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Original Research

Microbiological Hazard Analysis of Car Wash Wastewater

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Abstract

Wastewater from car washes may contain many pathogenic microorganisms that can pose a hazard to human health through inhalation of bioaerosols generated during car washing. The aim of this study was to investigate which types of bacteria are present in the wastewater. Over 30 types of bacteria were determined in the examined wastewater, with the cells number at the level 2.86-3.71x10^6 CFU/mL. The complete inhibition of bacterial growth was observed for D-cycloserine and for a mixture of antibiotics (polymyxin B sulfate, Ceftazidime and Acryflavine HCl). *E. coli* bacteria were used to determine how the composition of chemicals used to wash cars affects their survival. It was found that the foaming agents (surfactants) do not have an antibacterial effect, unlike the alkaline agents used to remove insects (Insect). The chemicals present in wastewater promote bacterial mutation in the adaptation process. As a result, despite increasing the concentration of Insect solution to 0.5% and extending the contact time to 30 min the amount of bacteria cells in the wastewater decreased only from 2.93x10^5 to 5.56x10^2 CFU/mL.

Keywords: carwash effluents, microbiological toxicity, public health, surface-active agents

Introduction

Professional car washes are a relatively quick and easy way to remove dirt from motor vehicles. However, the process itself requires the use of a large capacity of water and the application of chemicals that produce toxic wastewater [1]. Such wastewater contains significant amounts of surfactants [2], as well as chlorides, phosphates, acids, heavy metals and various organic substances such as oil, grease, asphalt and microorganisms [3].

According to the current regulations, wastewater and pollutants from car washes should be pre-treated in a clarifier and oil separator before being sent to the next stage of a municipal wastewater treatment plant [3]. In other solutions, the wastewater is treated in several stages and the recovered water is returned to washing [4, 5]. Sand filtration, membrane separation (such as micro- and ultrafiltration or reverse osmosis and nanofiltration) and the methods of chemical oxidation, coagulation and biological processes have been used primarily to treat such wastewater [3, 6].

Microbial contamination poses a significant issue in the treatment process since as microbes can exist even under the extreme environmental conditions [7]. Because of this, clarifiers and other components

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of a wastewater treatment plant can be a suitable place for microorganisms to thrive. These difficulties lead to possible microbial contamination of the washing equipment when attempting to recover the water from wastewater. This finding was confirmed by studies describing the presence of microorganisms such as *Cryptosporidium* and *Giardia* in the water reused [8].

During the car wash activities the washing solution is discharged under a high pressure, which promotes aerosolization of this solution. Airborne bioaerosols formed contain pathogenic microorganisms in their composition, which pose a potential threat to the health of users and employees of car washes [9]. Therefore, a control and risk assessment of car washing with reused water are of great importance [10]. It is also recommended to monitor the concentration of pathogenic microorganisms in the air surrounding washed cars [11].

The degree of health risks caused by microorganisms depends on the type of washing technology used. The most common are hand washing, gate washing or tunnel washing [12]. In the case of hand and gate washes, the user comes into a direct contact with the washed car, which encourages the inhalation of bioaerosol [12]. In automatic (tunnel) car washes, the contact with the spray water is substantially limited, however, these systems generally use a closed water circuit [12], thus, they may contain more microorganisms.

Due to a very limited number of scientific papers focused on the microbiological hazards in car washes, the present study was carried out to investigate the type of microorganisms present in the wastewater produced during car washing. In addition, literature data was used to indicate the potential health risk to users. The chemicals present in wastewater can promote bacterial mutation in the adaptation process, thus, the effect of antibiotics on their survival was studied. The influence of the cleaning agents used to wash cars on bacterial growth was also examined.

Material and Methods

Investigation of Wastewater

In the present study, the wastewater samples were taken from a settling tank where the untreated effluents from car washing were collected. The analyzed samples came from two manual car washes (M1 and M2) and one automatic car wash (A1). Samples A1 and M1 were from a metropolitan car wash and M2 from a car wash located in an agricultural region. The manual car washes were supplied with tap water, while the automatic car wash used the recycled water (clarifier, de-oiling, biological pre-treatment and sand filtration). Wastewater samples for microbiological testing were diluted 10-fold with sterile water.

Culture Media for Bacterial Cells

Bacterial cultures were carried out in the Petri dishes on appropriate media from BioMaxima (Lublin, Poland). Non-selective media (R2A LAB-AGAR™ and Standard Methods (Plate Count Agar) LAB-AGAR™) Columbia LAB-AGAR™ Base and three selective media (Chapman, Areomonas LAB-Agar™ BASE, and TTC Tergitol-7 LAB -AGAR™) were used. All types of cultures were performed in triplicate. Determined cell counts have been presented in the figures as an average value of CFU/mL, and the results spread have been indicated by error bars.

Determination of Microorganisms from Wastewater by MALDI-TOF MS

The diluted wastewater samples were inoculated onto selective media. The bacterial growth was carried out at 37ºC for 24 h. Pure bacterial colonies were then streaked onto Columbia LAB-AGAR™ Base non-selective medium and a reduction culture was performed. The pure bacterial cultures were transferred to a metal targeting plate (MALDI-TOF/MS sample plate) and dried after matrix application. A microbial identification was performed by mass spectrometry using a MALDI Biotyper®(MBT) instrument from Bruker (Bruker Daltonik GmbH, Bremen, Germany). Bacterial species were determined by comparing the mass spectrum of the microorganism under study with IVD's MBT mass spectra reference library of 4194 species.

Taxonomic Characteristics of the Isolated Bacteria

The 16S rRNA gene sequence from the NCBI/EZtaxon/Ribosomal Database Project (RPD) nucleotide sequence databases was used to evaluate the phylogenetic tree of bacteria identified in the wastewater using the BLAST (blastn) program (http://www.ncbi.nlm.nih.gov/BLAST/). All sequences were aligned using Clustal W. A phylogenetic tree was built following the maximum likelihood (ML) method using the MEGA software package version 11.

Physicochemical Composition of Washing Agents

In order to determine the impact of the cleaning agents on the bacterial viability, a foaming agent (0.5% Foam) and an insect remover (0.5% Insect) collected from hand washes (M1 and M2, respectively) were used.

The concentration of anionic and nonionic detergents in the diluted Foam solution (pH = 7.61) was 661 mg/L and 590 mg/L, respectively. With regards to the diluted Insect liquid (pH = 11.55), the concentration of anionic and nonionic detergents was significantly lower and amounted to 63 mg/L and 96 mg/L, respectively. The Insect agent contained also NaOH. A detergent content was determined using the HACH cuvette tests (LCK 334 - nonionic, LCK344 - anionic).

Physicochemical Composition of Wastewater

The Hach cuvette tests were applied for determination of surfactants concentration (LCK 334 – nonionic, LCK344 – anionic), chemical oxygen demand – COD (LCK 1014), total phosphorous (LCK 348) and chlorides (LCK 311). The pH and electrical conductivity were measured with a 6P Ultrameter (Myron L Company, USA). The turbidity of tested solutions was determined using a portable turbidity meter model 2100 AN IS (HACH, USA).

The Effect of Cleaning Agents on the Growth of *E. coli bacteria*

In the present study, bacterial strain Escherichia coli K12 (ATCC 29425) has been selected. Cell growth was carried out in EB broth (Enriched broth) from BioMaxima SA (Lublin, Poland).

To each of the diluted solutions of Foam and Insect (200 ml) was added 5 ml of EB broth with *E. coli* bacteria and 44 ml of NaCl (0.85%). The changes in the number of bacteria in the solutions were determined by taking samples for culture every 5-10 min. TTC Tergitol-7 LAB-AGAR™ selective lactose agar medium was used to determine cell counts. This medium was selected since it inhibits the growth of Gram-positive bacteria and limits the growth of *Proteus* spp. The complete growth of bacterial colonies was achieved after 24 hours in an incubator at 37ºC.

The Growth of Bacteria Inside Car Wash Installation

The effect of Foam detergent solution was carried out for water samples taken on manual car washes M3. The first water sample was taken from the wash nozzle at the beginning of the car wash (Rinsing S). The second sample was taken after washing the car with Foam detergent solution (10 min) and rinsing it with water (Rinsing F). The washing water was supplied by a nozzle at high pressure (over 10 MPa).

In order to determine the impact of the Insect solution on the bacterial viability inside carwash installation the pump-tank system was constructed. This system contained Plunger Pump model 3CP1221 (CAT PUMPS, England) connected to 5 L tank. The tank was filled with sterile water softened in the nanofiltration process (NF permeate) to which 50 ml of wastewater has been added form carwash M1. The solution was recirculated for two days, then removed and the plant was flushed several times with NF permeate. After rinsing, the permeate was recirculated intermittently through the system for a further two days, after which a water sample was taken (Pump). Insect solution was then added to the tank, obtaining a concentration of 0.5% (pH = 11.5). The solution was heated to 40ºC, and then, used to rinse the system for 30 minutes. Subsequently, a sample was taken for investigations (Insect).

For microbiological testing, 0.3 mL of the collected sample was transferred onto non-selective Plate Count Agar (LAB-AGAR™) media to determine cell counts (in triplicate). The complete growth of bacterial colonies was achieved after 24 hours in an incubator at 37ºC.

Confirmation the Antibacterial Properties of Antibiotics

The deactivation properties of antibiotics on Gram-positive and Gram-negative strains found in the tested wastewater from manual washer (M1 and M2) and automatic washer (A1) were evaluated.

The bacteria were cultured on a PCA agar medium to which a given antibiotic was added: ampicillin (5 mg/L), polymyxin B (50,000 UI/L), D-cycloserine (400 mg/L), vancomycin (10 mg/L) and a mixture containing polymyxin B sulfate (10 mg/L), Ceftazidime (20 mg/L) and Acryflavin HCl (5 mg/L). The antibiotics selected for the study provide a broad spectrum of action against bacteria present in the environment and are recommended for antibiotic resistance testing [13, 14].

Ampicillin inhibits the growth of Gram-positive and Gram-negative bacteria, however, has no biocidal activity against *Aeromonas* spp. and *Plesiomonas* spp. Polymyxin B inhibits the growth of Gram-negative bacteria but shows no activity against *B. cereus* and Gram-positive granulomas. In turn, D-cycloserine inhibits the growth of associated microflora present in the tested samples but shows no biocidal activity against *Clostridium perfringens*. Vancomycin inhibits Gram-positive bacteria and partially also Gram-negative bacteria. Finally, a mixture of polymyxin B sulfate, Ceftazidime and Acriflavine HCl has a broad spectrum of activity against Gram-positive and Gram-negative bacteria.

Results and Discussion

Effect of Car Wash Agents on Bacterial Survival Rate

The solutions used for washing cars contain various chemical components (surfactants, sodium hydroxide, organic acids, aldehydes) that could exhibit the antibacterial effects [15]. These substances not only disrupt a cell wall function but also cause the cell wall rupture [16]. Undoubtedly, it has a positive impact during the car wash activities, hence, the antibacterial effectiveness of active ingredients contained in the effluents from hand washing systems was tested. The car washing process has several stages. In the initial

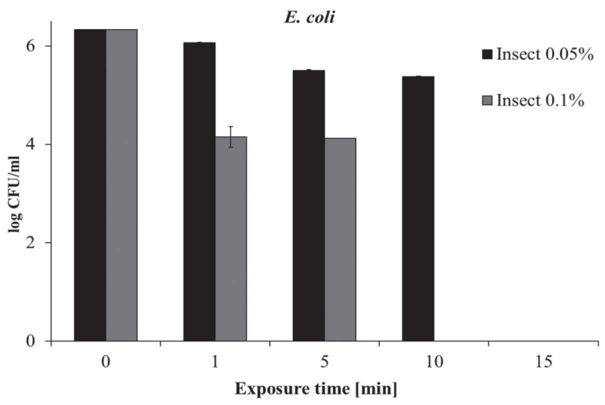

Fig. 1. Changes in the number of *E.coli* cells (log CFU/mL) after contact with Insect solution (0.05% and 0.1%) over time (min).

phase, the cleaning agents are used to wash rims and remove insect debris, followed by the main stage – foam washing.

The effect of these agents on the bacterial growth was studied for an *E. coli* model organism with the initial cells concentration of 1.5 x 10^6 CFU/mL. The impact of tested agents on the cell growth is shown in Fig. 1. Tested Insect solution had a strong toxic effect on *E. coli* cells. The 0.5% Insect solution (pH = 1 1.5) led, that to most of the cells dead below 5 min of contact. For lower concentrations, the solution still showed strong inhibitory effects (Fig. 1.), despite a lower pH value (e.g. 0.05% – pH = 10.2). The obtained result indicates that a significant part of the bacteria could be destroyed during washing with the Insect solution. However, it should be pointed out that only selected small areas such as car fronts or rims are washed with this agent.

The observed negative effect of alkaline solutions on *E. coli* growth has also been reported in the literature. It was found that a change in pH≥8 leads to alkalinisation of the cytoplasm and consequently, to changes in *E. coli* cell division and cell growth arrest [17], especially for pH>12 [18]. Moreover, in [19] inactivation of *E. coli* for a NaOH solution at pH = 11.5±0.1 was found after a time of 360 minutes. This is considerably longer than that shown in Fig. 2. It can be explained by the fact that the Insect liquid contained other agents, such as detergents, in addition to NaOH, which increases the observed negative effect on bacterial growth [15, 16].

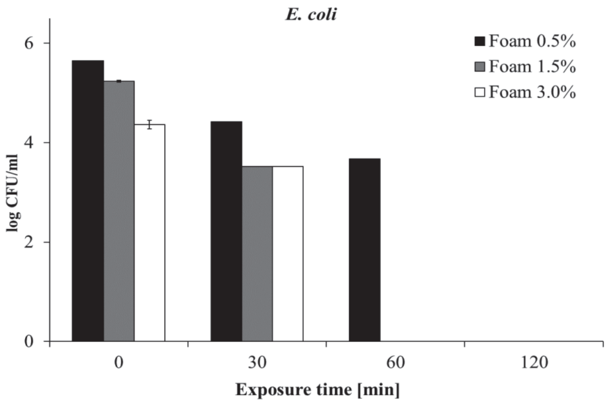

Fig. 2. Changes in the number of *E. coli* cells (log CFU/mL) after contact with Foam detergent solution (0.5%, 1.5%, 3.0%) over time (min).

When the initial stage is completed (or skipped), the entire car is washed with active foam solution. Tested Foam solution contains mainly anionic and nonionic surfactants, which can cause a cell membrane destruction [16]. However, for the concentration of the fluid used for car washing (0.5%), the effect was less prominent than that observed for Insect (Fig. 1). After 30 minutes (0.5% Foam), bacterial colonies decreased from 1.5 x 10^6 CFU/ml up to 2.67 x 10^4 CFU/ml. Increasing the concentration of Foam solution (1.5% and 3%) led a reduction in the number of bacterial colonies to 3.33 x 10^3 CFU/mL. Prolonged contact time increased the deactivation of *E. cells* coli and after 1 hour for 0.5% solution, the number of cells was 6.67 x 10^3 CFU/ml, and after 120 minutes no bacterial cells were found (Fig. 2.). However, such long contact times and concentrations above 0.5% are not used on carwash stations. Hence, it can be indicated that during car washing, the microorganisms on the car surface tend to be rinsed off. Therefore, their dispersion in the form of bio-aerosol may pose a risk to car wash users.

The results obtained in the present study indicate that the car washing agents will not cause the total elimination of bacteria, hence, their development in the installation of the car wash should be expected. The chemical cleaning solutions flow into a settling tank, where they are repeatedly diluted by the water used to wash the foam off the cars. In such a solution, the bacteria cells may find a favorable environment for their growth [20], where their number in the discharged wastewater will increase significantly. It also increases the microbiological risk if the reused water is subjected to even a simple treatment such as a sand filtration. Therefore, the treatment of wastewater from the car wash requires the use of several processes stages [21, 22].

Bacterial growth depends on the composition of the wastewater collected in the settling tank. The data obtained for the tested wastewater from three car washes are provided in Table 1. The pH value of the wastewater from the automatic car wash was 6.31, while the wastewater collected from the hand wash was poorly alkaline (pH = 7.45 - M1 and pH = 7.80 - M2). The pH values reported in other works also indicate that the wastewater from the car wash is the most common alkaline: 7.6-8.6 [23], 7-10 [24], >=7 [25].

In addition to surfactants, car wash wastewater also contains a significant amount of salt, as evidenced by the high values of electrical conductivity. For the analyzed wastewater it was equal to 2690 µS/cm (M1), 2495 µS/cm (M2) and 1232 µS/cm (A1). The higher

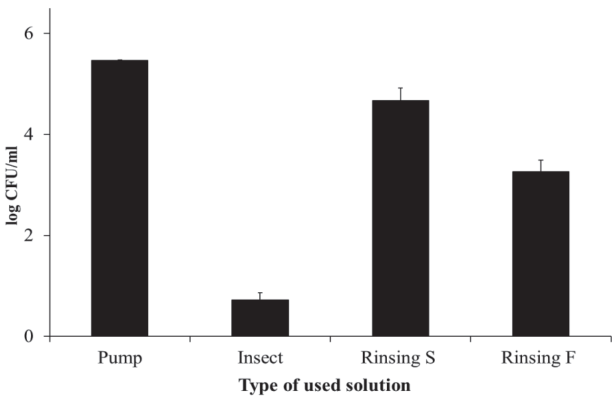

Fig. 3. Changes in the number of bacteria in the car wash installation. Pump - water after rinsing the pump, Insect - water from the pump after washing with 0.5% Insect, Rinsing S - water from the nozzle at the start of the car washing, Rinsing F - water from the nozzle after the car wash has been completed.

conductivity values for hand washes are due to the fact, that NaCl is used to regenerate water softeners. The parameters of wastewater presented in Table 1 confirm that they contained numerous hazard chemical components [26, 27]. The toxicity of wastewater contributes to the increased resistance of bacteria to the cleaning agents [28].

Consequently, this phenomenon can lead to contamination of the installation. Rinsing water, surfactant solutions and acidic or alkaline cleaning agents flowed through the washing nozzle. However, the test on a M3 carwash station confirmed that this did not prevent the growth of bacteria in installations, and its significant quantities (3.7±0.4 x 10^4 CFU/mL) was detected in water when the wash nozzle was activated. After initial rinsing, the car was washed with Foam solution, which has been rinsed with softened water. After these operations, the amount of bacteria in the water leaving the nozzle decreased to 1.46±0.3 x 10^3 CFU/mL (Fig. 3). Each washing programs ended with a flow of clean water, so that the detergents, which are harmful to bacteria, were removed from the washing system. The result is an intensive multiplication of resident bacteria in the system during the washing standstill.

Tests on the pump-tank system contaminated with car wastewater (M1) have shown, that after the introduction of bacteria into the installation, it is extremely difficult to remove them using multiple applications rinsing with sterile water. The NF permeate (75 S/cm) poured into the flushed-out installation contained a significant amount of bacteria (2.93±0.1 x 10^5 CFU/mL) after two days. Adding Insect solution (0.5%, pH = 11.5) to the permeate resulted in the reduction in that the number of bacteria cells to 5.56±1.2 x 10^2 CFU/mL after 30 min. This finding shows that the alkaline agent is more effective in removing bacteria than foaming agents (Fig. 3). However, despite a 5-fold increase in Insect concentration and an increase in contact time from 15 to 30 min, complete elimination of bacteria was not achieved, as was the case with *E. coli* (Fig. 2). This confirms that bacteria living in a toxic environment increase their resistance to harmful agents. As a result, they are expected to thrive in the car wash facilities, which may pose a risk to the users. For this reason, it is important to identify whether the bacterial strains that have developed show drug resistance in addition to chemical resistance.

Bacteria in Tested Wastewater

Bacteria are removed from the car surface of which common in the natural environment, and we have a contact with them e.g on the streets of the city. The chemicals and processes used to wash cars affect the water environment in the settling tank and thus, the type of microorganisms that may develop [30]. In such an environment, a new strains of bacteria more harmful to the public health may be formed. The study confirmed that the diverse chemical composition of the tested wastewater (Table 1) did not limit bacterial growth. The number of bacteria in the individual effluents was equal to 2.86±0.11 x 10^6 CFU/mL (A1), 3.71±0.29 x 10^6 CFU/mL (M1) and 3.28±0.1 x 10^6 CFU/mL (M2). Through the mass spectrometric identification of microorganisms, more than 30 types of bacteria could be determined in the examined wastewater, some of which, however, were only specific to a certain car wash (Fig. 4). The presented data (Table 2) indicate that the bacteria detected in the tested wastewater can cause many serious diseases, including incurable ones. Therefore, when using water reuse systems, ensuring microbiological purity is essentialThe performed study demonstrated (Fig. 4) the presence of following bacterial groups: *Firmicutes*, *Proteobacteria* and *Enterobacteria* in the car wash wastewater from the car wash. Worthy of note, these groups have also been

Table 1. Physicochemical properties of wastewater from: manual (M1 and M2) and automatic (A1) car washes.

Car wash	Conductivity [µS/cm]	pH	Turbidity [NTU]	COD [mg/l]	Anionic [mg/l]	Non-ionic [mg/l]	Cl^- [mg/l]	P_{total} [mg/l]
A1	*1232*	6.31	58.5	387	2.48	157	230	12.2
M1	*2690*	7.45	122	438	57.9	267	646	5.69
M2	*2495*	7.80	60.4	871	73.1	125	426	2.59

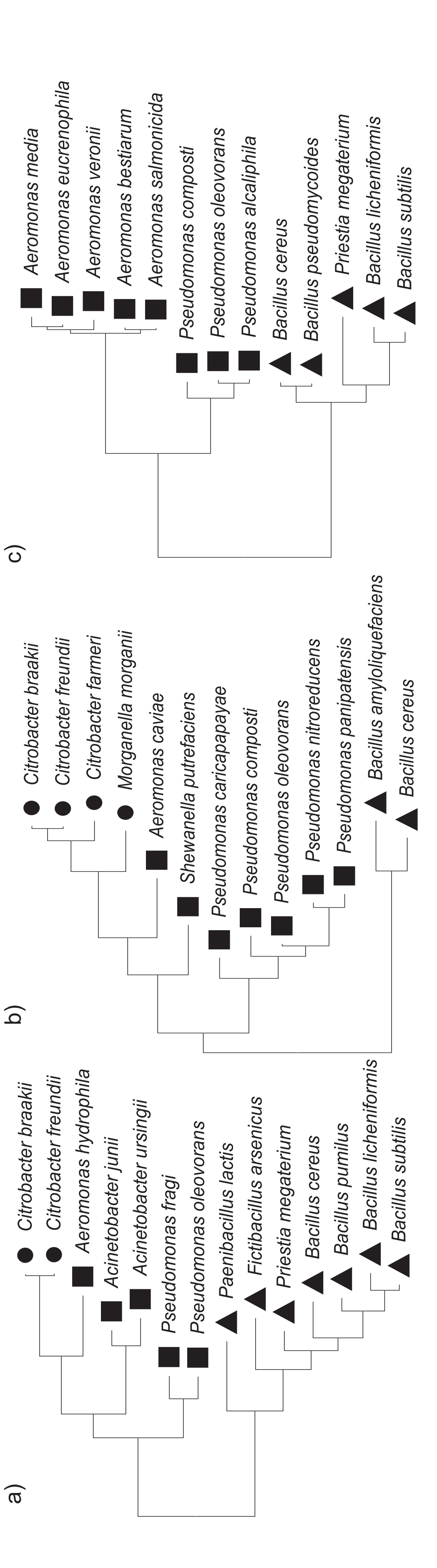

Fig. 4. Phylogenetic tree for bacterial cells from a car wash: a) automatic car wash “A1” b) manual car wash “ M1”, c) manual car wash “M2”. Bacterial groups: ▲- *Firmicutes*, ● – *Enterobacteria*, ■ - *Proteobacteria*

found in chemical plant wastewater [31], distinguishing it from the bacterial composition of typical municipal wastewater with *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* [32].

The latter are mainly found in the aquatic ecosystems and their growth is promoted by the presence of surfactants [20]. It can cause gastrointestinal diseases and carry antibiotic resistance genes [33]. For this reason, infections caused by *Aeromonas hydrophila*, which resulted from bioareosol inhalation, are difficult to treat [34].

The identified groups of microorganisms are consistent with those detected in carwash wastewater by other researchers [29, 23, 25]. In the above-mentioned works, in addition to *E. coli*, the presence of *Pseudomonas*, *Aeromonas*, *Bacteroidetes* and other bacteria has been reported. In the study [24], pathogenic bacteria of *Aeromonas species* (57%) and *Pseudomonas species* (43%) were found.

Pseudomonas spp. and *Aeromonas* spp. are metabolically versatile bacteria, which favor their adaptation to different environments [35]. *Pseudomonas* spp. are known to biodegrade hydrocarbons [36], allowing them to thrive in oil-contaminated car wash wastewater. These bacteria can also cause serious infections. *Pseudomonas oleovorans* bacteria cause hospital-acquired infections in patients and have associated to septicemia infections [37].

Bacteria of the *Citrobacter* spp. species can also use hydrocarbons as the sole carbon and energy source [38]. Among them, *Citrobacter freundii* is particularly dangerous, causing diarrhea [39] and infections of the urinary tract, respiratory tract and septicemia [40].

The *Firmicutes* group includes *Bacillus* spp. bacteria, whose growth is highly dependent on the physical and chemical parameters of the wastewater [41]. The detection of these bacteria in each of the effluents tested indicates that they find the favorable conditions for growth in the effluent from car washes. *Bacillus cereus* species cause gastrointestinal infections and eye infections [42]. The prevention of infections is hampered by the fact that some of them, like *Bacillus subtilis*, form the spore forms [43].

Results obtained in the present study confirmed that the car washes environment promotes the growth of bacteria that are dangerous to human health. In addition, the discharge of raw wastewater into surface water also endangers aquatic organisms [44]. For example, *Aeromonas* spp. bacteria cause fish diseases [45].

Effect of Antibiotics on Bacteria Detected in Car Wash Wastewater

In the case of bacterial infection, the primary treatment is antibiotic therapy. It is well known that the widespread use of antibiotics has led to ever-increasing bacterial drug resistance. The detected *Citrobacter* spp. in studies, in particular *Citrobacter freundii*, in contrast to *Klebsiella pneumoniae* and *Escherichia coli*,

Table 2. Diseases caused in humans by detected bacteria.

Microorganism	Diseases
Acinetobacter junii	Septicemia - neonates and pediatric oncology patients, immunocompromised adults [35].
Acinetobacter ursingii	Opportunistic pathogen in humans [46].
Aeromonas caviae	Inflammatory bowel disease [47].
Aeromonas hydrophila	Gastroenteritis and skin infections. Possible peritonitis, bacteremia, meningitis [48].
Aeromonas veronii	Gastroenteritis, causes inflammatory bowel disease [49].
Bacillus cereus	Food poisoning and severe eye infections. Pneumonia, sepsis, and central nervous system infections, especially in immunocompromised patients and newborns [42].
Bacillus subtilis	Forms resistant spores, making cleaning and disinfection difficult [43]. Present in the air, it irritates eyes, sinuses, throat and causes headaches [50]. Cases of hospital-acquired bacteremia in patients with cancer and hematological disorders [51]. In addition, *B. subtilis* produces an extracellular toxin (subtilisin) that causes allergies [52].
Bacillus licheniformis	Infections in immunocompromised patients [53].
Citrobacter freundii	It often causes nosocomial infections - diarrhea, urinary tract infections, wound infections, and respiratory tract infections [39]. It causes meningitis and septicemia [40].
Morganella morganii	An opportunistic pathogen that can cause bacteremia. It is the ninth most common cause of clinical infections in patients [55]. A pathogen that mainly causes surgical wounds and urinary tract infections [56].
Pseudomonas oleovorans	*P. oleovorans* is a pathogen found during human infections [36], particularly in cases of septicemia [57].

have become multidrug resistant [58]. One of the bacteria resistant to the commercial antibiotics is *Aeromonas hydrophila* which can be transmitted by bioareosols [59]. *Bacillus subtilis* strains are resistant to antibiotics used in oral bacteriotherapy [60]. *Aeromonas hydrophila* is another pathogen that shows resistance traits (e.g., to polymyxin B), while *Morganella morganii*, which causes many nosocomial infections, is resistant to some penicillins, such as benzylpenicillin, oxacillin, and amoxicillin [55]. An analysis of the literature has shown, that the bacteria detected in carwash wastewater can cause many diseases (Table 2). For this reason, the present study tested the effect of several commonly used antibiotics on the bacteria present in the wastewater. Analysis of the results shown in Figs 5-8 allowed to indicate that

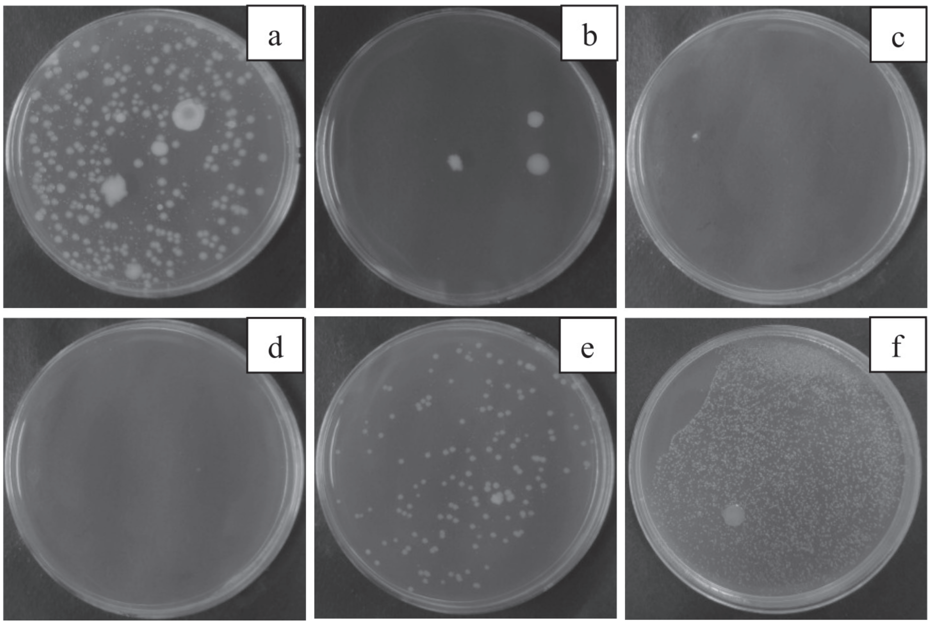

Fig. 5. Bacterial colonies on agar plate for car wash A1 a) no antibiotic addition b) polymyxin B addition c) antibiotic mixture: polymyxin B sulfate, ceftazidime and Acryflavine HCl, d) D-cycloserine addition e) ampicillin addition f) vancomycin addition.

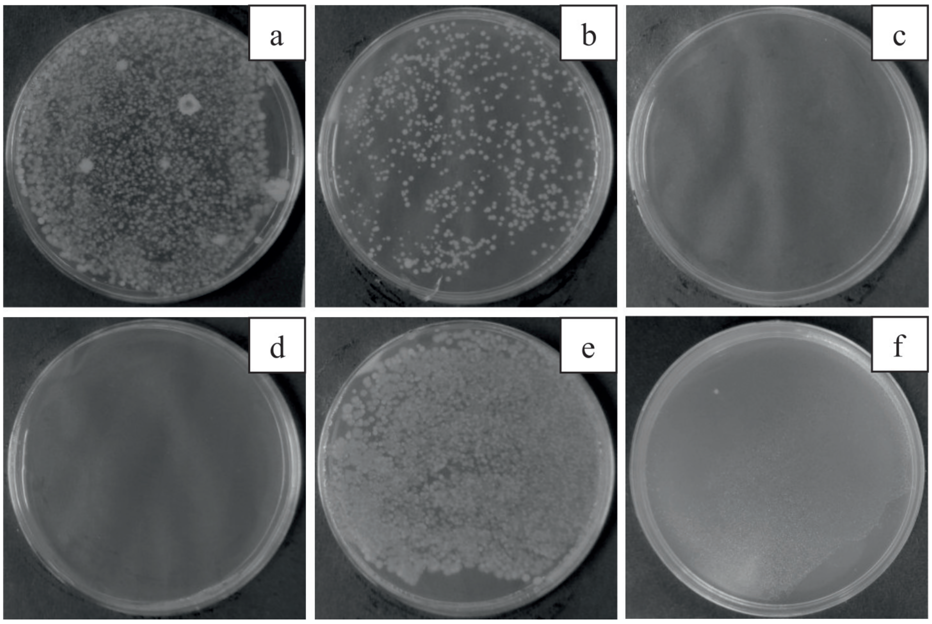

Fig. 6. Bacterial colonies on agar plate for car wash M1 a) no antibiotic addition b) polymyxin B addition c) antibiotic mixture: polymyxin B sulfate, ceftazidime and Acryflavine HCl, d) D-cycloserine addition e) ampicillin addition f) vancomycin addition.

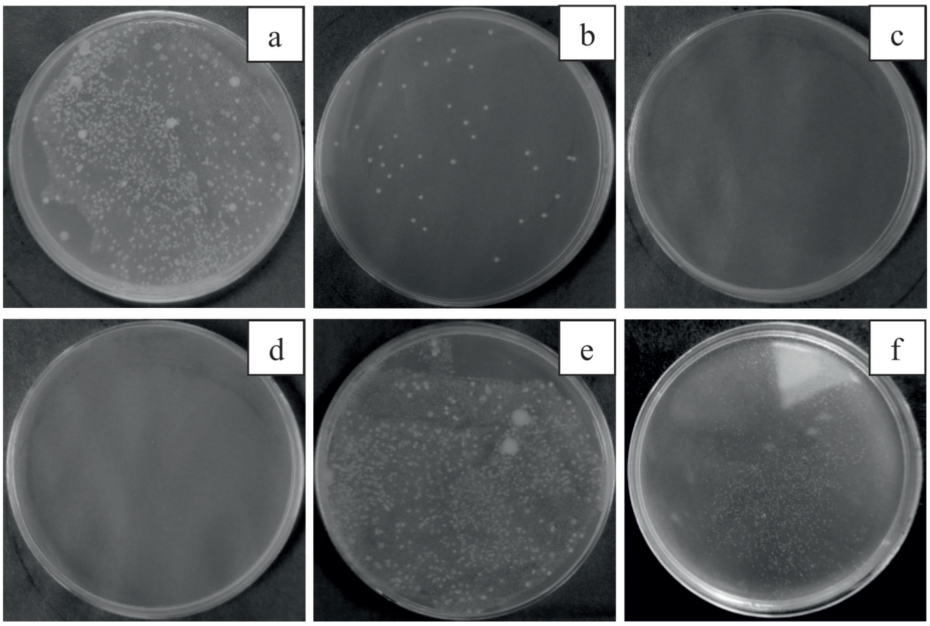

Fig. 7. Bacterial colonies on agar plate for car wash M2 a) no antibiotic addition b) polymyxin B addition c) antibiotic mixture: polymyxin B sulfate, ceftazidime and Acryflavine HCl, d) D-cycloserine addition e) ampicillin addition f) vancomycin addition.

some of the antibiotics only slightly reduced the bacteria growth.

The decreasing efficacy of the used drugs makes it important to raise awareness of the problem of the antibiotic resistance of the bacteria present in car wash wastewater. The tested wastewater showed some differences in bacterial composition (Fig. 3), therefore, differences in the effect of a specific type of antibiotic have been found (Figs 5-7). After 24 h of incubation, there were virtually no bacterial colonies on some media, while on others, on the contrary, their numbers were difficult to count. In each case, the complete inhibition of bacterial growth was observed for D-cycloserine and for a mixture of antibiotics (polymyxin B sulfate, Ceftazidime and Acryflavine HCl).

Polymyxins, as well as Ceftazidime are a class of antibiotics targeting Gram-negative bacterial infections. Acryflavin is known as an antibacterial and antiviral drug [61]. It is commonly used as a skin disinfectant for

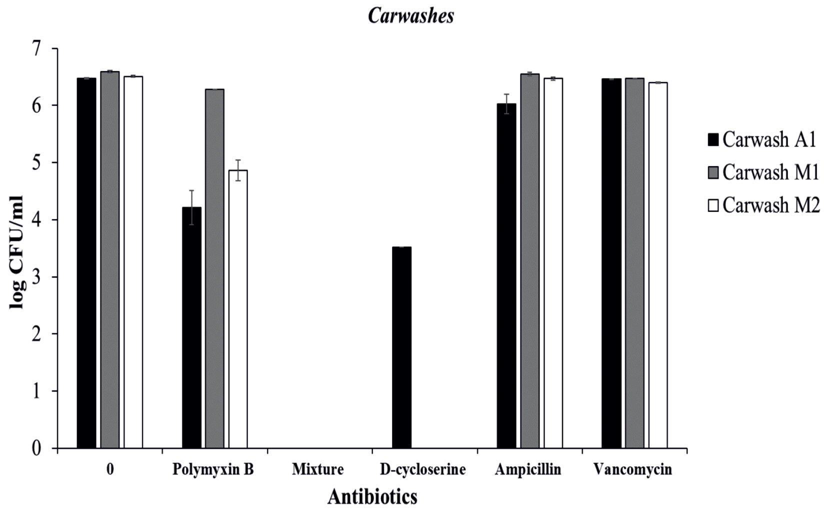

Fig. 8. Effectiveness of antibiotics against individual species of pathogenic microorganisms for each car wash station. (Mixture - Polymyxin B sulfate + Ceftazidime + Acryflavin HCl)

minor wounds, burns, and infected skin, and is effective against both Gram-positive and Gram-negative bacteria [62]. For these reasons, a mixture of these antibiotics has shown an effective broad spectrum of activity.

D-cycloserine inhibits the growth of most types of bacteria, however, has no significant antimicrobial effect against *C. perfringens*. The antibiotic inhibited the growth of all Gram-positive and Gram-negative bacteria present in the analyzed effluents from the three different car washes. D-cycloserine inhibits cell wall biosynthesis in susceptible strains of Gram-positive and Gram-negative bacteria and in *Mycobacterium tuberculosis*. Therefore, cycloserine is effective in treating acute urinary tract infections [63]. Its broad biocidal and bacteriostatic effects were confirmed by the results of ongoing studies (Figs 5-8).

Unexpectedly, a small inhibition of growth was achieved for vancomycin, which has a broad spectrum of activity against Gram-positive bacteria. In addition, the above-mentioned antibiotic is capable of inactivating methylcillin-resistant *Staphylococcus aureus* (MRSA) [64], a very dangerous pathogen spreading in the hospitals [65]. The application of vancomycin to isolates of *Bacillus species* led to the inactivation of these bacteria and the healing of ocular infections [66]. This is an important point, especially if the car wash operators do not use the safety glasses.

Nevertheless, Polymyxin B showed a partial effect on the bacteria. It is effective against Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Aerobacter*, *Influenzae* and *Shigella* [67]. However, the presence of Gram-positive bacteria in the analyzed wastewater was confirmed (Fig. 4). It has been shown that polymyxin B has no activity against such bacteria and anaerobes, *but the growth of Enterobacteriaceae and Citrobacter* spp. *bacteria detected in wastewater* [68]. Polymyxin B is also active against some *Aeromonas species* identified during the study [69]. However, some species, for example *Aeromonas jandaei* and *Aeromonas hydrophila*, show resistance to this type of antibiotic [69]. These species have also been detected in car wash wastewater, where *Morganella morganii* bacteria have shown resistance to polymyxin B [68]. For these reasons, although the use of the antibiotic significantly reduced the growth of bacteria in the wastewater it did not eliminate them completely (Figs 5-8). In the wastewater from carwashes A1 and M2, under the influence of the antibiotic, the number of bacteria decreased by 99%, but in the wastewater from washer M1, their number decreased slightly.

Ampicillin showed less effectiveness against bacteria present in wastewater. It is active against Gram-positive bacteria, including *Streptococcus pyogenes*, *Streptococcus pneumoniae*, as well as against some species such as *Streptococcus viridans*. The bacteria resistant to ampicillin are as follows**:** *Acinetobacter*, *Citrobacter* and *Pseudomonas* [70]. Ampicillin is effective against Enterococci, which include bacteria of the *Bacillus* spp. group (e.g., *B. cereus*, *B. subtilis*) present in the phylogenetic tree shown (Fig. 4). The efficacy of ampicillin against Gram-positive bacteria of the genus *Bacillus* spp. was confirmed in the study of Laskowski et al. [70]. Many types of bacterial species of this genus (*B. licheniformis*, *B. subtilis*, *B. pumilus*, *B. cereus*, *B. amyloliquefaciens* and *B. pseudomycoides*) were detected in the wastewater tested (Fig. 4). However, many bacterial colonies, presumably from Gram-negative group, were formed on the ampicillin-supplemented plates. The presence of such bacteria was confirmed in the tested wastewater, particularly from *Aeromonas* spp. (*A. hydropila*, *A. caviae*, *A. veronii*, *A. bestiarum*, *A. salmonieida*, *A. media*, *A. eucrenophila*). As a result, the number of bacteria under the influence of this antibiotic decreased from a few to a dozen percent.

The obtained results indicate that some of the bacteria detected in the effluents from car wash stations may be difficult to treat with commonly used drugs.

Conclusions

Wastewater from car washes contains many species of bacteria, most of which pose serious threats to human health and the aquatic environment. Bactericides added to washing liquors have a slight impact on reducing the growth of bacteria that have adapted to the composition of the effluent collected in the settling tank. Therefore, in order to ensure the effective treatment of water for post-washing, the plant must also be properly equipped to ensure the removal of microorganisms.

As a result of the adaptation, the bacteria present in the wastewater gained resistance to warm (40ºC) alkaline solutions (pH = 11.5). This indicates that despite the use of strong bactericides, car wash solutions may be colonised by bacteria.

The results of the phylogenetic analysis and the antibacterial tests performed indicate that some types of bacteria detected are virulent and resistant to several antibiotic classes. This finding suggests that inhaling bioaerosols generated during car washing can cause infections, which in some cases could even lead to sepsis. Currently, users are encouraged to wash their cars frequently, unfortunately without the knowledge of the microbiological risks involved.

It has been indicated that *Bacillus subtilis*, *Morganella morganii*, *Aeromonas hydrophila* and *Citrobacter freundii* were detected in the wastewater analyzed. The above-mentioned bacteria are hazardous to health due to their drug resistance.

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Conflict of Interest

The authors declare no conflict of interest.

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