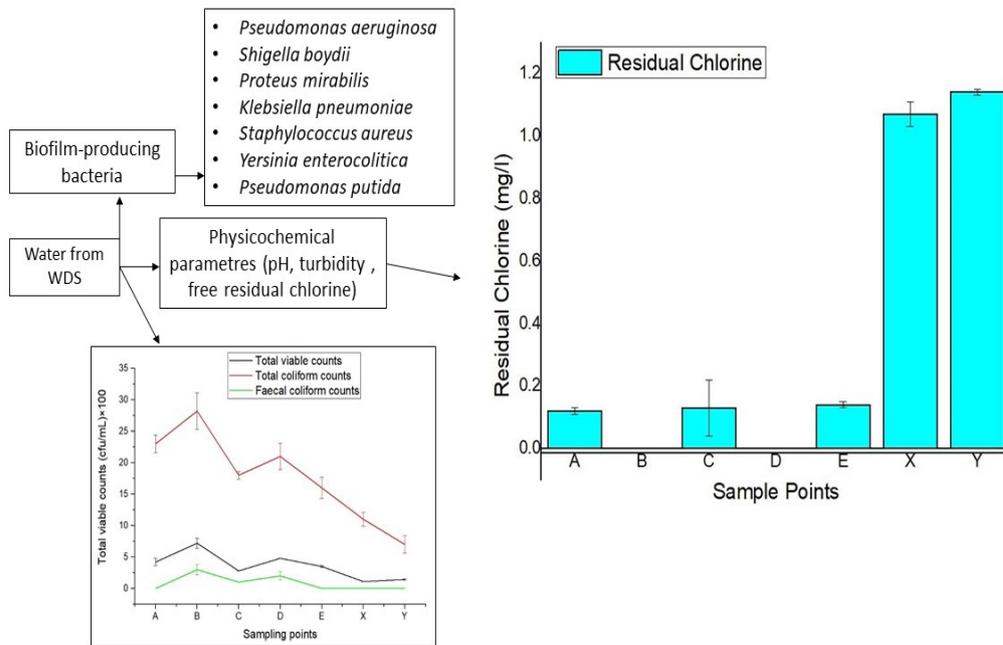


## Biofilm-forming bacteria and their antibiotic resistance in treated water supplies in Ilorin Metropolis, Nigeria

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### Highlights

- Eleven bacterial isolates were identified along the distribution system.
- Majority of them were biofilm formers, which showed stronger antibiotic resistance.
- Residual chlorine was not effective in some sampling points.
- Bacterial biofilm count should also be added to indicators of water.

RESEARCH ARTICLE

## Biofilm-forming bacteria and their antibiotic resistance in treated water supplies in Ilorin Metropolis, Nigeria

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**Abstract:** Several water-related disease outbreaks have been attributed to deficiencies in water distribution systems (WDS). This study determined the relationship between biofilm-producing bacteria and their antibiotic resistance in WDS in Ilorin Metropolis, Nigeria through the assessment of the quality of water, the virulence factors possessed and susceptibility to some antibiotics. The physicochemical and bacteriological quality of the water samples were analyzed using standard methods. A total of 60 samples were collected from seven different locations within Ilorin metropolis. The pH of the water samples ranged from 6.58 – 7.22, turbidity 0.14 – 2.07 NTU and free residual chlorine ranged from 0.00 – 1.14 mg/L. A total of eleven bacteria isolates were identified by biochemical and molecular methods. They included genera of *Pseudomonas*, *Escherichia*, *Staphylococcus*, *Klebsiella*, *Citrobacter*, *Shigella*, *Proteus*, *Yersinia* and *Serratia*. The total bacterial counts were  $1.10 - 7.20 \times 10^2$  cfu/mL. The isolates consisted of 72.7% biofilm producers using the tube method and 63.6% biofilm producers using the congo red method. Both biofilm and non-biofilm isolates displayed complete resistance to ceftriazone, amoxicillin, tetracycline, and cotrimoxazole. Biofilm producers showed stronger relationship with antibiotic resistance ( $p < 0.0001$ ) than non-biofilms, thus suggesting the use of bacterial biofilm counts as indicators of water quality.

**Keywords:** Biofilm; potable water; virulence; antibiotic resistance; water distribution systems.

### INTRODUCTION

The next vital resource after the air we breathe in is water. As a requisite for all forms of life on earth, it deserves continuous and adequate monitoring to ensure potability and safe consumption by the consumers because the quality of treated drinking water may become contaminated while distributing (Fish *et al.*, 2017). Mokuolu *et al.* (2017) reported that municipal water from treatment plants serves as the most secure drinking water source in many urban areas in Nigeria, however the quality of pipe-borne water has been compromised due to the unsustainable demographic growth and breaches in the integrity of distribution pipe network (Eniola *et al.*, 2015), which results from poor surveillance and maintenance practices by the government.

Biofilm is a heterogenous bacterial community consisting of slime matrix which protects the organisms

from desiccation, antibiotics, biocides, heavy metals and ultraviolet radiation (Flemming and Wingender, 2010). Naturally, bacteria dwell in several environmental concavities at regions between two phases, which can either be between water and air or a substratum. The growth of biofilms causes alteration in the turbidity, taste, color, and odor of water (Chandy and Angles, 2001) and even deterioration of residual disinfectants. They are capable of growing in the presence of disinfectants in water thereby making them resistant to antimicrobials and even to antibiotics (Khan *et al.*, 2016). It is, therefore, indicated that drinking water distribution systems (DWDS) could serve as an incubator for the growth and spread of antibiotic resistance opportunistic pathogens. Hence, there is a need to ascertain the relationship between biofilms and antibiotic resistance in DWDS.

In Ilorin, during the dry season, there is a high demand for treated water due to the drastic decrease in the level of groundwater sources. More so, little is known about the fate of these organisms, whether they could get into water at consumers' faucet to cause disease outbreaks. Furthermore, there are no recommended limits of bacterial biofilms in drinking water, unlike coliforms. The objective of this study thus was to determine antibiotic resistance in biofilm-producing bacteria isolated from various DWDS in Ilorin, Kwara State, Nigeria.

### MATERIALS AND METHODS

#### Sample collection

Samples for this study were obtained along water distribution networks from Fate, Basin, Tanke, Government Residential Area (G.R.A) and the University of Ilorin (Unilorin) communities within Ilorin which have their source from Agba Water Treatment Plant. Samples were also taken from Unilorin Water Treatment Plant. The sampling points A (Fate), B (Basin), C (Government Reservation Area) and D (Tanke) which are about two kilometers from each other were chosen based on the high population density of at least 20,000 inhabitants, the accessibility and usage of pipe-borne water.

A total of 60 water and swab samples were collected from household distribution taps and inner pipe walls respectively using standard methods as described by

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September *et al.* (2007). Treated water was also collected from Agba and Unilorin treatment sites, designated point X and Y, respectively, before entry into distribution pipelines. All samples were transferred to the Microbiology Laboratory, University of Ilorin under cold chain.

### Physicochemical analysis

The pH of water samples was determined using a calibrated pH meter, the temperature using mercury-bulb thermometer, turbidity using visible spectrophotometer (Canspec M105) at 460 nm wavelength, total dissolved solids (TDS) and electrical conductivity (EC) measured using the TDS and EC digital meter, respectively. Free chlorine residual was determined with the aid of a Lovibond comparator using its chlorine standard discs. All analysis was done in triplicates on each water sample collected.

### Bacteriological analysis

The total bacterial count was determined using standard pour plate technique (APHA, 2005). Total and faecal coliform bacteria were enumerated by the multiple tube fermentation test (APHA, 2005). Coliform count was obtained using the three tubes assay of the most probable number (MPN) technique. The presumptive, confirmatory and completed coliform test was carried out as described by Nwachukwu *et al.* (2013).

Pure bacterial isolates were characterized using cultural, cellular, biochemical, and 16S rRNA PCR techniques (Kolawole and Afolayan, 2017).

The tube and congo red agar method was employed for the detection of biofilm formation, and interpreted using Afreenish *et al.* (2011) protocol.

### Virulence screening of bacterial isolates

Bacterial isolates were screened for virulence based on the production of enzymes viz.: Haemolysin, lecithinase, lipase, proteinase, coagulase, and deoxyribonuclease (DNase) using the methods of Edberg *et al.* (2009) and Horn *et al.* (2016).

### Antibiotic susceptibility testing of the isolates

Antibiotic susceptibility test of the isolates was performed using the Kirby-Bauer disk diffusion method (Flores-encarnacion *et al.*, 2016). Results obtained were used to classify isolates using standard reference values of the Clinical and Laboratory Standard Institute (CLSI, 2016).

### Statistical analysis

All the data generated in this study were analyzed descriptively using MS Excel and Statistical Package for Social Sciences (SPSS version 16.0).

## RESULTS

### Physicochemical parameters

The physicochemical properties are presented in Table 1. The pH ranged from 6.58 - 7.22. Temperature ranged from 26 °C to 31 °C. The highest mean value of 31 °C was obtained in sample B while sample X had the lowest mean value of 26 °C. The turbidity of the water samples varied between 0.14 NTU and 2.07 NTU with the highest turbidity value recorded in sample D while the lowest value was reported in samples from point Y. Total dissolved solids ranged from 73.4 mg/L to 181.0 mg/L. Electrical conductivity ranged from 202.0 µS/cm to 290.6 µS/cm as observed in samples A and D, respectively. Free residual chlorine ranged from 0.00 mg/L to 1.14 mg/L obtained in sample Y.

### Bacteriological analysis

The total heterotrophic plate counts (THPC) of the water samples within the study period ranged from  $1.10 \times 10^2$  cfu/mL to  $7.20 \times 10^2$  cfu/mL in sample X and B, respectively. Total coliform counts ranged from 7.00 MPN/100 mL - 28.20 MPN/100 mL in samples Y and B respectively. Faecal coliforms were found in samples collected from sites B, C, and D with counts ranging from 0.00 MPN/100 mL to 3.00 MPN/100 mL. The zero counts were recorded in samples A, E, X and Y while sample B had the highest fecal coliform counts (Table 2).

**Table 1:** Physicochemical properties of water samples taken at different sampling points

Sampling points	Electrical conductivity (µS/cm)	Residual chlorine (mg/L)	Total dissolved solids (mg/L)	pH	Temperature (°C)	Turbidity (NTU)
A	202.00±2.55 <sup>a</sup>	0.12±0.01 <sup>b</sup>	128.80±4.67 <sup>c</sup>	7.22±0.07 <sup>c</sup>	28.00±0.45 <sup>bc</sup>	1.38±0.03 <sup>c</sup>
B	268.40±1.86 <sup>d</sup>	0.00±0.00 <sup>a</sup>	162.40±2.48 <sup>c</sup>	6.80±0.28 <sup>abc</sup>	31.00±0.55 <sup>d</sup>	1.24±0.09 <sup>c</sup>
C	279.40±0.68 <sup>c</sup>	0.13±0.09 <sup>b</sup>	129.00±1.27 <sup>c</sup>	6.64±0.13 <sup>ab</sup>	28.00±0.71 <sup>bc</sup>	0.61±0.02 <sup>b</sup>
D	290.60±0.68 <sup>f</sup>	0.00±0.00 <sup>a</sup>	181.00±0.71 <sup>f</sup>	6.88±0.19 <sup>abc</sup>	27.00±0.45 <sup>ab</sup>	2.07±0.17 <sup>d</sup>
E	265.40±1.72 <sup>d</sup>	0.14±0.01 <sup>b</sup>	143.00±0.71 <sup>d</sup>	7.10±0.16 <sup>bc</sup>	29.00±0.45 <sup>c</sup>	0.74±0.04 <sup>b</sup>
X	221.40±0.75 <sup>b</sup>	1.07±0.04 <sup>c</sup>	99.80±2.30 <sup>b</sup>	6.74±0.04 <sup>abc</sup>	26.00±0.71 <sup>a</sup>	0.81±0.18 <sup>b</sup>
Y	235.40±0.00 <sup>c</sup>	1.14±0.01 <sup>d</sup>	73.40±0.75 <sup>a</sup>	6.58±0.02 <sup>a</sup>	28.00±0.00 <sup>bc</sup>	0.14±0.01 <sup>a</sup>

Key = Mean ± Standard error, ANOVA, DMRT (Duncan multiple range test) (n = 5). A, B, C, D, E, X and Y = Fate, Basin, Government Reservation Area, Tanke, Unilorin, Agba dam and Unilorin dam respectively. Values with different superscripts within same column are significantly different and those with the same superscripts have no significant difference at  $p < 0.05$ .

**Table 2:** Bacteriological counts of the water samples

Sampling Points	Total viable counts ( $\times 10^2$ cfu/mL)	Total coliform counts (MPN/100 mL)	Faecal coliform counts (MPN/100 mL)
A	4.20 $\pm$ 0.58 <sup>cd</sup>	23.00 $\pm$ 1.4 <sup>d</sup>	0.00 $\pm$ 0.00 <sup>a</sup>
B	7.20 $\pm$ 0.8 <sup>e</sup>	28.20 $\pm$ 2.9 <sup>e</sup>	3.00 $\pm$ 0.80 <sup>b</sup>
C	2.80 $\pm$ 0.02 <sup>b</sup>	18.00 $\pm$ 0.61 <sup>cd</sup>	1.00 $\pm$ 0.00 <sup>ab</sup>
D	4.80 $\pm$ 0.04 <sup>d</sup>	21.00 $\pm$ 2.07 <sup>cd</sup>	2.00 $\pm$ 0.70 <sup>b</sup>
E	3.50 $\pm$ 0.18 <sup>bc</sup>	16.00 $\pm$ 1.7 <sup>bc</sup>	0.00 $\pm$ 0.00 <sup>a</sup>
X	1.10 $\pm$ 0.01 <sup>a</sup>	11.00 $\pm$ 1.10 <sup>ab</sup>	0.00 $\pm$ 0.00 <sup>a</sup>
Y	1.42 $\pm$ 0.07 <sup>a</sup>	7.00 $\pm$ 1.4 <sup>a</sup>	0.00 $\pm$ 0.00 <sup>a</sup>

Mean  $\pm$  Standard error, ANOVA, DMRT (Duncan multiple range test) (n = 5). A, B, C, D, E, X and Y = Fate, Basin, G.R.A, Tanke, Unilorin, Agba dam and Unilorin dam respectively. Values with different superscripts within same column are significantly different and those with the same superscripts have no significant difference at P < 0.05

**Table 3:** Distribution of bacterial isolates of various sources of water distribution system

Isolates	Distribution points	Inner pipe walls	Treatment sites
<i>P. aeruginosa</i>	A, B, C, D, E	A, B, D, E	X, Y
<i>P. cichori</i>	B, C, D	-	-
<i>S. marcescens</i>	A, D	A	-
<i>E. coli</i>	A, B, D	B, C, D	-
<i>S. boydii</i>	B, C, D, E	B, C, D, E	-
<i>P. mirabilis</i>	A, D	A, C, D	-
<i>K. pneumoniae</i>	A, B, C	B, C	-
<i>S. aureus</i>	A, B, C, E	A, B, C, D, E	X, Y
<i>Y. enterocolitica</i>	A, B, C, E	E	-
<i>P. putida</i>	A, B, C	-	-
<i>C. freundii</i>	B, E	-	X

Key: - = Absent; A, B, C, D, E, X and Y = Fate, Basin, G.R.A, Tanke, Unilorin, Agba dam and Unilorin dam, respectively.

### Bacterial species isolated from all samples

A total of eleven bacterial species were isolated from all water and swab samples analyzed. They are: *Escherichia coli* strain ASRM93, *Pseudomonas aeruginosa* strain SMVIT-1, *Pseudomonas putida* strain R1-72, *Pseudomonas cichorri* strain PCI, *Shigella boydii*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Staphylococcus aureus*, *Serratia marcescens* and *Proteus mirabilis*. The distribution of each isolates in various sampled points is shown in Table 3.

### Biofilm screening

#### Tube method (TM)

Eleven bacterial isolates were screened for biofilm production using the tube method. Of these, eight were tested positive while three were non-biofilm formers. Of the eight biofilm-forming isolates, four produced strong biofilms while the other four were observed as weak biofilm producers (Table 4).

#### Congo red agar (CRA) method

Using CRA method for the detection of biofilms, seven isolates produced biofilms while four did not. Of the seven biofilm formers, five produced strong biofilms while two were weak biofilm producers (Table 4).

### Virulence characteristics of bacterial isolates

Five of the isolates (*Pseudomonas aeruginosa*, *Shigella boydii*, *Proteus mirabilis*, *Staphylococcus aureus* and *Pseudomonas putida*) produced haemolysin and proteinase, three (*Klebsiella pneumoniae*, *Pseudomonas putida* and *Staphylococcus aureus*) produced Lipase and Lecithinase, two (*Shigella boydii* and *Pseudomonas aeruginosa*) produced DNase while only one (*Serratia marcescens*) was positive for lecithinase.

### Antibiotic susceptibility test

The diameter of microbial zone of inhibition was determined and used to calculate the number of isolates in percentage. The potency of antibiotics used was also recorded (Table 5).

**Table 4:** Biofilm screening outcome for the isolates

Isolates			Observation		Inference
	CRA	TM	CRA	TM	
S <sub>4</sub>	++	++	Black colonies	Visible film in tube	True positive
S <sub>9</sub>	-	-	Pink colonies	No visible film in tube	True negative
S <sub>6</sub>	-	++	Pink colonies	Visible film in tube	False positive
W <sub>5</sub>	-	-	Pink colonies	No visible film in tube	True negative
W <sub>6</sub>	+	++	Dark growth without dry crystalline morphology	Visible film in tube	True positive
W <sub>7</sub>	++	+	Black colonies	Very less visible film in tube	True positive
S <sub>8</sub>	++	+	Black colonies	Very less visible film in tube	True positive
W <sub>1</sub>	++	+	Black colonies	Very less visible film in tube	True positive
S <sub>2</sub>	+	+	Dark growth without dry crystalline morphology	Very less visible film in tube	True positive
S <sub>11</sub>	++	++	Black colonies	Visible film in tube	True positive
W <sub>3</sub>	-	-	Pink colonies	No visible film in tube	True negative

Key: - = Negative (non-biofilm), + = Weak biofilm, ++ = Strong biofilm, CRA = Congo red agar method, TM = Tube method; S<sub>4</sub>, S<sub>9</sub>, S<sub>6</sub>, W<sub>5</sub>, W<sub>6</sub>, W<sub>7</sub>, S<sub>8</sub>, W<sub>1</sub>, S<sub>2</sub>, S<sub>11</sub> and W<sub>3</sub> = *Pseudomonas aeruginosa*, *Pseudomonas cichorri*, *Serratia marcescens*, *Escherichia coli*, *Shigella boydii*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Pseudomonas putida* and *Citrobacter freundii*, respectively.

**Table 5:** Antibiotic susceptibility pattern of the isolates

Antibiotics	Disc potency (µg)	No. of Isolates (%)					
		Resistant		Sensitive		Intermediate	
		Biofilm	Non-biofilm	Biofilm	Non-biofilm	Biofilm	Non-biofilm
AUG	30	28.6	25	28.6	25	42.9	50
CRO	30	100	50	0	0	0	50
NIT	200	71.4	25	0	25	28.6	50
GEN	10	28.6	0	42.9	100	28.6	0
COT	25	71.4	75	14.3	0	14.3	25
OFL	5	0	0	85.7	50	14.3	50
AMX	25	71.4	75	14.3	0	14.3	25
CPX	10	0	0	100	75	0	25
TET	30	85.7	75	14.3	0	0	25
PFX	5	0	0	42.9	75	57.1	25

Key: AUG = Augmentin, CRO = Ceftriazone, NIT = Nitrofurantoin, GEN = Gentamicin, COT = Co-trimoxazole, OFL = Ofloxacin, AMX = Amoxicillin, CPX = Ciprofloxacin, TET = Tetracycline and PFX = Pefloxacin.

## DISCUSSION

The study found that the pH of water samples fell within the values recommended by WHO (2006) guidelines for drinking water quality (i.e. 6.5 – 8.5).

Temperature, which determines the chemical reaction rates in water, also conformed to values reported by Adegboyega *et al.* (2015).

The turbidity was also under the acceptable limits (5 NTU) by the WHO (2006), thus confirming the quality of drinking water supply. The results obtained revealed that low turbidity values corresponded to low bacteriological counts and vice versa. This is also confirmed from the reports of Oparaocha *et al.* (2010) and Agbabiaka *et al.* (2014).

Total dissolved solids (TDS) ranged from 73.4 - 181

mg/L which were in accordance with the findings of Adesoji and Ogunjobi (2013) in a similar experiment elsewhere, also fall within the WHO (2010) recommended permissible limits of 500 mg/L.

The electrical conductivity ranges from 202.0 - 290.6 µS/cm, which is in consonance with the acceptable limits of 0 – 1000 µS/cm of the WHO (2010). High electrical conductivity in water results from a higher amount of impurities and vice versa thus indicative of purity level.

All sampled points along distribution systems did not conform to the residual chlorine standard set by WHO (2000). This is thus indicative of bacterial regrowth and post-treatment contamination in the water distribution systems. A similar trend has been reported by Sule *et al.* (2009).

The total bacterial count aligns with those reported

by Eniola *et al.* (2015). However, this higher bacterial count is a clear indication of bacterial regrowth and post-treatment failure/contamination. The total coliform counts did not conform to the WHO standards of zero coliform counts in 100 mL sample, thus it is a clear reflection of contamination and poor water infrastructure set in place. Faecal coliform counts ranged from 0.00 MPN/100 mL to 3.00 MPN/100 mL. The detection of faecal coliforms and *E. coli* among the isolates is strong evidence of faecal contamination. Agbabiaka *et al.* (2014) also recorded higher faecal coliform counts of about 0.0 -  $2.3 \times 10^2$  cfu/mL in a similar study of assessment of public water supplies. The bacterial isolates include: *Escherichia coli* strain ASRM93, *Pseudomonas aeruginosa* strain SMVIT-1, *Pseudomonas putida* strain R1-72, *Pseudomonas cichorri* strain PCI and others include; *Staphylococcus aureus*, *Serratia marcescens*, *Shigella boydii*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Citrobacter freundii* and *Proteus mirabilis*, all of which have been previously implicated in gastroenteritis (Nwidu *et al.*, 2008).

The isolation of some bacterial species from inner pipe walls indicates a gradual microbial deterioration of drinking water quality which is in agreement with the findings of Liu *et al.* (2014). Wingender and Flemming (2011) also explained that the developmental phase of biofilms involves a process of temporary attachment and detachment.

The Tube and Congo red agar (CRA) methods employed for the detection of biofilm production correlated significantly ( $p < 0.05$ ) and was in agreement with Jain and Agarwal (2009).

Most of the bacteria isolated showed varying levels of virulence from the production of enzymes and their resistance to selected antibiotics. Analysis of virulence revealed that most biofilm-producing isolates possessed more virulent traits than the non-biofilms as there was a significant relationship between biofilms and the specific virulence parameters suggesting that biofilm formation contributes greatly to virulence. This corresponds with the findings of Pimenta *et al.* (2003) who reported that the ability to form biofilms increases the degree of virulence in many bacterial species.

Antibiotic susceptibility screening indicated that both biofilm and non-biofilm isolates displayed 100% resistance to ceftriazone and 75% to tetracycline, cotrimoxazole, and amoxicillin in agreement with the findings of Flores-encarnacion *et al.* (2016) who reported the resistance of bacteria in water pipes to the first-line antibiotics. The overall susceptibility for both biofilm and non-biofilm isolates was observed for Ciprofloxacin (100%) and Gentamicin (100%) respectively. Statistical evidence shows that both biofilm and non-biofilm producing bacteria had a relationship with antibiotic resistance ( $p < 0.05$ ), although biofilms had a stronger relationship ( $p < 0.0001$ ) indicating that biofilm formation seriously contributes to antibiotic resistance.

Andersson and Hughes (2010) similarly reported that the emergence of antibiotic resistant bacteria is not only due to physiological factors like biofilm formation,

but also depends on genetic factors like horizontal gene transfer (HGT) rate. Jaglic and Cervinkova (2012) and Morente *et al.* (2013) indicated that other mechanisms like multi-drug efflux pump and modification of cell wall also induce antibiotic resistance in bacteria. Khan *et al.* (2016) suggested that in (WDS), the presence of disinfectant resistant genes in most bacteria e.g. *qac* (Quaternary ammonium compounds) genes also delivers a corresponding antibiotic resistance. This study, therefore, suggests that other factors (beyond biofilm formation) and mechanisms are also involved in the antibiotic resistance phenomenon, thus this needs further studies to establish the unknown factors contributing to antibiotic resistance.

## CONCLUSION

Biofilm formation coupled with other predisposing factors in WDS accounts for the presence of antibiotic-resistant bacteria at consumers' end. The quality of water supplied was also found compromised as pathogens were present in the water and on pipe walls in consumers' faucets. Poor sanitary conditions, inadequate treatment, and post-treatment contamination seem accountable for the poor water quality. Biofilms in water can be removed using nutrient salts which aggregate them into large clusters that can then be filtered.

Going by the findings of this study, it becomes necessary that bacterial biofilm counts should be added to the indicators of water quality as they could indicate poor water quality and the presence of virulent bacteria in water.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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