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The Identification of Selective Pathogenic Microbial Community Biofilms in Different Distribution Pipeline Materials and Their Disinfection Kinetics

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Abstract: Biofilms in water distribution lines strongly affect water safety as they are the main carriers of pathogens. The current study investigated the biofilm formation and identification of selected pathogens in different distribution pipeline materials and their disinfection method in an annular reactor (AR). Initially, the quality of the flowing water from each pipeline material was analyzed, i.e., pH, TDS, EC, turbidity, and salinity; then, the biofilm formation was monitored for each material, i.e., ABS, PC, PVC, PP, and HDPE. Further, the disinfection kinetics of biofilm at different chlorine doses, i.e., 0.5, 1.0, 1.5, and 2.0 mg/L, was investigated. The selected pathogens, i.e., *E. coli*, *Pseudomonas*, *Shigella*, *Salmonella* sp., and *Vibrio* sp. were identified in biofilms formed in different pipeline materials. The disinfection kinetics results showed that a chlorine dose of 2.0 mg/L was the most effective in disinfecting selected pathogens. Following the disinfection kinetics, it was observed that *Salmonella* sp. was disinfected within 7 days, whereas other pathogenic biofilms were disinfected within 14 days. The efficacy of chlorine disinfection was affected by the types of pipeline materials. The study outcomes could provide insights into biofilms' disinfection method and the selection of suitable pipeline materials to ensure drinking water safety.

Keywords: pathogen; biofilm; water pipeline; disinfection; kinetics

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1. Introduction

Contaminated drinking water is detrimental to public health and may contain pathogens and microorganisms, including bacteria, fungi, protozoa, and viruses [1]. Around 5% of microorganism biomass in drinking water distribution systems (DWDSs) usually originates at the inner surface of water pipes, in which various microbes present in colony form adhere to each other, grow under nutrients, and develop into heterogeneous cell aggregates called biofilms [2,3]. The build up of biofilms in DWDSs is a critical concern because the survival of microorganisms in the form of deposited biofilms is more challenging to control due to their high concentration and complex nature. Moreover, intermittent water supply (IWS) in low- to middle-income countries compromises drinking water quality as polluted water enters the drinking water line due to back pressure when the drinking water supply is closed and allows biofilms to be nourished [4]. The drinking water is distributed to the end

users through long water pipes in which biofilm is formed, hosting various pathogens [5], which survive and continuously spread their population on the biofilms and in flowing drinking water [6]. These pathogens include *E. coli* (a fecal contamination indicator), *Campylobacter* spp. (an opportunistic environmental-origin bacteria), *Salmonella*, Cryptosporidium, Giardia, *Legionella* spp., *Pseudomonas aeruginosa*, and enteric viruses, including adenoviruses, rotaviruses, noroviruses, and parasitic protozoa including cryptosporidium parvum [7]. The growth of biofilm depends on the biological and chemical properties of the water, the material from which the pipe is made [8], temperature, hydraulic environment, stagnation time or water age, and biocides for the supply of drinking water [9,10].

Biofilm formation in water pipes depends on the type and properties of pipe materials, i.e., polyvinyl chloride (PVC), un-plasticized polyvinyl chloride (UPVC) [11], high-density polyethylene (HDPE), medium-density polyethylene (MDPE) [11], polypropylene (PP), acrylonitrile–butadiene–styrene (ABS), etc. [12]. The plastic-based pipes' surface roughness normally ranges between 0.002 and 0.007 mm, which is also a considerable factor of biofilm formation because of microbial adherence in the porous and rough channels and buildup in the biofilms [13]. The PVC raw materials are derived from oil and salt. The reported roughness of PVC pipes is around 0.0033 mm [14]. UPVC is manufactured using eco-friendly and virgin un-plasticized compounds. MDPE is a common plastic with a uniquely specific density and roughness of 0.003. It has very high shock- and drop-resistance properties, is less notch-sensitive, and is far more crack-resistant than HDPE. The raw material used is HDPE pellets made from virgin polyethylene granulates and recycled HDPE, and the highest roughness of HDPE is 432 $\pm$ 76 nm [15]. The ABS pipe roughness is around 0.003 and pipe fittings are made from a thermoplastic resin of ABS.

The PP has a high flexural strength due to its semi-crystalline nature, melting point of 130–170 °C, relatively slippery surface, and roughness of 69 $\pm$ 5 nm [15]. PP is very resistant to absorbing moisture and various bases and acids [15,16]. PP piping systems are made of high molecular weight resins of copolymer or homo-polymer PP materials, and PP pipes are around nine times lighter than iron and 36% lighter than hard PVCs. Proline® combines high chemical resistance with toughness and strength at operating temperatures higher than the PVC and polycarbonate (PC) heat deflection temperature (HDT) of 140 °C (284 °F) at 0.46 MPa (66 PSI). The PCs are strong, stiff, hard, tough, transparent engineering thermoplastics that can maintain rigidity up to 140 °C and toughness down to −20 °C or special grades even lower. The PC is a polyester made of carbonic acid and bisphenol-A condensation.

A biofilm's slow development can take years to complete. It starts when planktonic cells—free-living bacteria—stick to surfaces in particular environmental circumstances. These first colonizing microorganisms, mainly bacteria, start to increase and then alter the surface to provide more attachment sites for the colonization of other microorganisms. Various factors influence the first adherence of planktonic cells to surfaces in DWDS. These include the material, diameter, roughness of the pipe, temperature, pH, and organic matter concentration of the source water [13,17,18]. In addition, the bacterial cells' intrinsic properties, such as their capacity to create an extracellular matrix and hydrodynamic circumstances, include elements like flow and shear stress [13,17,19]. The removal and disinfection process is quite difficult due to the multiplex medium of biofilms. Also, higher doses of disinfectant are ineffective against biofilm development. The issue related to the disinfection of biofilm is its inability to penetrate deep into the biofilms.

Biofilm isolation and identification have been accomplished by applying culture-dependent procedures and low-profiling molecular biology techniques [20]. Biofilm removal can be obtained using various methods, i.e., chlorine, coal filtration, activated carbon, ozone-activated carbon and UV disinfection, and ozonation [21] and nanomaterials [11]. However, chlorine-based chemical disinfection is the most often utilized technique. Because biofilms are a multiplex medium, the removal and disinfection process is highly challenging. Higher disinfectant dosages are also ineffective at preventing the formation of biofilms. It is challenging to effectively and continuously remove and disinfect biofilms from DWDS

because different distribution pipe materials act differently on biofilm growth and removal mechanisms, and these aspects need critical studies and scientific data on the selection of pipe materials and disinfection types, dosages, and encountered mechanisms [22].

Considering the required research information on biofilm formation in different pipe materials and their disinfection kinetics, this study aimed to isolate and identify the selective pathogenic bacterial community in bulk water and biofilm formed on different materials for pipelines and to investigate the efficacy of chlorine disinfection (sodium hypochlorite (NaOCl) [22] against the colonized pathogens in biofilm. Initially, pathogenic bacteria were identified in the biofilm through the selective agar method; then, different chlorine doses (0.5, 1.0, 1.5, and 2.0 mg/L) were applied to the biofilm to reduce the bacterial load. This study also investigated suitable materials for pipelines that promote lower biofilm growth in water supply distribution lines.

2. Materials and Methods

2.1. Biofilm Annular Reactor Setup

Two annular reactors (ARs) (Model 1320 LJ. BioSurface Technologies Corporation, Bozeman, MT, USA) (shown in Figure S1) were operated at room temperature at Mehran University of Engineering and Technology, Jamshoro, Pakistan, for biofilm generation in a dark environment. Each AR consisted of a stationary outer cylinder and a rotating inner cylinder. The 18.75 cm^2 slides of HDPE, PVC, PP, PC, and ABS materials were mounted on the inner cylinders of the ARs. The process fluid circulated between the two cylinders and a variable speed motor and was passed from the inner cylinder where the different material slides were mounted. The rotational speed of the inner cylinder was set at 30 rpm (with Reynold number RE = 960 and shear stress of 0.007 N/m^2) to simulate the drinking water system's real pipe flow conditions and shear stress [23]. The ARs were operated twice following similar conditions with each selected pipeline material for reproducible results.

The operation time for each reactor setup was three months for the biofilm buildup and testing chlorine disinfection at 0.5, 1.0, 1.5, and 2.0 mg/L doses; the process scheme is mentioned in Table 1 and Table S1 in the Supplementary Materials. Initially, a two-month (eight-week) period was given for acclimatization without disinfectant so the biofilms could grow and achieve a steady-state condition. Later, each chlorine dosage for each setup was applied for four weeks. Two dosage concentrations, i.e., 0.5 and 1.0 mg/L, were tested simultaneously during the first batch, while the other two dosage concentrations, i.e., 1.5 and 2.0 mg/L, were tested after completion of the first batch. Before each setup, the reactors were cleaned and sterilized to avoid any contamination [24,25].

Table 1. Operation of the annular reactor at different dosages of chlorine.

S. No.	Dosage (mg/L)	Acclimatization (Weeks)	Disinfectant	Dosage Time (Weeks)
1	0.5	8	Chlorine	4
2	1.0	8	Chlorine	4
3	1.5	8	Chlorine	4
4	2.0	8	Chlorine	4

2.2. Sample Collection and Physicochemical Analysis of Inlet Water and Outlet Water

The inlet and outlet water samples were collected in sterile plastic bags every week before the removal of slides from the ARs in each setup. Since no free chlorine was detected in inlet water, sodium thiosulfate was only added to the collected outlet water samples to neutralize any residual chlorine if present. All the glassware was washed and rinsed with distilled water, then sterilized at 121 °C for 15 min. Physicochemical parameters of the flowing bulk, including pH, temperature, total dissolved solids (TDS), and electric conductivity (EC), were analyzed through multi-parameter 3630 IDS (Xylem Analytics, Weilheim, Germany). At the same time, turbidity was analyzed through Lovibond TB 210 IR (Lovibond, Dortmund, Germany) [12]. The bulk water's total organic carbon (TOC) was obtained using a TOC analyzer (Shimadzu, Kyoto, Japan).

2.3. Biofilm Sampling

Biofilms were extracted from one slide of each pipe material each week for four subsequent weeks to measure the effects of disinfection. A sterilized metal scraper scraped the slides in a sterile 50 mL falcon tube containing 50 mL autoclaved phosphate buffer saline (PBS), and 0.1% *w*/*v* sodium thiosulfate was added to neutralize any residual chlorine. The biofilm suspension was homogenized by vertexing for 45 min [26].

2.4. Bacterial Load in Bulk Water and Biofilm

Selective pathogen detection in the bulk water and biofilm samples was performed on five different selective media agars, i.e., R2A (Oxoid Limited, Thermo Fisher Scientific Inc., Loughborough, UK) [26], XLD (Oxoid Limited, Thermo Fisher Scientific Inc., Loughborough, UK) [27], cetrimide (Oxoid Limited, Thermo Fisher Scientific Inc., Loughborough, UK), and thiosulfate–citrate–bile salts–sucrose (TCBS, Oxoid Limited, Thermo Fisher Scientific Inc., Loughborough, UK). These agars were used for heterotrophic plate counts (HPC) and *Salmonella*/*Shigella*, *Pseudomonas*, and *Vibrio* detection and identification. The 100 µL serially diluted samples of the inlet water samples, outlet bulk water, and biofilm suspension samples were plated on agar plates, and all solid agar media plates were incubated at 37 °C for 24 h except R2A plates, which were incubated at 20 °C for five days. After incubation time, bacterial colonies were counted and recorded as CFU/100 mL for bulk water, whereas biofilm colony counts were recorded as CFU/cm^2 as the number of CFU/mL.

2.5. Bacterial Viability Tests

To qualitatively analyze the chlorine disinfection bacterial viability (live or dead staining) of the biofilm samples in different water distribution materials, the LIVE/DEAD BacLight bacterial viability kit (by Thermofisher Scientific, Waltham, MA, USA) was used [12,28]. The BacLight Bacterial Viability Kit contained SYTO 9 and propidium iodide, which were stained on live/dead bacterial cells and could be analyzed via fluorescence microscopy. Initially, around 1 mL of biofilm culture was scraped from each pipe material, which underwent different disinfection doses, and, later, the scraped culture was centrifuged at 10,000× *g* for 10 min. The supernatant was discarded, the centrifuged bacterial pellets were washed twice with a PBS buffer, and the last bacterial pellets were dissolved in 1 mL PBS. To each sample of 1 mL, 3 µL of a staining dye mixture was added, and samples were incubated at room temperature in the dark for 20 min. Finally, 5 µL of the stained bacterial suspension was taken on a glass slide and covered with a coverslip. The glass slides were analyzed in the fluorescence microscope (Zeiss Axio Scope. A1 Carl Zeiss, Jena, Germany) using the appropriate fluorescence filters. To investigate the bacterial reduction, the disinfection kinetics were performed for different chlorine dosages, i.e., 0.5, 1.0, 1.5, and 2.0 mg/L for different pipe materials. The pseudo first-order reaction for disinfection kinetics was used to find the kinetic rate:

$$N(t) = N_0 \times e^{-kT} \quad (1)$$

where N = the bacterial concentration at time t, N_0 = initial bacterial concentration at time 0, T = contact time (min), and k = inactivation rate constant.

3. Results

3.1. Physicochemical Analysis of Inlet and Outlet Water Samples of ARs

The observed physicochemical parameters of the bulk water samples are given in Table 2. The appearance of the bulk water sample remained consistently colorless and clear throughout the experiment. The recorded pH levels were within the normal range, i.e., between 7.5 and 8.2. This pH stability indicates that the system effectively maintained its neutral to slightly alkaline conditions, which is typically desirable for water treatment processes. Turbidity measurements ranging from 5 to 6 NTU demonstrated that the water

remained relatively clear, with low levels of suspended particles. This suggests that the treatment process effectively removed particulate matter, contributing to the clarity of the bulk water. Total dissolved solids (TDS) varied within the 334–1053 mg/L range. While TDS levels did fluctuate, they remained within acceptable limits for treated water quality. The variations may be attributed to seasonal changes in the source water composition. The average total organic carbon (TOC) value of the bulk water was around 8 mg/L, indicating traces of organic/particulate matter in the typical range, which may have resulted as a potential nutrient source of microbial colonies in biofilms.

Table 2. Physicochemical parameter analysis of bulk water samples before and after different chlorine doses (BD and AD).

Dose	Water	Collection Time	Temp. (°C)	pH	TDS (mg/L)	EC (µS/cm)	Salinity (mg/L)	Turbidity (NTU)
0.5 mg/L	Inlet	BD	31 ± 1	7.9 ± 0.38	425.1 ± 137.7	654 ± 211.9	0.2 ± 0.18	11.6 ± 7.4
		AD	30 ± 1	7.9 ± 0.05	383.7 ± 16.5	590.3 ± 25.4	0.3 ± 0.1	4 ± 0.42
	Outlet	BD	30 ± 0	8.1 ± 0	351 ± 23.9	540 ± 36.8	0.15 ± 0.07	3.1 ± 0.6
		AD	30 ± 1	7.9 ± 0.1	370.5 ± 19.2	570 ± 29.6	0.3 ± 0.1	3 ± 0.3
1.0 mg/L	Inlet	BD	31 ± 1	7.9 ± 0.38	425.1 ± 137.7	654 ± 211.9	0.2 ± 0.18	11.6 ± 7.4
		AD	30 ± 1	7.9 ± 0.05	383.7 ± 16.5	590.3 ± 25.4	0.3 ± 0.1	4 ± 0.42
	Outlet	BD	30 ± 0	7.95 ± 0.2	352.95 ± 23.9	543 ± 36.8	0.15 ± 0.07	2.55 ± 0.47
		AD	30 ± 1	7.9 ± 0.1	372.0 ± 18	572.3 ± 28	0.26 ± 0.05	2.78 ± 0.4
1.5 mg/L	Inlet	BD	25 ± 4.2	8.1 ± 0.1	555.7 ± 61.6	855 ± 94.8	0.45 ± 0.07	4.86 ± 1.27
		AD	22.3 ± 1.53	7.9 ± 0.06	596.5 ± 61.5	917.7 ± 94.7	0.46 ± 0.06	7.03 ± 1.06
	Outlet	BD	22 ± 0	8.1 ± 0	596.1 ± 0	917 ± 0	0.5 ± 0	3.78 ± 0
		AD	22.3 ± 1.53	7.9 ± 0	720.4 ± 1.71.4	1108.3 ± 263.7	0.47 ± 0.06	5.38 ± 1.19
2.0 mg/L	Inlet	BD	19.8 ± 0.21	8 ± 0	646.1 ± 16.5	994 ± 25.5	0.45 ± 0.07	5.9 ± 0.81
		AD	19.7 ± 0.7	8.07 ± 0.1	849.9 ± 192.1	1307.7 ± 295.5	0.53 ± 0.06	3.88 ± 0.87
	Outlet	BD	19.8 ± 0	8.1 ± 0	637 ± 0	980 ± 0	0.4 ± 0	3.92 ± 0
		AD	19.8 ± 0.8	7.9 ± 0.1	756.4 ± 139.2	1163.7 ± 214.1	0.53 ± 0.11	3.85 ± 1.14

The electric conductivity (EC) values ranged from 514 to 1621 µS/cm, indicating the presence of dissolved ions in the water. These variations could be attributed to changes in the water source or treatment conditions. Despite these fluctuations, the conductivity values were within acceptable ranges. Salinity, with a range of 0.2 to 0.6 mg/L, remained relatively stable, signifying that the treatment process effectively controlled salinity levels, keeping them well below levels of concern. Temperature fluctuations were observed, with an initial measurement of 32 °C during the first month of operation and a decrease to 19 °C during the winter. This decline was attributed to seasonal temperature variations and remained within permissible limits for the treated water. The data in Table 2 provides a detailed overview of these parameters over time. Furthermore, the free chlorine was detected and recorded before and after the dose to comply with the WHO-recommended free chlorine value. The free chlorine results are shown in Table 3, which showed traces of free chlorine.

Table 3. Analyzed free residual chlorine concentrations in water samples after passing from the annular reactor.

Disinfection Dose	Free Residual Chlorine			
	Week 1	Week 2	Week 3	Week 4
0.5 mg/L	0	0	0	0
1 mg/L	0.0	0.01	0.04	0.1
1.5 mg/L	0.4	0.10	0.17	0.20
2 mg/L	0.22	0.37	0.48	0.69

3.2. Bacterial Contamination in Bulk Water Samples

The bacterial counts of pathogenic bacteria, including HPC, *Pseudomonas*, *Salmonella*, *Shigella*, *Vibrio*, and *E. coli*, in bulk water samples at both the inlet and outlet points exhibited a notable decrease following each chlorine dosage, as shown in Figure 1. A paired *t*-test analysis assessed the statistical significance of the differences between the bacterial counts in the inlet and outlet water samples, disregarding variations over time. The results of this analysis are shown in Table 4, revealing statistically significant differences in the counts of specific bacteria at varying chlorine doses. At a chlorine dose of 1.0 mg/L, a significant difference in the counts of pseudomonas was observed (p-value = 0.05). Similarly, at a chlorine dose of 1.5 mg/L, *Salmonella* and *Shigella* exhibited significant differences in counts, with p-values of 0.04 and 0.05, respectively. Finally, at a chlorine dose of 2.0 mg/L, significant differences were noted in the HPC, *Pseudomonas*, and *Shigella* counts, with p-values of 0.05, 0.04, and 0.05, respectively. These findings suggest that a high chlorine dose significantly impacted the reduction of pathogenic bacterial counts in the water samples. The results support the effectiveness of chlorine as a disinfection agent in mitigating the presence of pathogens, particularly *Pseudomonas*, *Salmonella*, and *Shigella*, across different dosage levels. This underscores the importance of chlorine dosing in maintaining water safety and preventing potential health risks associated with these pathogens.

Table 4. Statistical significance between identified pathogen counts of the inlet and outlet bulk water samples concerning dose concentration and irrespective of time.

Dosage	HPC	*Pseudomonas*	*Salmonella*	*Shigella*	*Vibrio*	*E. coli*
0.5 mg/L	0.10	0.19	0.07	0.13	0.06	0.08
1.0 mg/L	0.16	0.05	0.12	0.07	0.09	0.09
1.5 mg/L	0.06	0.16	0.04	0.05	0.06	0.07
2.0 mg/L	0.05	0.04	0.06	0.05	0.06	0.06

The overall bacterial counts, irrespective of the specific pathogens, were evaluated using paired *t*-test analyses to observe significant differences between the inlet and outlet water samples at various time points and chlorine dosage levels. At the initial point (day 0), when no disinfectants were present in the AR setups and with chlorine dosages of 1.0 mg/L and 1.5 mg/L, significant differences were observed in overall bacterial counts between the inlet and outlet water samples. However, as the study progressed, significant differences in overall bacterial counts were consistently detected at multiple times, i.e., at 0, 7, 14, and 21 days following disinfection across various chlorine dosages, i.e., 0.5, 1.0, 1.5, and 2.0 mg/L; detailed results are shown in Table 5. These findings suggest that chlorine as a disinfectant had a substantial and sustained impact on reducing overall bacterial counts in the treated water. The results highlight the effectiveness of chlorine as a disinfection agent in controlling bacterial populations in AR setups. The presence of significant differences in bacterial counts at different times of disinfection underscores the ability of chlorine to provide continued protection against bacterial proliferation, contributing to water quality maintenance over an extended period. These findings have implications for water treatment and disinfection strategies, emphasizing the importance of chlorine dosing in ensuring the microbial safety of water supplies, particularly in situations where overall bacterial counts need to be controlled and minimized.

Table 5. Statistical analysis of the pathogen counts for inlet and outlet bulk water samples concerning time and dosage concentration and irrespective of pathogen type.

Time (Days)	0.5 mg/L	1.0 mg/L	1.5 mg/L	2.0 mg/L
0	0.8226	0.0028	0.0266	0.1318
7	0.0183	0.0101	0.0033	0.0091
14	0.0062	0.0051	0.0066	0.0090
21	0.0065	0.0054	0.0490	0.0098

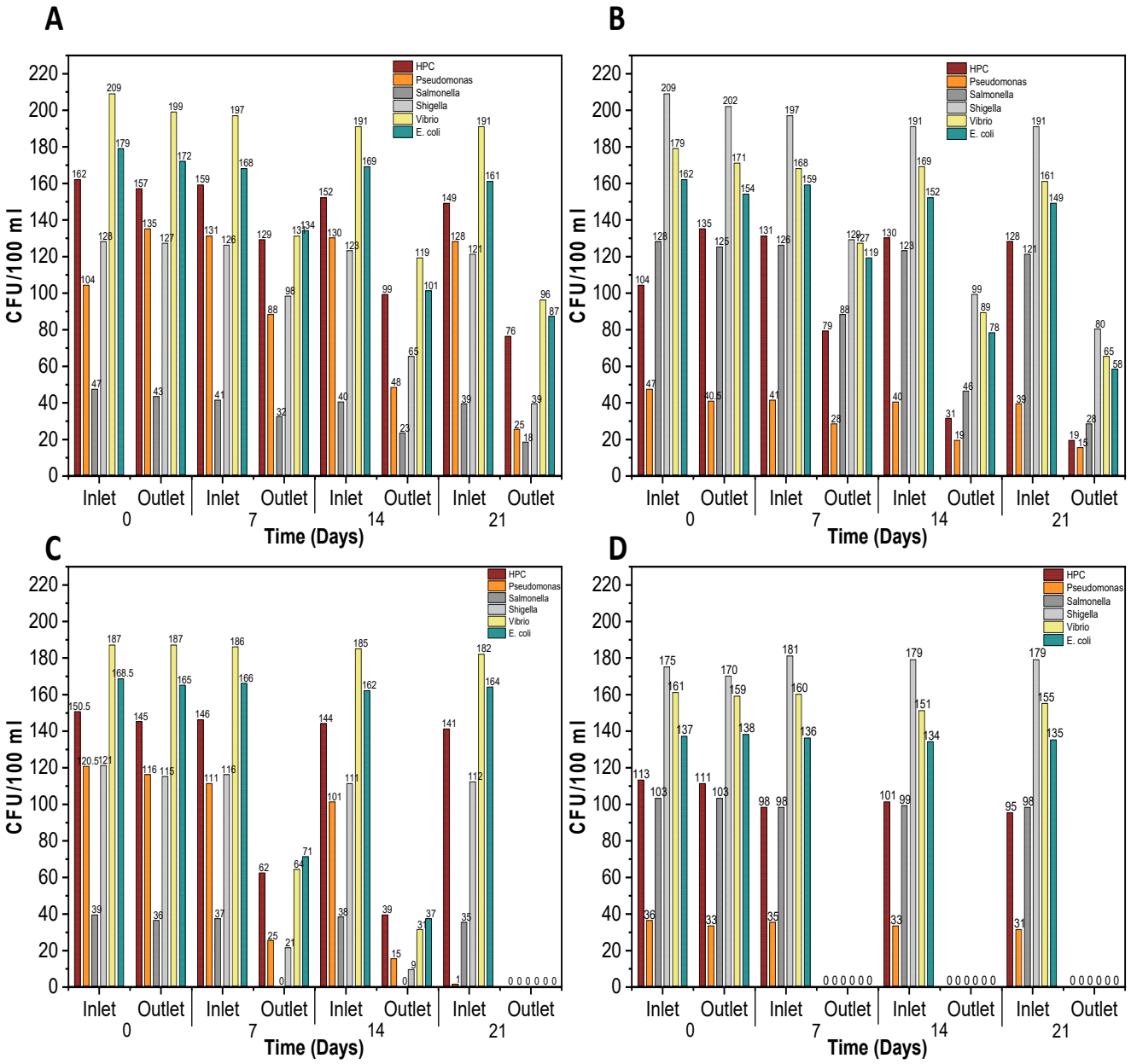

Figure 1. Bacterial counts in the inlet and outlet bulk water from different pipe material slides and after different chlorine disinfection doses at (**A**) 0.5 mg/L, (**B**) 1.0 mg/L, (**C**) 1.5 mg/L, and (**D**) 2.0 mg/L.

The disinfection kinetics and disinfection efficiency of HPC, *Pseudomonas*, *Salmonella*, *Shigella*, *Vibrio*, and *E. coli* in the outlet water of four AR setups showed that the chlorine dosage of 2.0 mg/L was the most efficient dosage to disinfect the planktonic cells in the outlet water sample as no bacterial counts of these pathogens were observed in the plate count method, as shown in Figure S2. Chlorine disinfection was introduced to the biofilm ARs passing water for the disinfection and inhibition of the grown microbial colonies. As water passed through the system, the disinfection process sought to lower bacterial counts of the outlet water, i.e., compared to inlet counts, i.e., due to disinfection and/or inhibition. Effective disinfection depends on the contact of the chlorine disinfectant with the water and biofilms. There may be variations in the residual chlorine concentration across the distribution system, which refers to the quantity of chlorine left in the water after disinfection. Chlorine decay affects its ability to suppress bacterial development depending on several factors, including water temperature, flow rates, and pipe material. The mean

values of four inlet samples at different time intervals and one outlet sample before chlorine dosage were considered as control samples, representing whole bacterial counts without disinfection. The salmonella sp was most efficiently removed from the bulk water sample at 1.5 and 2.0 mg/L doses, i.e., after 7 days. The removal rate for salmonella at 0.5 and 1.0 mg/L was 0.01285 and 0.01817 log CFU after 7 days [29]. The highest removal rates at given doses were for pseudomonas, which were most efficiently removed at a 0.5 mg/L concentration among all the tested bacterial pathogens with 0.02013 log CFUs after 7 days. HPCs were efficiently removed at 1.0 mg/L with a removal rate of 0.02532 log CFUs per 7 days; at 1.5 mg/L, salmonella and shigella were most efficiently removed with a rate of 0.04752 log CFUs per 7 days.

3.3. Disinfection of Pathogenic Bacteria in Biofilm

The bacterial counts of five selected pathogens were assessed in CFU/cm^2 for biofilms through the bacterial plate count method, i.e., after the biofilm sample collection. Two ARs were operated twice in parallel for the disinfection kinetics (four reactor setups for 0.5, 1.0, 1.5, and 2.0 mg/L) by following different time sequences in each pipe material. For each configuration, the log CFU/cm^2 counts of selected HPC, *Pseudomonas*, *Salmonella*, *Shigella*, and *Vibrio cholera* were assessed on HDPE, PVC, PP, PC, and ABS slides, i.e., before and after disinfection. The lowest counts (CFU/cm^2) were observed for each selected pathogen at chlorine doses of 1.5 mg/L and 2.0 mg/L, as shown in Figures 2 and 3.

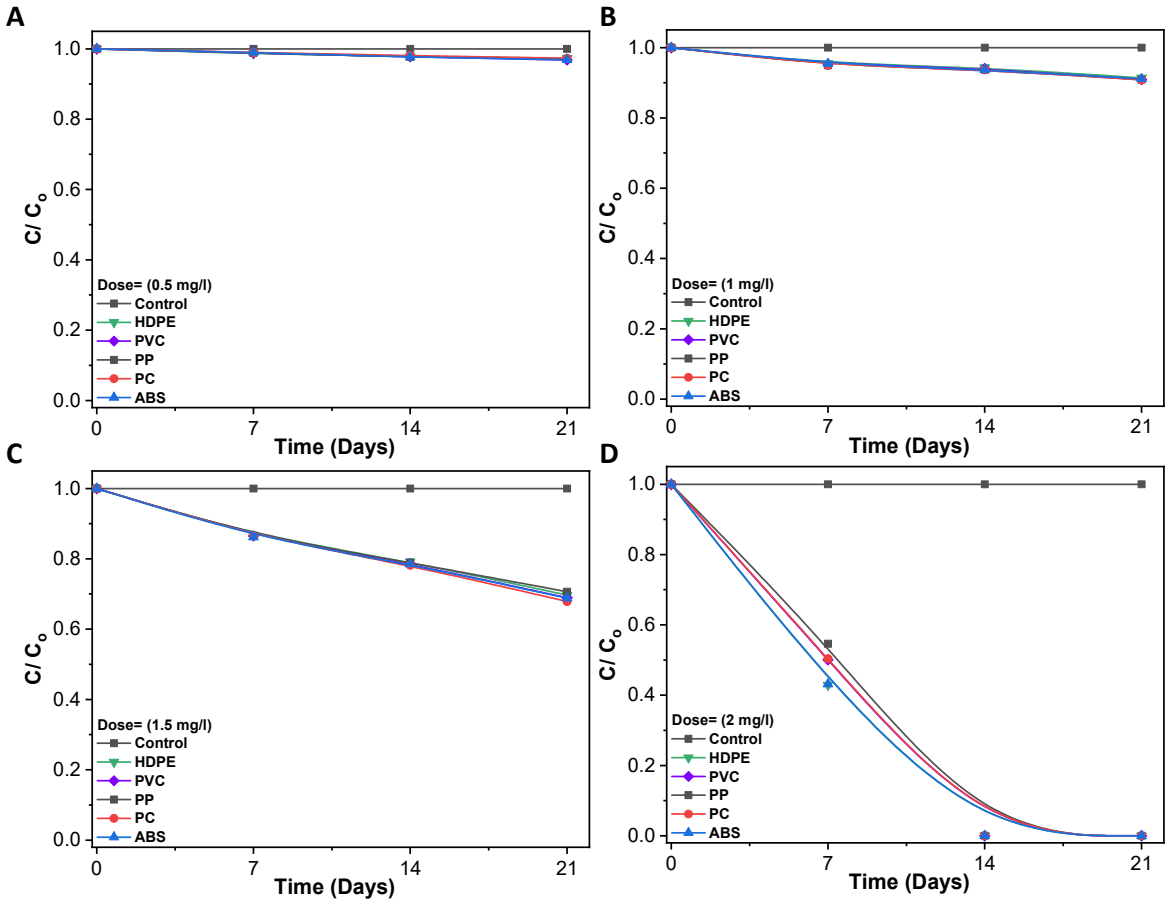

Figure 2. *Pseudomonas* reduction in biofilm at different chlorine doses of (**A**) 0.5 mg/L, (**B**) 1.0 mg/L, (**C**) 1.5 mg/L, and (**D**) 2.0 mg/L.

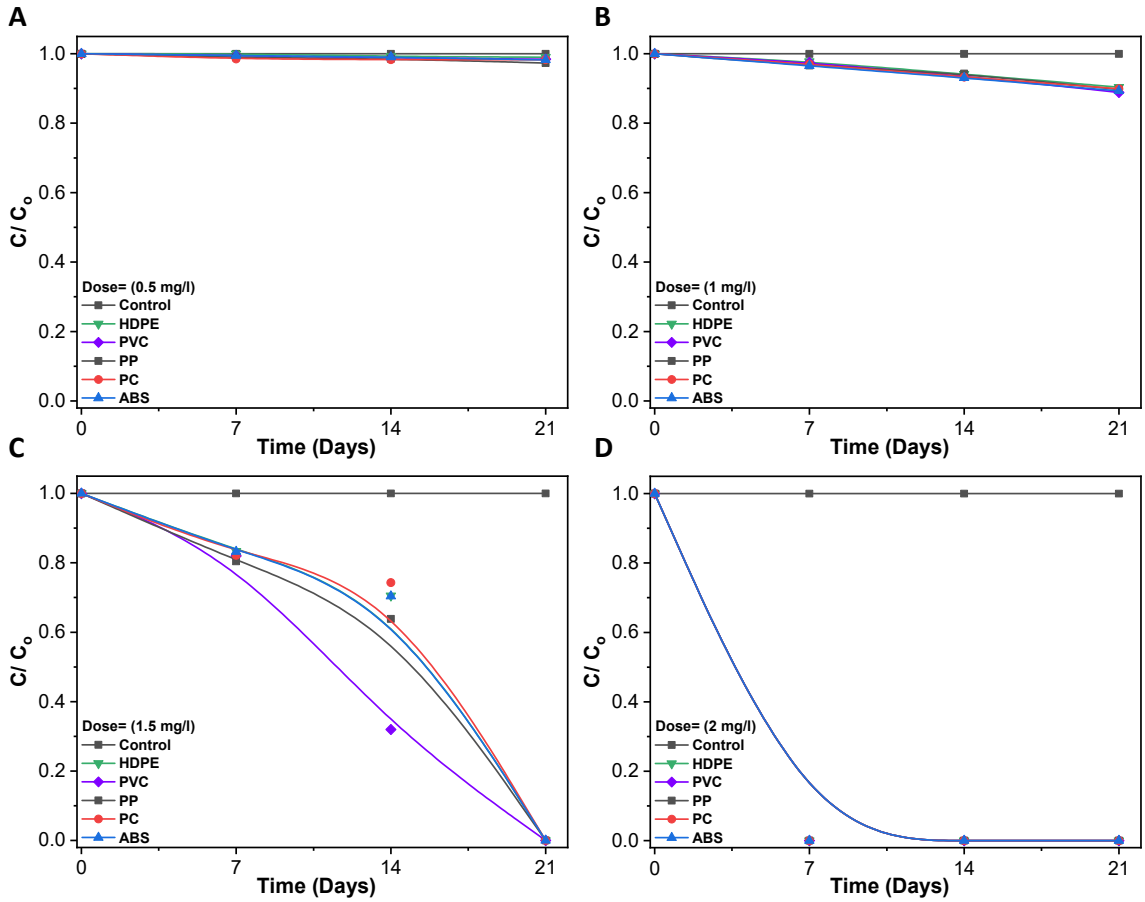

Figure 3. *Salmonella* reduction in biofilm at different chlorine doses of (**A**) 0.5 mg/L, (**B**) 1.0 mg/L, (**C**) 1.5 mg/L, and (**D**) 2.0 mg/L.

The counts (log CFUs/cm^2) of HPC, *Pseudomonas*, *Salmonella*, *Shigella*, and *Vibrio cholerae* in all plastic material biofilm samples at varying concentrations were affected by the disinfection contact time and disinfectant concentration, as shown in Figures 2–5. The details based on each bacterial count in log values are shown in Tables S2 and S3. For example, a gradual reduction in HPC counts was found in all disinfectant concentrations evaluated over time (from day 7 to day 21) (Supplementary Material Tables S2 and S3). However, biofilms were least affected by a 0.5 mg/L disinfectant concentration, whereas biofilms were most affected by a 2.0 mg/L disinfectant concentration, as shown in Figure S3. The log CFUs/cm^2 counts of *Pseudomonas*, *Salmonella*, *Shigella*, and *Vibrio cholerae* are shown in Figures 2–5, respectively, for various disinfectant concentrations and time intervals. In contrast, the biofilms' formation in and removal from various pipe materials were not different.

The obtained disinfection kinetics results shown in Figures 2–5 and stated in Tables S2 and S3 suggested that a 2.0 mg/L dose was the most efficient for removing biofilms, which could completely remove the bacterial contamination in biofilms on different pipe materials within 21-day time intervals.

3.4. Bacterial Viability Live/Dead Fluorescence Staining

The bacterial viability test was conducted to check the live and dead bacterial cells (red stains in fluorescence microscopy) after 21 days of chlorine dosage in the outlet bulk water samples. Clear dead cells were observed at all concentrations. However, more dead cells than live cells were observed at 1.5 and 2.0 mg/L chlorine concentrations, as shown in Figure 6A [23].

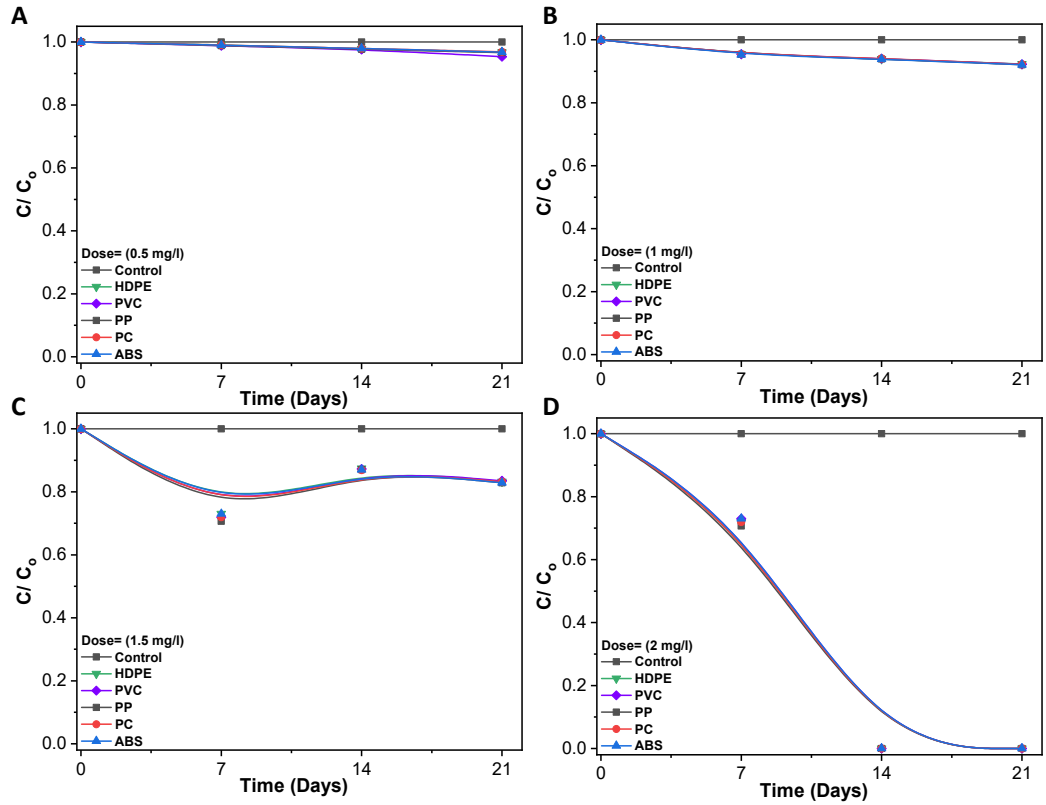

Figure 4. *Shigella* reduction in biofilm at different chlorine doses of (**A**) 0.5 mg/L, (**B**) 1.0 mg/L, (**C**) 1.5 mg/L, and (**D**) 2.0 mg/L.

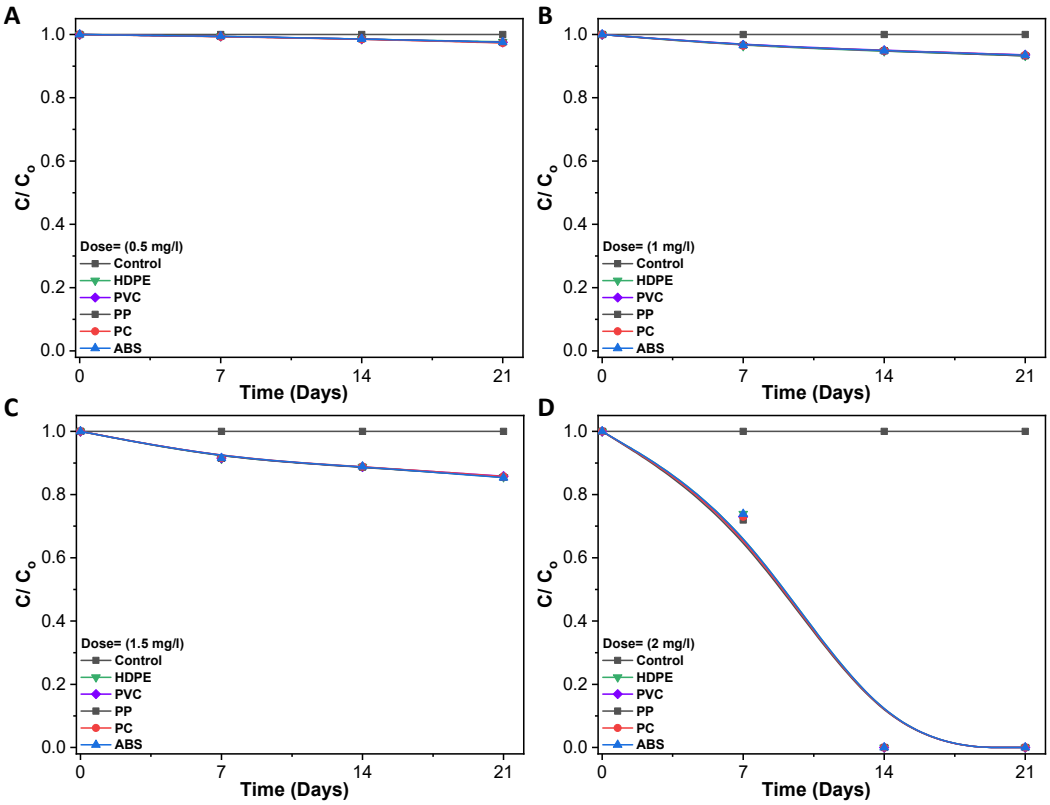

Figure 5. *Vibrio Cholera* reduction in biofilm at different chlorine doses of (**A**) 0.5 mg/L, (**B**) 1.0 mg/L, (**C**) 1.5 mg/L, and (**D**) 2.0 mg/L.

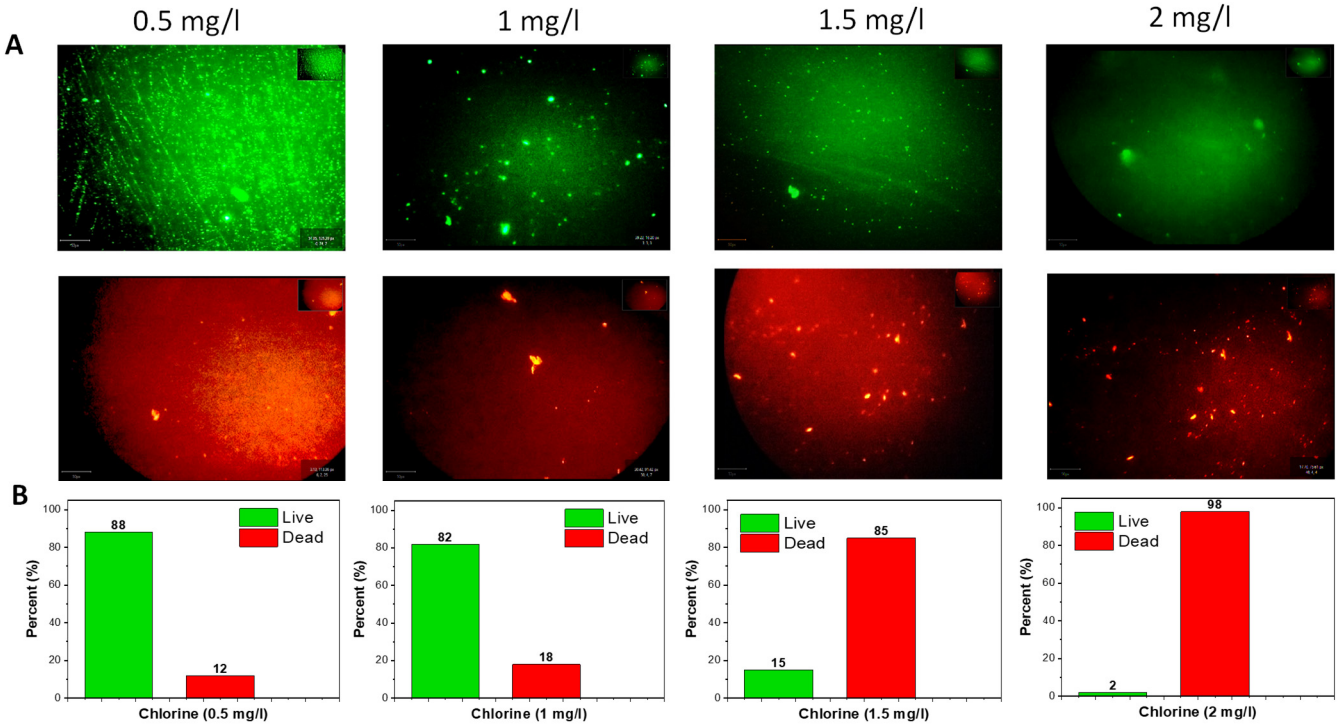

Figure 6. (**A**) Fluorescence staining images of the samples' live/dead bacterial colonies from the biofilms grown on different pipe material slides after 21 days of chlorine disinfection and (**B**) percentage of live/dead stained bacterial colonies at each chlorine dose.

With a 0.5 mg/L dose applied, only a 12% reduction was observed in bacterial cell counts, and exposure to a 1.0 mg/L chlorine concentration for 14–21 days was slightly effective. The present findings resemble those of a prior investigation by [15], which showed that the chlorine disinfectant inactivation effect on heterotrophic bacteria was higher than that of free chlorine. It was observed that the number of viable bacteria grew as the amount of chlorine residue reduced, and, when increasing the chlorine concentration, the bacterial viability decreased with continued disinfection and availability of the residual chlorine. However, some dead cells were observed at 1.5 and 2.0 mg/L chlorine concentrations compared to live cells. Exposure to a 2.0 mg/L chlorine concentration within 21 days of disinfection treatment showed the complete disinfection of bacterial colonies in biofilms, with an estimated reduction of 98%. It is observed that when increasing the chlorine dose, the percentage of the live bacterial density is reduced, and there is a significant increase in the dead bacteria, as shown in Figure 6B. These results are comparable to those of another study that demonstrated full removal of pathogens after 24 h of treatment, which did not grow again when the chlorine concentration was raised to 2.0 mg/L [30–32].

4. Discussion

After identifying the selected pathogens on the slides of the different pipe materials, the effectiveness of chlorine disinfection was evaluated following the different chlorine doses and contact times for each pipeline material. The counts (log CFU/cm^2) of HPC, *Pseudomonas*, *Salmonella*, *Shigella*, and *Vibrio cholera* in all pipeline material biofilm samples at varied concentrations were affected by the disinfection contact time and disinfectant concentration. For example, in all disinfectant doses, the removal of all selected bacterial counts was observed over time (from day 0 to day 21). The bacterial counts initially at time 0 days (before disinfection) were very high, i.e., HPC 5.32 log CFU/cm^2, *Pseudomonas* 4.03 log CFU/cm^2, *salmonella* 4.68 log CFU/cm^2, *shigella* 5.34 log CFU/cm^2, and *Vibrio cholera* 5.35 log CFU/cm^2 (Tables S2 and S3). The disinfectant dose of 0.5 mg/L showed the least effect on removing all counted bacteria concerning time and pipe material. A chlorine

disinfectant dose of 2.0 mg/L achieved the maximum effect on total bacterial reduction of selected pathogens (Figures 2–6). Previous research demonstrates that plastic-based pipe materials are rough; microorganisms can cling to them and form biofilms, inhibiting bacterial development. Germs tend to colonize less readily on smoother surfaces than on rougher ones. Decreased roughness reduces the space where organic and inorganic particles can accumulate, lowering nutrient availability for bacterial development [2,3,14].

The disinfection kinetics results in Figures 2–5 revealed that a dose of 2.0 mg/L was the most effective for removing biofilms; *salmonella* sp. was removed 7 days after disinfection, whereas all other tested bacteria, i.e., *Pseudomonas*, Shigella, and *Vibrio* sp. biofilms, were removed within 14 days of contact time with 2.0 mg/L. This investigation showed that a 2.0 mg/L disinfectant dose might eliminate biofilms. Chlorine levels in drinking water are deemed safe for human consumption for the rest of one's life if they do not exceed the WHO's recommended guideline value of 4–5 mg/L [27]. However, some research indicates that bacteria become resistant to chlorine at elevated doses [23].

The fluorescence images of live/dead bacterial cells and colonies in Figure 6 confirmed chlorine disinfection in qualitative and quantitative terms and also confirmed an optimum dose of 2 mg/L for the removal and disinfection of all the selected pathogens in the biofilm with the appearance of red-stained dead cells/colonies.

The compiled results showed that at 2.0 mg/L chlorine disinfectant, the maximum kinetic rate of *Pseudomonas* elimination effectiveness was 0.846 log CFU/cm^2 per 7 days. The HDPE and ABS pipe material had the maximum removal rate of *Pseudomonas* at 2.0 mg/L with 0.84 log. CFU/cm^2 removal per 7 days, and the PP pipe material had the lowest removal rate of *Pseudomonas*, with 0.60 log. CFU/cm^2 removal per 7 days. The removal efficiency was different for *salmonella* counts on different pipe materials, i.e., *salmonella* could be completely removed from PVC pipe material at 1.5 mg/L within 14 days. However, it was completely removed from all other pipe materials at a disinfectant concentration of 2.0 mg/L, which could disinfect *salmonella* in 21 days. In HDPE, PVC, PP, PC, and ABS pipe materials, salmonella's disinfection rates at 1.5 mg/L were 0.00239, 0.01345, 0.00329, 0.00144, and 0.00239 log. CFU/cm^2 removal per 7 days, respectively. PVC pipe material had the maximum removal rate of *salmonella* at 1.5 mg/L with 0.013 log. The CFU/cm^2 removal per 7 days and PC pipe material for HDPE and ABS pipe materials had the lowest removal rate. The PP pipe material had the highest *Shigella* removal rate of 2.0 mg/L, 0.04 log. CFU/cm^2 removal per 7 days; 2.0 mg/L for the PP pipe material had the maximum reduction rate for *Vibrio cholerae* at 0.041 log. CFU/cm^2 removal per 7 days, whereas ABS and HDPE pipe materials had the lowest reduction rate for *Vibrio cholera*—0.04 log and 0.04 CFU/cm^2 removal per 7 days. Another study shows that the plastic pipe materials used in water distribution systems can impact how well chlorine disinfects. Some plastics, such as PP and PVC, are generally compatible and have little effect on chlorine's effectiveness. On the other hand, substances such as HDPE could show varying resistance to chlorine, which could impact the effectiveness of disinfection. Moreover, certain plastics' compositions, such as ABS, may cause chemical reactions with chlorine that could compromise the material's efficiency.

5. Conclusions

This research investigated the identification of selected pathogens in biofilms formed on different pipe materials and their disinfection kinetics at different chlorine doses. This study showed the presence of several Gram-negative bacteria (*Pseudomonas*, *Salmonella*, *Shigella*, and *Vibrio cholera*) in the biofilm. The observations showed that different pipe materials (PVC, PC, HDPE, ABS, and PP) exhibited minor variations in the bacterial communities of the biofilms as growth for all the selected pathogens was observed on each pipe material. All the physicochemical parameters of the flowing water (used for biofilm growth in each material) were analyzed, and the analyses showed that all the parameters were under the permissible limits of WHO, EPA, and USEPA guidelines.

Furthermore, the correlation of biofilm between different pipe materials and inlet and outlet water samples was evaluated. Different chlorine doses were applied to disinfect selected pathogen colonies in the biofilms, i.e., 0.5, 1.0, 1.5, and 2.0 mg/L; the best biofilm disinfection rate was found with a concentration of 2.0 mg/L. Among these materials, PVC, ABS, and PC demonstrated superior performance as suitable pipe materials in drinking water distribution systems regarding their effectiveness in removing biofilms at a chlorine dose of 2.0 mg/L.

The study has provided in-depth information on the pathogen colonies in biofilms and their chlorination in different plastic pipe materials. However, this study must be extended to a real drinking water distribution system to assess different hydrological and fluid dynamics aspects to obtain satisfactory results and practical applications of suitable pipe material(s) used in the water distribution network.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/w15234099/s1: Figure S1: Annular reactors (ARs) (Model 1320 LJ. BioSurface Technologies Corporation, USA); Table S1: Operation time, reactor setups, and sample collection details; Figure S2: Bacterial reduction before and after disinfection in biofilm samples; Figure S3: Bacterial reduction before and after disinfection in bulk water samples after disinfection with a 2.0 mg/L dose; Table S2: Bacterial reduction (log CFu/cm^2) with a 0.5 mg/L and 1.0 mg/L chlorine dose in biofilm samples; Table S3: Bacterial reduction (log CFu/cm^2) with a 1.5 mg/L and 2.0 mg/L chlorine dose.

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