is iterated with boxes of different widths to build a graph $\ln(N(\varepsilon))/\ln(\varepsilon)$.

3. Results

3.1. Detachment of the Biofilm

3.1.1. The Effect of Sonication Time on the Efficiency of Biofilm Detachment

The duration of the ultrasound is an important parameter in detaching the biofilm from the surface of coupons. Due to the strong bond between the surface of the material and bacteria, external disturbances can cause severe cell damage. Too long exposure to ultrasound can reduce cell viability, while with shorter sonication time, detachment of bacterial cells is reduced.

Research has shown that the highest number of microorganisms, regardless of the counting method, was recorded using a 60 s sonication time. The mean values of ATP(o) and ATP(z) after 60 s of sonication were 22,303 and 8902 RLU/mL/cm², respectively. The difference between total and extracellular ATP, indicating the number of live microorganisms, was on average 13,401 RLU/mL/cm² (Figure 1). In the remaining cases, the average number of live microorganisms was: 4256 RLU/mL/cm² for 30 s and 7130 RLU/mL/cm² for 75 s.

Figure 1. Average ATP value (a) and average number of microorganisms measurement by flow cytometry (b).

The quantitative analysis of the biofilm determined by flow cytometry with the SYBRGreen fluorescent dye showed slight differences in the obtained values. For 30, 60 and 75 s sonication the mean values were: 9,168,305, 9,067,320 and 8,873,200 particles/mL/cm², respectively. However, the number of living microorganisms was the highest in the case of 60 s sonication, being 8,832,095 particles/mL/cm² (for 30 and 75 s it was: 1,696,297 and 853,157 particles/mL/cm², respectively (Figure 1).

The highest number of microorganisms determined using standard HPC methods was also recorded for the 60 s sonication (Figure 2). R2A agar compared to A agar stimulated the growth of a higher number of mesophilic and psychrophilic bacteria (the results obtained for both substrates differed by two orders of magnitude). The low nutrients in the medium give higher counts than the highly nutritious medium. The effect of ultrasound could damage the bacterial cells of the biofilm, and therefore the longer incubation time (7 days) could provide better conditions for the development of damaged and stressed microorganisms. The average number of mesophilic and psychrophilic bacteria recorded in the sample subjected to 60 s sonication was 14,600 and 12,800 CFU/mL/cm², respectively (Figure 2).

Figure 2. Average number of mesophilic and psychrophilic bacteria on A agar (a) and R2A agar (b).

The optimal ultrasonic parameters should make as many cells as possible transfer and maximize their activity. After analyzing the obtained results, it was found that the optimal time enabling effective separation of the biofilm from the coupons, while not causing disintegration of bacterial cells, is 60 s.

The minimum, maximum, and average values of the quantitative analysis of microorganisms detached from the surface of the material depending on the sonication time are presented in Table 1.

Table 1. Summary of the results of the quantitative analysis of the biofilm depending on the used sonication time (n = 9).

Value	The Ultrasound Duration											
	30 s				60 s				75 s			
	ATP ¹	FC ²	HPC ³ Mesophilic Bacteria	HPC ⁴ Psychrophilic Bacteria	ATP ¹	FC ²	HPC ³ Mesophilic Bacteria	HPC ⁴ Psychrophilic Bacteria	ATP ¹	FC ²	HPC ³ Mesophilic Bacteria	HPC ⁴ Psychrophilic Bacteria
MIN	9265	7,687,180	6200	1700	19,500	7,795,600	11,500	12,000	12,270	8,000,540	10,600	3200
MAX	14,888	10,950,105	10,300	3100	24,724	10,106,660	17,700	13,600	19,414	9,989,095	14,900	5700
M	12,328	9,168,305	8167	2400	22,303	9,067,320	14,600	12,800	16,428	8,873,200	12,750	4450
Me	12,830	8,867,630	8000	2400	22,685	9,299,700	14,600	12,800	17,601	8,629,964	12,750	4450
SD	2845	1,652,112	2055	990	2633	1,172,924	4384	1131	3714	1,016,347	3041	1768

¹ ATP bioluminescence assay—ATP(o), (RLU/mL/cm²); ² Flow cytometry analysis with dye SYBRGreen, (particles/mL/cm²); ³ Number of mesophilic bacteria (R2A agar), (CFU/mL/cm²); ⁴ Number of psychrophilic bacteria (R2A agar), (CFU/mL/cm²).

3.1.2. The Effect of Physiological Solutions on the Viability of the Biofilm Microorganisms

A study by Waller et al. [10] showed that the method of sonication in PBS solution was characterized by high efficiency of separating the biofilm from the surface of the tested materials [10]. Whereas Beo-Hansen et al. [42] recommend collecting the attached biofilm bacteria using a sterile cotton swab. Therefore, in this work, it was decided to carry out a further stage of the research consisting of testing various salt solutions (i.e., Ringer's and PBS solutions). The obtained test results allowed the determination of which of the used solutions is more effective in maintaining the viability of microorganisms (this is especially important in the case of HPC methods). The ultrasound duration was 60 s.

The obtained results indicate that the applied saline solutions had an impact on the viability of detached bacterial cells. The ATP bioluminescence assay showed significantly higher the average values of ATP(o) (22,205 RLU/mL/cm²) and ATP(z) (9755 RLU/mL/cm²) for the Ringer's solution. The number of living organisms, in this case, was: 12,450 RLU/mL/cm² (Figure 3). These values were comparable with the results obtained in the previous stage of the research, which proves the correctness and repeatability of the analyzes performed.

Figure 3. Average ATP value (a) and average number of microorganisms measurement by flow cytometry (b) using different methods of biofilm detachment.

Then, the biofilm was detached using a sterile cotton swab using Ringer's solution and 60 s sonication (method II). On the basis of the obtained results, it was found that method II was most effective in detaching bacterial cells from the coupon (the average value of ATP(o) was 22,650 RLU/mL/cm², while ATP(z) was 11,807 RLU/mL/cm²) (Figure 3). The

number of living organisms was: 10,845 RLU/mL/cm². The results were similar to the measurements made for the variant: Ringer's solution + sonication for 60 s.

The quantitative analysis of the biofilm using flow cytometry shows that the highest number of microorganisms was recorded in method II (similar to the ATP analysis) (Figure 3). As a result of the use of the PBS solution, lower mean values were obtained (the total number of living and dead microorganisms was 5,115,830 particles/mL/cm²), which confirms the usefulness of Ringer's solution in the quantification of biofilm.

As in the earlier stage of the study, the number of bacteria grown on A agar was two orders of magnitude lower than on R2A agar. The number of mesophilic bacteria obtained on agar A was similar in all variants (35–30 CFU/mL/cm²) (Figure 4). On the other hand, the highest number of psychrophilic bacteria was recorded for the variant with Ringer's solution and 60-s exposure to ultrasound (80 CFU/mL/cm²) (Figure 4). The results of the standard heterotrophic plate count method on R2A agar obtained by method 2 (using a sterile swab) were lower than in the other variants (the average value of mesophilic and psychrophilic bacteria was: 1150 and 1950 CFU/mL/cm² (Figure 4).

Figure 4. Average number of mesophilic and psychrophilic bacteria on A agar (a) and R2A agar (b) and using different methods of biofilm detachment.

The minimum, maximum and average values of the quantitative analysis of microorganisms depending on the method of biofilm detachment are presented in Table 2.

Table 2. Summary of the results of the quantitative analysis of the biofilm depending on the method of detachment used (n = 9).

Value	PBS Solution				Ringer's Solution				Swab + Ringer's Solution			
	ATP ¹	FC ²	HPC ³ Mesophilic Bacteria	HPC ⁴ Psychrophilic Bacteria	ATP ¹	FC ²	HPC ³ Mesophilic Bacteria	HPC ⁴ Psychrophilic Bacteria	ATP ¹	FC ²	HPC ³ Mesophilic Bacteria	HPC ⁴ Psychrophilic Bacteria
MIN	9490	3,318,150	8100	9100	18,709	7,192,950	10,000	20,000	20,666	6,607,820	950	1680
MAX	13,100	6,499,650	9800	10,600	25,835	9,558,085	14,000	24,500	25,403	8,123,380	1500	2170
M	11,620	5,115,830	9100	9750	22,205	8,270,675	11,500	22,000	22,650	7,300,500	1150	1950
Me	12,269	5,529,690	9400	9550	22,070	8,060,990	10,500	21,500	21,880	7,170,300	1000	2000
SD	1891	1,630,627	889	770	3565	1,196,429	2179	2291	2461	766,123	304	249

¹ ATP bioluminescence assay—ATP(o), (RLU/mL/cm²); ² Flow cytometry analysis with dye SYBRGreen, (particles/mL/cm²); ³ Number of mesophilic bacteria (R2A agar), (CFU/mL/cm²); ⁴ Number of psychrophilic bacteria (R2A agar), (CFU/mL/cm²).

3.2. Characterization of the Sterile Coupon and Coupon with Biofilm

The galvanized steel control sample had an irregular structure with a large number of overlapping lumps of various sizes. The surface of the tested material, due to its roughness, promotes the formation of biofilm, but may also hinder its detachment. SEM photos of the installation material, taken after 40 days of contact with water, confirm the presence of biofilm (Figure 5). The test sample was completely covered with biofilm with a complex structure.

Figure 5. The structure of the sterile coupon (a) and coupon with formed biofilm (b).

Table 3 presents the results of the fractal analysis containing the measurement of the fractal dimension D value and the total height of the roughness profile. Both parameters were measured for the surfaces of the sterile coupon and the coupon with the biofilm formed.

Table 3. Morphology of the surface of sterile coupon and coupon with formed biofilm.

Parameters	Sterile Coupon	Coupon with Biofilm
Fractal dimension $D \pm$ standard error (-)	1.23 ± 0.016	1.18 ± 0.015
Total height of the roughness profile $Pt \pm$ standard error (μm)	83.6 ± 6.2	393.9 ± 23.4

The increase in the fractal dimension of the surface of this sterile coupon ($D = 1.23$) compared to the fractal dimension of the surface of the coupon with biofilm ($D = 1.18$) indicates the presence of bacterial cells on this surface. The biofilm stratification is visible on a scanned fragment of the material surface (Figure 6). Moreover, the analysis of the shape of the profile lines and a definite change in the total height of the roughness profiles indicate a significant presence of microorganisms on the tested coupon (Table 3, Figure 7). On the basis of the total height of the roughness profile, the thickness of the biofilm layer can be estimated to be around 300 μm (the difference between the total height of the roughness profile of the biofilm material and the reference material).

Figure 6. The surface of the sterile coupon (a) and coupon with biofilm (b).

Figure 7. Representative profile lines for the sterile coupon (a) and coupon with biofilm (b).

4. Discussion

The presented techniques of quantitative determination of microorganisms using three different microbiological methods allowed the demonstration of their advantages and drawbacks. The HPC methods (Agar A and R2A) in determining the microbiological quality of drinking water do not reflect the exact and real number of microorganisms, as a significant part of bacteria does not proliferate well in laboratory conditions (only specific fractions of the microbial populations were detected, which usually constitute <1% of all bacteria) [45,46]. This study showed that R2A agar stimulated the growth of a higher number of microorganisms compared to A agar. Similar results were obtained in the studies [47,48]. The composition of Agar R (with reduced peptone, yeast extract and dextrose level) combined with an extended incubation time stimulates the growth of a higher number of heterotrophic bacteria [47]. Therefore, for future studies, the use of R2A medium for microbial culture should be considered. Thus, the selection of an appropriate growth medium is an essential element in microbial cultures as a foundational diagnostic method. Whereas, the drawback of this method is the relatively long time required to obtain a result (up to 7 days). Moreover, the culture methods are labor-intensive, time-consuming and very complex procedures which also generate significant costs [49]. Besides, any changes in incubation conditions affect the number and composition of cultured bacteria [46,50]. Normative values for HPC for drinking water are required according to legislation. However, in some countries, maximum HPC values are being replaced by the “no abnormal change” (NAC) guideline, while Canada and Australia have excluded their upper limit of HPC in drinking water [46]. Flow cytometry (FC) is an advanced technique used to detect and measure populations of cells. For the reason that FC allows for quick (results obtained within 15 min) and accurate determination of the number of bacteria in the analyzed samples [46], the technology has applications in a number of fields, including drinking water [49,51,52]. The FC may also be used to measure the

number of viable cells in the entire population, e.g., to assess the effectiveness of chemical disinfection [53]. This is possible due to the quantitative and qualitative characteristics (cell counting, cell sorting, determining cell characteristics and function) of the tested cells based on the content of nucleic acids in the cells. This allows the analysis of mixed populations of microorganisms that occur in water [54]. The discrepancies in the obtained data may result from the properties of the fluorochromes used for analysis. Literature shows many comparisons between data obtained from FC and HPC and indicates that FC is more appropriate as a method for determining the microbiological quality of drinking water, thus contest the further use of the HPC method for such application [46,55–58]. Furthermore, a notable alternative method is the ATP test, a process of rapidly measuring actively growing microorganisms through ATP detection (a molecule found in and around living cells) in the water sample. Studies published by [59,60] confirm the effectiveness of this method. Since ATP is quantified by measuring the light produced through its reaction with the naturally occurring enzyme luciferase using a luminometer, the amount of light produced is directly proportional to the amount of ATP present in the sample. Therefore time is the main limitation as fluorescence is highly unstable. Compared to the cytometric method, the ATP test does not require large outlays, experience in operating the apparatus or interpreting the results. The presence and multiplication of microorganisms, including opportunistic pathogens, in water systems is an increasing threat to public health [49]. The ability to establish an effective method to analyze biofilm is a key issue today. DWDS installations are constructed of various materials, creating complex environments that differ chemically, microbiologically and spatially, this highly affects the survival and development of biofilms. All used pipe materials, depending on the present conditions on the spot, may stimulate or inhibit the growth of microorganisms. Most of the studies in the literature, describe the methods of determining the composition of biofilm while there is still no effective and simple method regarding the procedure on effective acquisition and collection of the material under actual conditions. In view of the fact that the access to the actual water pipes is limited, the process of biofilm detaching directly from the surface of the installation material is the most complicated and complex stage of the entire study. Moreover, it is extremely difficult to collect a biofilm sample from a selected surface without damaging its original structure. The method presented in this study, consisting of the physical separation of the biofilm from the material surface with the use of ultrasound, may be useful in tests carried out on samples of materials (plates) prepared on a laboratory scale. However, in real conditions, this method most likely might be inadequate and impractical. Thus, the method with the use of a sterile swab allows for quick biofilm sampling from the surface of the pipes (from internal installations or water supply networks). Therefore, the practical application of this method, requires a tailored flexible form with rectangular holes of given dimensions. The flexibility of the element allows to place it on the inner surface of the pipe and collect the biofilm using a cotton swab. In the future, it is planned to develop semi-technical scale testing on samples of various materials (coupons) placed in such intended installation. However, the use of a sterile swab generates difficulties related to the effectiveness of washing out microorganism cells from it. Many factors, including ultrasound parameters and duration of the sonication process, result in the effectiveness of separating the biofilm from the installation material. The sonicator used in this study was 28 W, while the results of other studies presented in the literature show that the lower power (6.5, 13 and 39 W) provide a higher microbial growth rate than the higher power (78, 97.5 and 130 W) [33]. It was found that the number of living microorganisms depends on the time spend under ultrasound treatment. Furthermore, a longer duration of ultrasound treatment destroys more active cells of the biofilm. Therefore, it was established that the optimal treatment time was 1 min. Peng et al., within 1–2 min of ultrasound treatment, obtained an average of 5.67×10^7 CFU/mL, and by increasing the time up to 3–5 min and 7 min, the number of living and metabolically active microorganisms decreased to 38% and 70%, respectively. This tendency is also confirmed by other studies [61]. Thus, the precise

selection of ultrasound treatment parameters indicates a positive effect on the increasing amount of detached bacterial cells from the biofilm.

5. Conclusions

Drinking water safety is a serious problem worldwide and biological stability is a criterion for guaranteeing good water quality. The quantitative analysis of biofilm can be used not only to assess the microbiological stability of tap water but also at its basis can assess: the degree of colonization of materials by bacterial cells, the rate of biofilm formation on the surfaces of pipes and determine their composition and number.

Detaching the biofilm from the surface of the installation material is the first stage of the analysis but at the same time the most complicated and problematic. Although biofilm has a much higher cell density than the DWDS waterline, detachment of cells is not an easy task. Based on the research, it was found that:

- the most effective method of detaching bacterial cells from the surface of the coupons is a mechanical method using a sterile cotton swab, which is then subjected to ultrasound;
- the optimal time of exposure to ultrasound was 60 s (for the sonicator power of 28 W). At that time, the highest values obtained by the ATP bioluminescence assay, flow cytometry methods and the HPC method were recorded, while at the same time guaranteeing the least destruction of the bacterial cell);
- the use of buffered saline (Ringer's solution) allowed to extend the viability of the microorganisms detached from the coupon surface compared to the PBS solution.

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