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Heterotrophic Plate Count for Bottled Water Safety Management

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Abstract: Heterotrophic bacteria are able to form biofilms in water processing systems, adhering to pipe materials and colonizing surfaces. The aim of our research was to identify the critical points in the process of bottled water production at which controls can be applied to prevent, reduce, or eliminate water safety hazards. Microbiological monitoring was conducted using the plate count method and luminometry. To identify the bacterial isolates, we used polyphasic identification based on biochemical tests and molecular analysis using ribosomal RNA. The heterotrophic plate counts were higher in the water filtration station, ultrafiltration (UV) disinfection station, and holding tank. At these points of the industrial process, the water is stagnant or there is poor flow. Molecular analysis identified the bacterial isolates as belonging to *Acinetobacter*, *Agrobacterium*, *Aeromonas*, *Brevundimonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Pantoea*, and *Rhizobium* genera. Bacterial isolates showed various levels of biofilm formation, and the best adhesion properties were exhibited by the *Aeromonas hydrophila* and *Citrobacter freundii* strains.

Keywords: bottled water; technology; biofilm; heterotrophic plate count; bacteria

1. Introduction

The quality of drinking water is a worldwide concern and has a great impact on human health. In Europe, consumer demand for mineral water is increasing, including for bottled waters containing varying amounts of minerals, such as magnesium, calcium, potassium, or sodium. Estimated per capita, the consumption of bottled water in the European Union increased from 43 L in 2003 to 119 L in 2018 [1]. Mineral waters are recommended for patients with immune-system deficiencies and kidney disorders, as well as for those with urinary and heart diseases. It is therefore important to make sure the water is safe to drink, otherwise bacteria in the water may harm consumers. Moreover, bottled water is often shared between persons, and as a consequence microorganisms including opportunistic or true pathogens may be transferred by this route [2,3].

Bottled waters may be derived from natural spring water or from processed water. Spring water may be bottled directly, without any processing, or it may be subjected to a number of processes to modify its physical, chemical, biological, and microbiological properties. The type of treatment procedure depends on the origin and quality of the raw source [4]. Often, particulate matter is congregated with coagulants, then the water is mixed with clarifiers. Larger particles of sediment and smaller particles that are left after clarification are directed to the filtration system. Finally, various disinfection methods (ultrafiltration, UV) may be used [2].

Water quality does not depend solely on the raw water. It is also affected by the formation of biofilms in installation systems. Dissolved organic compounds in drinking water are responsible for the growth of heterotrophic bacteria, which colonize installation materials. Heterotrophs are microorganisms that require organic carbon for proliferation. This term includes bacteria and fungi capable of growing in a

water environment. They are therefore counted among the normal, saprophytic water microorganisms. However, under certain circumstances pathogenic or opportunistic microorganisms can also appear from diverse sources. The main source of heterotrophs is raw water. Microbial cells can also enter water networks during the repair or replacement of sections of the installation, as well as during failures that lower pressure. As a consequence, biofilms may harbor and support the proliferation of pathogenic cells, thereby contributing to the spread of waterborne diseases [3,5].

Heterotrophic plate count (HPC) is a parameter that quantifies the formation of biofilm in water systems. It has been widely adopted as a standard and simple technique for microbiological testing and safety management of drinking water [6]. The HPC method has a long history in drinking water microbiology. At the end of the nineteenth century, HPC was used as an indicator of the proper functioning of water treatment systems (filtration, disinfection), and as an indirect indicator of water safety. In the twentieth century, the use of HPC as a safety indicator declined due to the application of fecal indicators, namely coliforms and enterococci. Nevertheless, HPC measurements and limits are still included in the water regulations and guidelines of many countries [4].

Bottled water is a specific environment for the multiplication of microorganisms. It may contain natural or added carbon dioxide, which restricts microbial growth. However, no long-acting disinfectants are present. Moreover, the final product is often exposed to elevated (room) temperatures, for a few days or weeks before consumption. The type of packaging used, such as plastic bottles, can also affect the development of spoilage microorganisms [7]. Therefore, the levels of HPC recovered from bottled water in the distribution period can be significantly higher than those found in water from industrial systems before or shortly after bottling [4,8].

Given the importance of ensuring the safety of drinking water and identifying potential microbial contaminants, this study was carried out to assess the microbiological quality of bottled water and of the industrial distribution system. To our knowledge, there have been only a few previous studies on the bacteriological quality of both the installation lines and the final bottled water [9,10]. In particular, we analyzed the critical points that influence the microbial quality of processed water. Since the bacteriological quality of drinking water is highly dependent on the bacterial species present, the dominant heterotrophic bacteria were identified along with their capacity for biofilm formation.

2. Materials and Methods

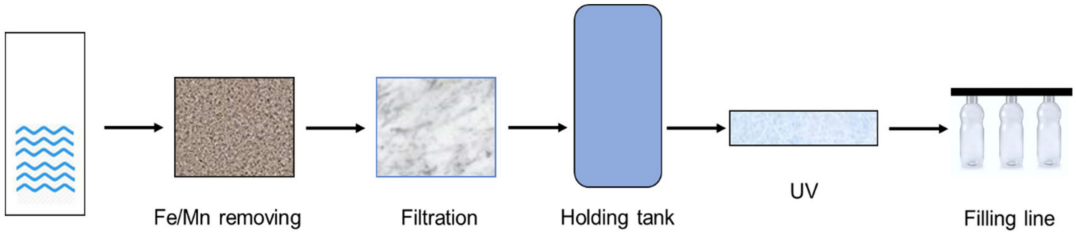
2.1. Water Analysis

In total, 30 samples of spring bottled water (volume 500–1500 mL) were subjected to HPC analysis: 10 samples of still mineral water, 10 samples of lightly carbonated water (<1500 mg/L CO_2) and 10 samples of carbonated water (>1500 mg/L CO_2). All the samples had been packaged in polyethylene terephthalate (PET) bottles and sourced from a single Polish manufacturer during two summer months. All cleaning and sanitization procedures in the plant were conducted according to detailed procedures and an internal CIP (Cleaning In Place) program.

All the bottled waters were stored at 20–25 °C for at least two weeks after bottling. At the same time as the bottled water was being produced, 23 samples of processed water were also collected. The processed water was taken from six different collection stages: (i) raw water; (ii) water after Fe/Mn removal; (iii) water after microfiltration; (iv) water from a holding tank; (v) water after UV disinfection; (vi) water before filling (bottling) (Table 1, Figure 1). In this technological process, carbonization was performed just before filling.

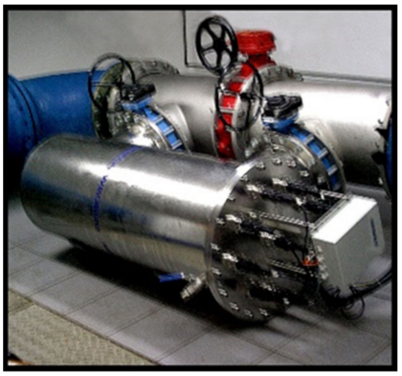
Table 1. Processed water samples used in the research.

Collection Stage	No of Samples
Raw water	3
Clarification (Fe/Mn removal)	3
Filtration	3
Holding tank	5
UV station	5
Filling	4



(**a**)

(**b**)



(**c**)

Figure 1. Bottled water processing: (**a**) general scheme; (**b**) holding tank; (**c**) UV station.

For HPC analysis, the samples were agitated by vortexing, filtered (0.45 µm-pore-size filter, Merck KGaA, Darmstadt, Germany), plated on R2 Agar medium (Merck KGaA, Darmstadt, Germany), and incubated for 2 or 3 days at two different temperatures, 37 °C and 22 °C, respectively. All the tested samples were also analyzed for sanitary indicators (coliforms/*Escherichia coli*, enterococci, *Pseudomonas aeruginosa*) [11]. The agar media and incubation conditions for the inoculated plates are shown in Table 2.

Table 2. Agar media and incubation conditions.

Detection	Agar Medium	Temperature [°C]	Time [Day]
HPC	R2 Agar, TSA Agar	22, 37	3, 2
Coliforms/*E. coli*	Chromocult Coliform Agar	37	2
Enterococci	Slanetz-Bartley Agar	37	2
P. aeruginosa	Cetrimide Agar	37	2
Pseudomonas /*Aeromonas* sp.	GSP Agar	25	1

2.2. HPC Isolation and Identification

Characteristic slimy colonies were picked up from the agar plates, restreaked to ensure purity, and cultivated on TSA slants (Merck KGaA, Darmstadt, Germany) at 22 °C. The following

biochemical methods were used to identify the bacteria: Gram staining, the aminopeptidase test (Bactident® Aminopeptidase, Merck KGaA, Darmstadt, Germany), the oxidase test (Bactident® Oxidase, Merck KGaA, Darmstadt, Germany), and the catalase test (Bactident® Catalase, Merck KGaA, Darmstadt, Germany). The growth and morphology of the bacteria were also analyzed on agar plates and under a microscope. Identification of isolates was confirmed using the PCR technique described previously [12]. The 16S rRNA gene sequences were compared with those obtained from the NCBI database, using the program BLASTN 2.2.27 + (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were deposited in the GenBank with accession numbers.

2.3. Assessment of Bacterial Adhesion

Bacterial adherence was evaluated under laboratory conditions. Experiments were conducted using white glass as the reference hydrophilic material (Star Frost 76 × 26 mm, Knittel Glass, Germany). Sterile glass carriers were placed in sterile 25 mL Erlenmeyer flasks with 20 mL of 50-fold diluted buffered peptone water (Merck KGaA, Darmstadt, Germany). The amount of bacterial inoculum (0.1 mL) was standardized densitometrically (1°McF). The samples were incubated at 22 °C with agitation on a laboratory shaker (135 rpm) for 6 days. Cell adhesion to the carriers was evaluated using luminometric sampling pens (Merck KGaA, Darmstadt, Germany). The measurements were expressed in RLU/cm^2 using a HY-LiTE2 luminometer (Merck KGaA, Darmstadt, Germany) [13,14].

2.4. Statistical Methods

The results were calculated as the means and standard deviations in the data from three independent tests. Analysis of variance (ANOVA) was used to examine the differences between group means representing the adhesion results (OriginLab Corporation, Northampton, USA). The results were compared to those for the control samples (without inoculation). Values with letters show statistically significant differences: a, $p \geq 0.05$; b, $0.005 < p < 0.05$; c, $p < 0.005$.

3. Results and Discussion

3.1. Bacteriological Analysis of Processed Water and Bottled Water

The results of bacteriological analysis of the processed water are presented in Figure 2. Sand filtration was found to have a negative effect on the HPC, with the number of detected bacteria increasing by up to 100-fold. In addition, more HPC bacteria were detected in the holding tank and UV station. At these points in the technological process, the water is stagnant or the flow is very poor. This suggests that that water processing may promote growth of the detected HPC. The WHO reports that elevated HPC levels are particularly likely to occur in the various parts of distribution systems where the water is stagnant [15]. A similar tendency has been observed by Wang et al. [16], who found that the filtration processes in advanced drinking water treatment systems promote the development of HPC bacteria considerably. Hydrodynamics impacts the interactions between free microbial cells and regulates biofilm formation in drinking water systems. It can either promote biofilm formation (in the case of water stagnation or weak flow) or induce bacteria to escape from the biofilm matrix (in the case of rapid velocity and turbulence) [17,18].

The HPC results indicate that the numbers of bacterial colonies in all the tested types of bottled water exceeded 100 CFU/100 mL after incubation at both 22 °C and 37 °C (Figure 3). The HPC determined at 22 °C was between 450 and 266 CFU/100 mL, for still water and carbonated water, respectively. Therefore, carbonation contributed to a slight reduction in the number of microorganisms determined by the plate count method at 22 °C. Smaller differences were noted at 37 °C.

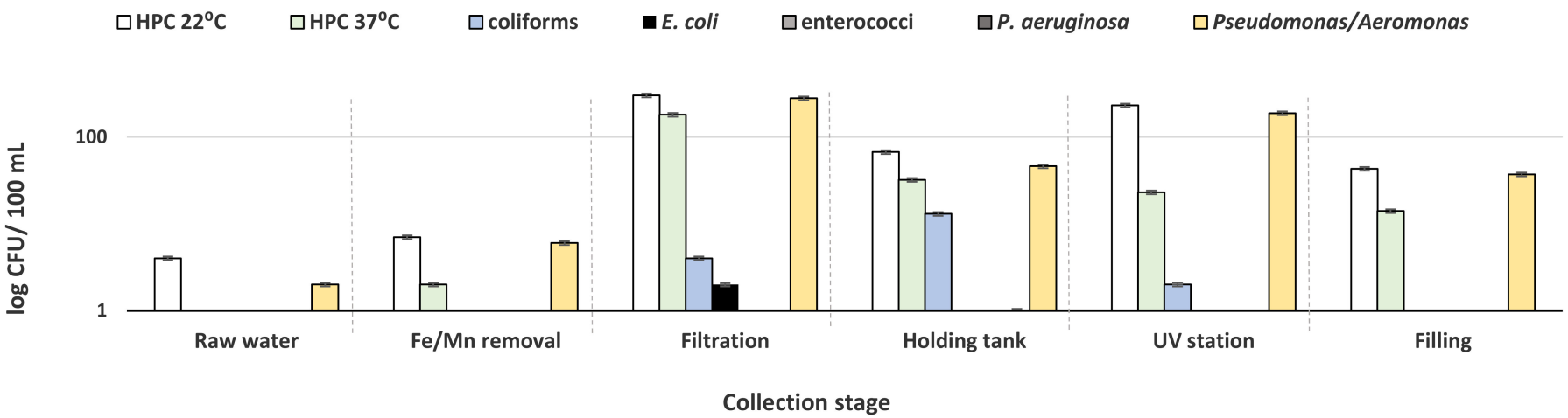


Figure 2. Bacteriological analysis of processed water.

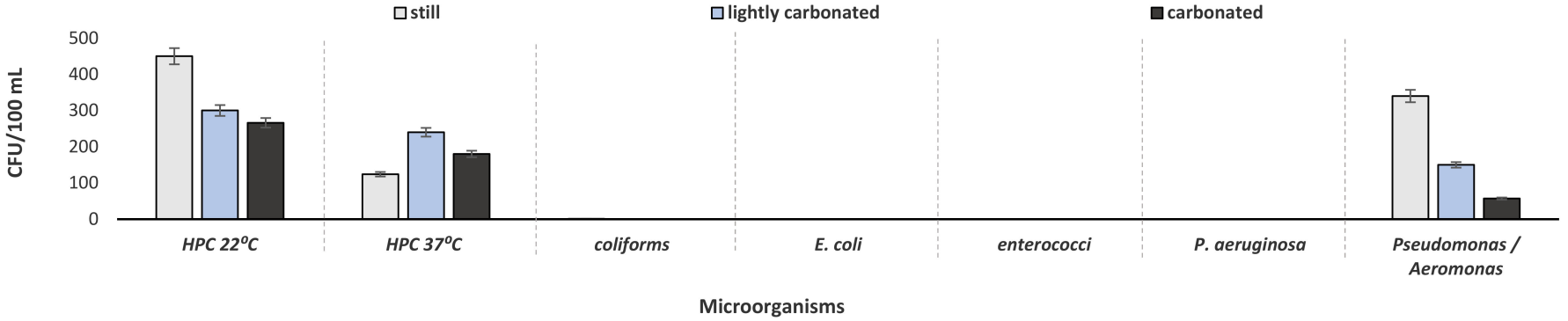
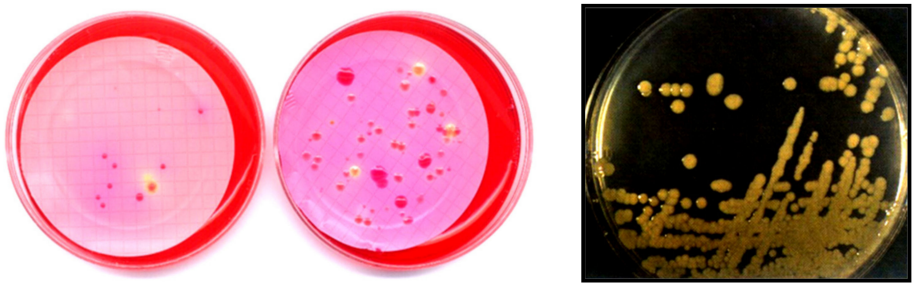


Figure 3. Bacteriological analysis of bottled water.

At 22 °C, the HPC results are described as being of little sanitary value, but are a good indication of the efficiency of water treatment. However, some HPC increases at 22 °C may be attributed to the aerobic bacteria *Aeromonas* sp. or *Pseudomonas* sp., often associated with opportunistic infections. It was confirmed by the results on GSP agar. At 37 °C, the HPC results are formed mainly by indicator organisms, namely facultative anaerobes (coliforms) and other Enterobacteriaceae [4].

The utility of carbon dioxide in extending the shelf-lives of some food products has been well established [19]. Increased CO_2 levels are beneficial for reducing microbial growth directly, although facultative anaerobes such as coliform bacteria favor growth in an atmosphere high in CO_2 and proliferate due to the reduction in levels of competing aerobic microorganisms. Our study therefore confirms that CO_2 treatment may contribute to bacterial destruction, and was most effective against aerobic bacteria growing at 22 °C [20].

The typical microbiota found in the process and bottled water were Gram-negative rods belonging to *Pseudomonas*/*Aeromonas* genera. On TSA agar, the bacterial colonies varied from small (2 mm) and regular to large (6 mm) with irregular structures and edges. On the GSP agar, the bacteria were able to grow as regular red (*Pseudomonas* sp.) or yellow (*Aeromonas* sp.) colonies (Figure 4a). In total, 20 different bacterial isolates were obtained from the samples. The bacterial morphotypes were diverse, from small circular colonies to larger (4–5 mm) irregular colonies. Some of the colonies had a characteristic slimy appearance (Figure 4b).



(**a**) (**b**)

Figure 4. Growth of bacteria on: (**a**) GSP agar; (**b**) TSA agar.

The bacterial isolates were identified as Gram-negative bacteria. Ten bacterial monocultures forming characteristic slimy colonies were selected for molecular identification. The nucleotide sequences of the isolates were deposited in the GenBank (presented with accession numbers in Table 3).

Table 3. Bacterial strains isolated from the processed water system.

Isolate	Cell Shape	Gram	Oxidase	GenBank Number
A. johnsonii	[1] R	[2] N	N	KT751294
A. hydrophila	R	N	[3] P	KC756842
A. tumefaciens	R	N	P	KJ719245
B. vesicularis	R	N	P	KT751295
C. freundii	R	N	N	KJ995856
E. aerogenes	R	N	N	KJ995857
E. soli	R	N	N	KJ995858
K. oxytoca	R	N	N	KJ995859
P. agglomerans	R	N	N	KJ995860
R. giardinii	R	N	P	KT751297

[1] R—rods; [2] N—negative; [3] P—positive.

The bacterial isolates were identified as Acinetobacter johnsonii, Aeromonas hydrophila, Agrobacterium tumefaciens, Brevundimonas vesicularis, Citrobacter freundii, Enterobacter aerogenes, E. soli, Klebsiella oxytoca, Pantoea agglomerans, and Rhizobium giardinii. Some of isolates were

typical microbiota commonly found in water systems. Vaz-Moreira et al. [21] confirmed the presence in drinking water systems of the following Gram-negative rods: Blastomonas, Brevundimonas, Sphingomonas, Acinetobacter, Aeromonas, Enterobacter, and Pseudomonas. The following strains from the Enterobacteriaceae family were also detected: Citrobacter, Enterobacter, Klebsiella, and Pantoea. Some of these are opportunistic pathogens responsible for numerous infections [22]. These bacteria were detected in process/bottled water, although E. coli (a standard fecal contamination indicator) was not. Non-enteric Gram-negative rods were also detected in the processed water and bottled water. Agrobacterium and Brevundimonas originate in plants and soil [23,24]. A. hydrophila frequently colonizes water installation surfaces, ventilation systems, as well as medical equipment [13]. These bacteria can cause opportunistic infections (endocarditis, bacteremia, meningitis, respiratory and urinary tract infections) which are often difficult to treat. Despite their various taxonomies and biochemical characteristics, the bacterial isolates were able to form slimy colonies on the agar plates. It seems that this ability may facilitate aggregation and adhesion, and therefore stimulate biofilm formation in water installation systems.

3.2. Adhesion Abilities

Bacterial adhesion to the glass carriers was assessed in minimal medium. According to the literature, biofilm formation may be stimulated in water environments poor in organic carbon sources [25]. Adhesion ability is usually correlated strongly with the ability to form biofilm. Of course, initial adhesion does not ensure the ability of the tested drinking water-isolated bacteria to form mature biofilms, but it may make it much easier. Other important events, including phenotypic and genetic switching during biofilm development, the production of extracellular polymeric substances, and water stagnation, as well as the kind of abiotic surface, also play a significant role in biofilm formation and differentiation [26].

The physicochemical properties of the surface are an important factor in the adhesion of bacteria to inert surfaces. The degree of hydrophobicity, in particular, is correlated with biofilm formation. More hydrophilic surfaces usually reduce the growth of biofilms. In our study, we chose a glass surface for adhesion tests. Due to its high hydrophilicity, glass is similar to stainless steel, another inert surface commonly used in the food industry. However, it is worth noting that all surfaces are modified rapidly by immersion in water and adsorption of conditioning films, which change the properties of these materials [27].

In our study, the adhesion samples on glass were incubated with agitation on a laboratory shaker. The assumption was made that if bacteria have the ability to form biofilm on hydrophilic surfaces in water with regular flow, then under industrial conditions, where water may be stagnant, the formation of biofilms will be even more likely.

Figure 5 shows the results of cell adhesion expressed in Relative Light Units per cm^2 (RLU/cm^2). For each of the tested bacterial strains, various levels of cell adhesion can be observed. The RLU values are the highest for A. hydrophila and C. freundii at 138–142 RLU/cm^2. For the other strains, the RLU results are significantly lower, from 28 to 102 RLU/cm^2. In this monitored technological process, CO_2 saturation occured in the final stage before the filling of processed water, so it did not affect biofilm formation in the installation pipes. Therefore, the influence of carbon dioxide on biofilm formation was not studied in this research.

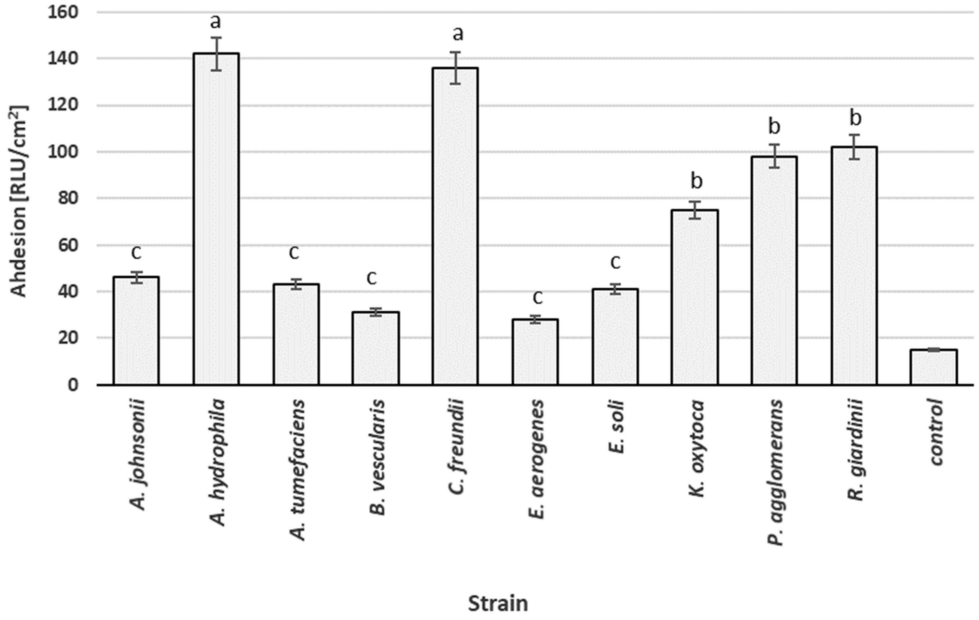


Figure 5. Adhesion of bacterial isolates to a glass surface after 6 days of incubation.

4. Conclusions

Biofilm formation by heterotrophic bacteria in various water distribution systems can lead to the contamination of drinking water and the transmission of various opportunistic pathogens. The WHO recommends the use of molecular techniques to identify specific heterotrophs of potential concern for human health [28]. The best strategy for water industries is to monitor HPC levels regularly. Most producers conduct regular scheduled reviews, and also test in case of failures. Such monitoring has a formal structure based on the international standard ISO 9000. In this study, HPC testing was used to identify the critical points in the production of bottled drinking water responsible for biofilm formation and contamination of the final products. Microbiological monitoring was conducted using the plate count method and luminometry. The identification methodology used in this study can be used for differential identification of microbiota in specific water environments. More research is needed to explore the potential role of the HPC method in reducing or preventing the colonization of water systems and products by harmful microorganisms.

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Conflicts of Interest: The authors declare no conflict of interest.

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