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Microbiological assessment of biofilm formation on different water storage containers

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Abstract
The provision of safe drinking water, particularly in developing nations, remains a significant challenge. These issues are both diverse and serious. Furthermore, scarcity has led to the practice of storing water in containers for future use, which can result in the development of biofilms over extended periods of time. This study seeks to monitor the formation of biofilms in various types of water storage containers by examining viable and total coliform counts. Additionally, the study aims to isolate and characterize the organisms that may be responsible for biofilm formation. The containers used for water storage include polyethylene, plastic, glass, rubber, galvanized steel, aluminium, stainless steel, and clay. Weekly sampling and analysis were conducted, measuring pH, viable and total coliform counts through plate count and Most Probable Number techniques, and weighing the biofilm formed in each container. The findings revealed that polyethylene exhibited the highest level of biofilm formation, weighing 0.090 g, while galvanized and stainless steel exhibited the lowest level, weighing 0.010 g. Statistical analysis demonstrated a significant difference between polyethylene and galvanized steel, as well as between aluminium and stainless steel, with p-values below 0.05. However, there was no statistical difference between glass and plastic, or between plastic and rubber. The biofilm forming organism in the various water storage containers was identified as *Pseudomonas aeruginosa*. These findings provide further evidence that pathogenic bacteria can survive in biofilms within water storage systems for several weeks, even in unfavorable conditions, posing a potential risk to consumers of such water.

Keywords: Biofilm, microbial cells, stored water, storage containers, viable counts, *Pseudomonas aeruginosa*

1. Introduction
Water is a valuable resource that is essential for human health, food security, and the environment (WHO/UNICEF, 2000 and Mir *et al.* 2013) [1, 2]. It plays a vital role in sustainable development, socio-economic growth, energy and food production, ecological stability, and human survival (WHO/UNICEF, 2000 and Mir *et al.* 2013, WHO, 2012, UNDP, 2006) [1, 4]. As the global population increases, it becomes increasingly important to manage water resources effectively to meet the diverse demands of households, agriculture, industry, and the environment. Failure to meet these demands often leads to conflicts over water (WHO/UNICEF, 2000, WHO, 2012, WHO/UNICEF, 2006) [1, 3, 5]. Developing countries face significant challenges in ensuring adequate water supply (Gadgil, 1998) [6]. To address this issue, people have started storing water in containers for future use, collecting surface or groundwater, or implementing rainwater harvesting (Momba and Notshe, 2003, Van der Merwe *et al.* 2013) [7, 8]. However, water stored in containers can become contaminated due to poor handling, unclean containers, unhygienic practices, and natural environmental pollutants (Van der Merwe *et al.* 2013) [8]. When this happens, contaminants may interact with the container surfaces and form biofilms. These biofilms, as previous studies have shown, can develop on various surfaces and are commonly found in natural, industrial, and hospital settings (Arampatzi *et al.* 2011, Hall-Stoodley *et al.* 2004, Ma *et al.* 2009) [9, 10, 11].
Microorganisms like bacteria can stick to different surfaces and form groups called biofilms (Oliveira *et al.* 2015) [12]. This helps them survive in tough environments and spread to new places (Arampatzi *et al.* 2011, Hall-Stoodley *et al.* 2004, Oliveira *et al.* 2015) [9, 10, 12]. Each biofilm is unique, but they all have a slimy coating that helps them stick together and trap

food. The bacteria in biofilms change how they grow and behave, which makes them hard to kill with medicine or the body's defenses. Biofilms can break apart and spread to other surfaces. The different types of bacteria in a biofilm can also help each other stay strong. Other things from the environment or the body can also affect the structure of a biofilm (Tolker-Nielsen and Molin, 2000, Donlan, 2002, Costerton, 1999, Sanchez *et al.* 2013, Dötsch *et al.* 2012, Sauer *et al.* 2002, Anderson GG and O'Toole, 2008, Mah T-FC and O'Toole, 2001, Fux *et al.* 2005, Characklis *et al.* 1981) [13, 14, 15, 16, 17, 18, 19, 20, 21, 22].

As previously mentioned, the manner in which water is stored has a significant impact on its quality (Van der Merwe *et al.* 2013) [8]. Previous research conducted by esteemed scholars such as Jagals *et al.* (2003) [24] and van der Merwe *et al.* (2013) [8] suggests that water stored outside its natural habitat is more vulnerable to environmental influences and contamination. Notably, authors such as Jagals *et al.* (2003) [24], Momba and Kaleni (2002) [25], Momba and Notshe (2003) [7], and van der Merwe *et al.* (2013) [8] have extensively studied the microbial quality of water stored in small household containers, consistently finding high levels of objectionable organisms that pose a threat to human consumption. Similarly, studies conducted by Momba and Kaleni (2002) [25] and Momba and Notshe (2003) [7] demonstrate that plastic-based containers are more conducive to bacterial incorporation into biofilms on their inner surfaces compared to metal-based containers. These investigations also reveal that plastic containers have a greater tendency to incorporate faecal coliforms into biofilm structures. In fact, van der Merwe *et al.* (2013) [8] highlight the role of these biofilms as reservoirs for pathogenic microorganisms, which, through growth and detachment, contribute to the majority of planktonic cells in the aqueous environment. This is a matter of great concern, as the storage of untreated water that may be contaminated with pathogens such as *P. aeruginosa, S. epidermidis, E. coli, S. aureus, E. cloacae*, and *K. pneumoniae* can create an ideal environment for microbial proliferation and biofilm formation (Ma *et al.* 2009, Fux *et al.* 2005, Parsek and Singh, 2003) [11, 21, 26]. The present study was designed to observe and quantify the formation of biofilms in various water storage containers, utilizing viable and total coliform counts as indicators. Additionally, the study aimed to isolate and characterize organisms that may play a role in biofilm formation.

2. Materials and Methods

2.1. Sample collection and storage

The water sample was collected from the tap (groundwater, pumped to the overhead tank) located near the research centre Govt. Model Science College Rewa (M.P.). The water samples were collected in a sterilized polyethylene, plastic, glass, rubber (polyvinyl chloride), galvanized steel, aluminium, stainless steel and clay containers. A treated water in a polyethylene container served as control. The samples were labeled and transported immediately to laboratory of research centre.

2.2. pH determination

The pH of the different water samples was determined on the first day and after eight weeks of storage using the method described by Okpo *et al.* (2006) [5] with some modification. The pH meter (pHS 25) was standardized with buffer solution of pH 4, 7 and 9.14 and to avoid cross contamination of the samples, the electrode tip was rinsed with the water to be tested, before taking measurements. pH meter reading was taken, when the display becomes stable and the results of each measurement recorded accordingly.

2.3. Most probable number counts

The water samples underwent analysis to detect the presence of coliforms using the Most Probable Number (MPN) technique as previously described by Packiyam *et al.* (2016) [27] and Okore (2009) [28]. The analysis followed the three-tube MPN method to assess coliforms in the water both initially and after eight weeks of storage. The presence of acid is indicated by a change in the medium's color, while the presence of gas is identified by gas bubbles collected in the inverted Durham tube within the medium. The total number of coliforms is determined by counting the tubes that exhibit a positive reaction (color change and gas production) and comparing the pattern of positive results (the number of tubes showing growth at each dilution) with established statistical tables. If the initial test yields a negative result, no further testing is conducted, and the water source is deemed microbiologically safe. However, if any tube within the series displays both acid and gas, the water is considered unsafe, and a confirmed test is performed on the tube that exhibits a positive reaction.

2.4. Enumeration of viable cell

Viable aerobic count was performed on all samples using spread plate technique on Plate count agar (Lab M, UK) and Sabouraud dextrose agar (Lab M, UK) as previously described (Razvi *et al.* 2014, Adeola *et al.* 2012, Cheesbrough, 2006) [29, 30, 31]. The PCA plates were incubated at 37 °C for 24 hours while SDA plates at 25°C for 5 days. Uninoculated plates containing only the sterile media were used as blank to compare the different samples. After the incubation period, discrete colonies were counted using a colony counter and the total aerobic counts expressed as CFU/mL.

2.5. Isolation and identification of microbial isolates

Tests for the isolation of possible microbial contaminants were conducted after eight weeks of storage on all sample containers. A sterile swab sticks was used to swab the inner walls of each container, and the swab from each container was thereafter streaked on the surfaces of Mannitol salt agar (HIMEDIA, India), Salmonella shigella agar (Lab M, UK), MacConkey agar (Lab M, UK), and cetrimide nutrient agar in duplicate. After overnight incubation at 37°C, colonies were identified and characterized using colony characteristics, gram reaction of the organisms and biochemical test following standard procedure (Cheesbrough, 2002, Sandle, 2016) [32, 33].

2.6. Biofilm quantification

Quantification was performed by taking the weight of the empty containers before the start of experiment and after 8 weeks of storage. The difference between initial and final weight was recorded as weight of the biofilm formed.

2.7. Statistical analysis

Statistical analysis using one-way analysis of variance method to compare the results obtained in all the containers was performed. A p-value of less than or equal to 0.05 is

considered to be statistically significant (Alemu *et al.* 2012) [34].

3. Results

3.1. pH determination

The results of pH measurement are presented in Fig. 1. The pH of the control sample increased from 6.89 at the start to 8.24 on the eight weeks of the study. pH level of the water samples in all eight container types was 5.81 at the start of experiment and on the final week, it had changed to 6.36, 6.38, 6.84, 6.89, 7.24, 7.87, 8.04, 8.23 respectively for polyethylene, clay, glass, plastic, rubber, stainless steel, aluminum, galvanized steel containers.

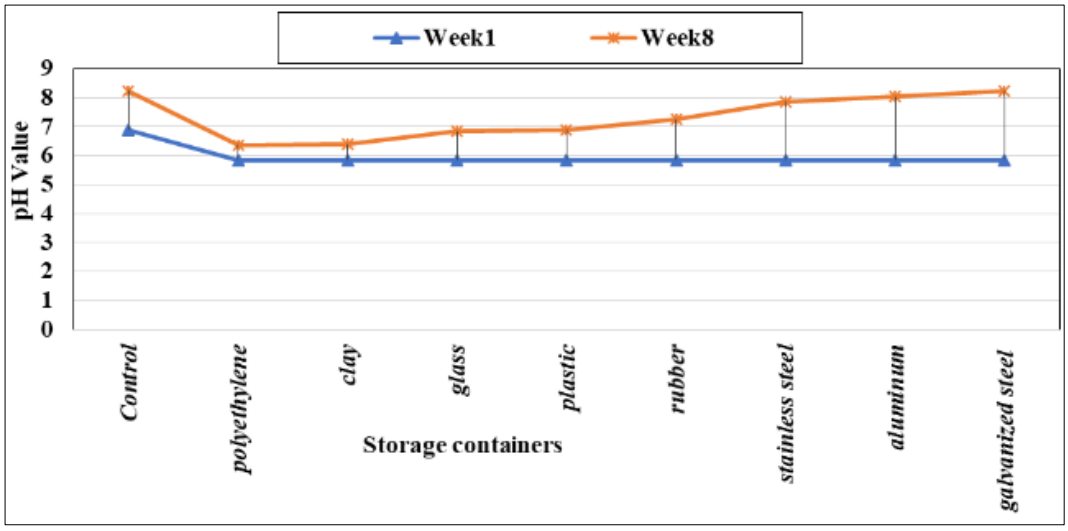

Fig 1: Graph analysis of pH level of water stored in different waters containers during week 1 & 8 of the study

3.2. Most probable number

Results of the statistical value of Most Probable Number (MPN) per 100 mL of water for both the control and water samples at week 1 and week 8 of the study using three tubes of different dilution is presented in Table 1.

Table 1: Most probable number per 100 mL of water for each container before and after week 8 of storage using three tubes of each dilution

S. No.	Storage container	Week 1			MPN/ 100 mL	Week 8			MPN/ 100 mL
		10 mL	1 mL	0.1 mL		10 mL	1 mL	0.1 mL	
1.	Control	---	+--	---	3	---	+--	---	3
2.	Polyethylene	+++	++-	++-	210	+++	++-	+++	290
3.	Clay	+++	++-	++-	210	++-	+++	+++	53
4.	Glass	+++	++-	++-	210	++-	++-	+++	42
5.	Plastic	+++	++-	++-	210	++-	++-	+--	28
6.	Rubber	+++	++-	++-	210	++-	++-	+--	28
7.	Stainless steel	+++	++-	++-	210	++-	+--	+--	20
8.	Aluminum	+++	++-	++-	210	+--	+++	++-	24
9.	Galvanized steel	+++	++-	++-	210	+--	+--	---	7.3

+ = gas production, - = No gas production

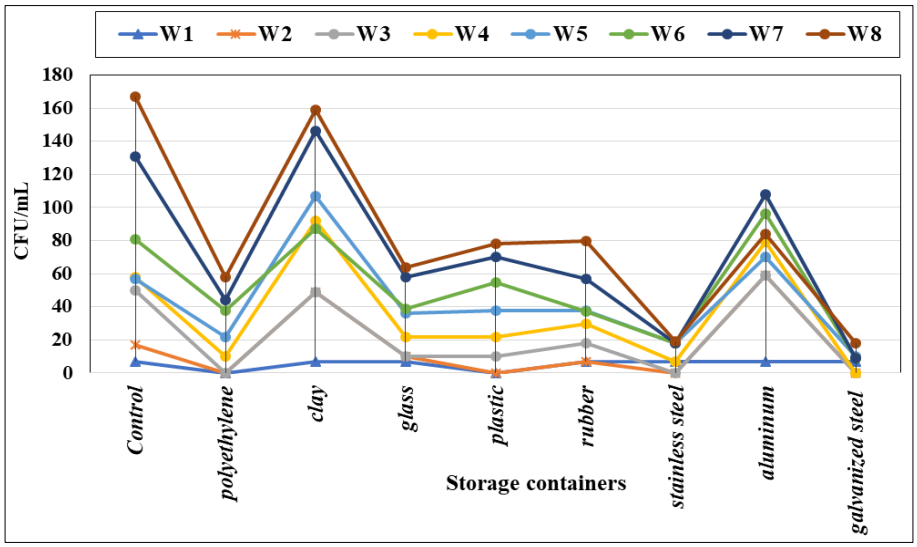

Fig 2: Graph analysis of total viable cell count of water stored in different containers during week 1 & 8 of the study.

3.3. Microbial enumeration

Microbial enumeration tests are required to demonstrate the quality of water under acceptable hygienic conditions and data obtained from the first week up to the eight weeks are presented in Fig. 2. The samples analysed were uncontaminated with fungi as shown from the Sabouraud dextrose agar (SDA) plates. This is obviously due to the absence of planktonic fungi in the water tested. However, there was the presence of viable and potentially pathogenic microorganisms, *Pseudomonas aeruginosa* in all the water samples tested with exception of the control.

3.4. Biofilm determination: Fig. 3 shows the weight of biofilm formed in each of the water storage container, which was determined by difference between the final weight of the container after the water was discarded on the eight weeks of storage and the initial weight of the empty container before the experiment commenced. The result of biofilm formed after the end of the study showed that water stored in control container (treated water), glass, plastic and rubber (PVC), polyethylene had the weights as 0.002 g, 0.04 g, 0.03 g and 0.05 g, 0.09 g respectively, while biofilm formed in galvanized steel, stainless steel aluminum and clay containers had the weights of 0.01 g, 0.010 g, 0.050 g and 0.070 g respectively.

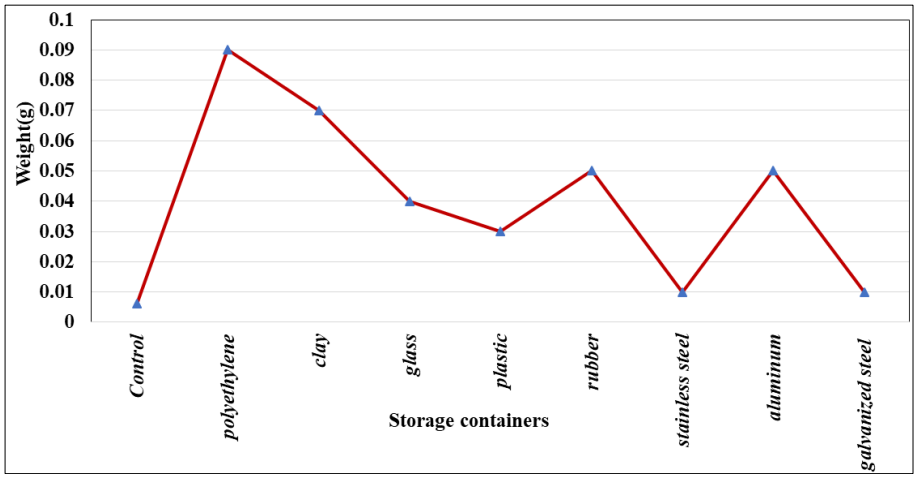

Fig 3: Graph analysis of weight of biofilms forms in different containers after 8 weeks of storage

4. Discussion

The process of biofilm formation initiates when microorganisms, like bacteria, come into contact with a suitable surface and goes through a series of events to adapt to different nutritional and environmental conditions (Oliveira *et al.* 2015, Jamal *et al.* 2015) [12, 35]. In this particular study, our objective was to observe and document the growth and development of biofilms in various water storage containers. We used total aerobic counts as indicators to monitor the process and also isolated and characterized any contaminant organisms present. The water sample we examined was collected from the tap, specifically groundwater pumped into an overhead tank. We then distributed equal amounts of this water onto sterilized containers made of different materials such as polyethylene, plastic, glass, rubber (polyvinyl chloride), galvanized steel, aluminum, stainless steel, and clay. As a control, we used treated water in a polyethylene container.

The findings of this study reveal the widespread presence of microorganisms in nature and highlight the influence of different water storage containers on the pH of the stored water. Galvanized and stainless steel containers were found to have the highest pH values, while polyethylene containers had the lowest. Figure 1 illustrates that the pH of polymer containers was close to neutral, which is an optimal range for the growth of most microorganisms. This aligns with a previous study conducted by Baird (2007) [36]. The presence of *P. aeruginosa* suggests potential contamination before storage, potentially originating from water sources, dust deposits, or handling processes. Microorganisms naturally present in water systems strive to form a biofilm, creating favorable conditions for survival and reproduction. According to Jamal *et al.* (2015) [35], increased attachment occurs when there are higher critical levels in flow velocity, water temperature, or nutrient concentrations. Even relatively low nutrient levels, such as 2,000 micrograms per liter on an agar plate or 0.5 micrograms per liter in purified water systems, can support the flourishing of a microbial community (An YH and Friedman, 1998, Lehtola *et al.* 2007) [37, 38]. *P. aeruginosa* may have an advantage over other organisms due to locomotor structures on their cell surfaces, such as flagella, pili, fimbriae, proteins, or polysaccharides (Jamal *et al.* 2015) [35].

The findings displayed in Table 1 indicate that the polyethylene container contained a significantly greater quantity of bacteria compared to the other containers, which potentially facilitated the development of a more substantial biofilm. Previous research has demonstrated that biofilms within drinking water systems can act as reservoirs for various organisms, including Helicobacter pylori, Legionellae species, Mycobacterium avium, and free-living protozoa, which are increasingly acknowledged for their ability to harbor pathogens (Lehtola *et al.* 2007, Watson *et al.* 2004, Mackay *et al.* 1998, Rogers and Keevil, 1992) [38, 39, 40, 41].

The water stored in various types of containers, such as polymer, glass, aluminum, and clay, showed growth in microbial colonies, except for galvanized steel and stainless steel containers. Among these, aluminum and clay containers had the highest growth, with 60 and 50 colony forming units per milliliter (CFU/mL) respectively. This finding supports a previous study by Rajagopal *et al.* (2013) [42] which also highlighted the ability of clay to support microbial growth. In week three, the bacterial count in water

stored in polyethylene containers increased from 20 to 50 CFU/mL, while glass, plastic, and rubber containers showed minimal growth of 10 to 20 CFU/mL. Weeks 4, 5, 6, 7, and 8 showed an increase in total viable count in all materials, but polyethylene, aluminum, and clay containers exhibited higher growth, consistent with the findings of Maggy and Kaleni (2002) [25]. The growth observed in water stored in polymer containers like plastic, rubber (PVC), polyethylene, and glass can be attributed to the leaching of materials used in their production, which may have provided nutrients for the organisms, as previously reported (Rogers *et al.* 1994) [43]. It was noted that water stored in galvanized and stainless-steel containers showed limited growth at a dilution of 1/100 during the 4th and 3rd week respectively. Water stored in aluminum containers showed growth throughout the 8 weeks, with the peak growth of 110 CFU/mL occurring in week 7. According to Tang and Cooney (1998) [44], the added paint materials in galvanized and stainless-steel containers may have contributed to the decrease in the number of colonies recorded in this study compared to aluminum containers. Statistically, there was no significant difference in CFU/mL obtained from water in control and glass containers, as their p-value was greater than 0.05. Similarly, there was no significant difference between plastic, rubber, aluminum, and clay containers, as their p-value was also greater than 0.05 (statistically insignificant). However, there was a significant difference observed when comparing polyethylene, galvanized steel, stainless steel, and aluminum containers, as their p-values were less than 0.050.

In this study, the formation of biofilm varied among different containers. This could be attributed to the characteristics and composition of the containers, as mentioned in a previous report (Verran and Whitehead, 2005) [45]. Figure 3 shows that biofilm formation was highest in polyethylene containers. This could be because the hydrophobic surfaces of these polymers enhance cell adhesion and, consequently, biofilm formation, as previously observed (Zeng *et al.* 2015) [46]. The higher biofilm formation may also be influenced by the release of nutrients from the container materials, which can support bacterial growth (Rummel *et al.* 2017) [47]. On the other hand, the galvanized and stainless-steel containers exhibited the lowest level of biofilm formation. This could be due to the hydrophilic nature of their surfaces, which requires a longer exposure time for microbial attachment to occur (Momba and Kaleni, 2002) [25].

5. Conclusion

The findings of the current study have confirmed that the quality of water can be affected by long-term storage, leading to an increase in the number of viable cells on water storage containers. It has been determined that *Pseudomonas aeruginosa* is the organism responsible for the formation of biofilm in various types of water storage containers. It is recommended that galvanized steel or stainless-steel containers be used for long-term storage of drinking water, as other materials such as aluminium, clay, glass, and polymer containers have the potential to support biofilm formation, which could be harmful to public health. Considering that water stored in containers in a domestic setting is more susceptible to environmental factors and potential nutrient contamination compared to water in closed pipe distribution systems, it is logical to assume that biofilm-like substances can accumulate in these containers. Therefore, it is advised not to store untreated water for extended periods of time, as cells within a biofilm possess unique physiological characteristics that enable them to adapt to their surroundings in terms of nutrient availability, oxygen supply, and interaction with waste products. This distinct state can result in the tolerance and development of persistent and dormant cells, posing a risk to consumers of such water.

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