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Evaluation of Different Salts and Heavy Metal Concentrations on Bacterial Biofilm from Selected Surface and Borehole Water Samples

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Abstract: Biofilms in drinking water systems can serve as significant environmental reservoirs for pathogenic bacteria associated with gastro-enteric diseases. The evaluation of the effects of different salts and metal concentrations on bacterial biofilm from surface and borehole water samples was conducted. Water samples were collected, from 10 selected water sources of economic importance, aseptically using sterile containers. The physicochemical properties were investigated before the biofilm generation process. The collected water samples were allowed to stand in a secluded environment for four (4) weeks at 27°C±2°C for biofilm generation. The isolates were characterized culturally, morphologically, biochemically and molecularly. The isolates were identified as *Stenotrophomonas pavanii, Stenotrophomonas maltophilia, Chromobacterium violaceum, Bacillus cereus, Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa*. It was observed that the isolates exhibited growth at a wide range of temperature, salts, pH, and metal salt concentrations. To determine the metal tolerance of the isolates, different concentrations (0.05, 0.1, 0.5, and 1.0%) of four metal salts; ferrous chloride ($FeCl_2$), zinc chloride ($ZnCl_2$), calcium chloride ($CaCl_2$), and magnesium oxide (MgO) was used in nutrient broth. Their ability to grow in medium containing different salt (NaCl) concentrations was also evaluated. Different concentrations of NaCl ranging from 2.0% to 10.0% were used in nutrient broth seeded with 0.1ml of the inocula and incubated at 37°C for 48 and 24hours respectively. Growth was measured in terms of OD at 660 nm using spectrophotometer. Results showed a decline in the growth of the isolates with percentage increase in concentrations of all the metal salts. The result of the effect of NaCl salt on growth showed a decrease in growth with an increase in NaCl concentration from 2% to 10%. For *Stenotrophomonas pavanii*, $FeCl_2$ is negatively correlated with pH r=-.998 but there is positive correlation between $CaCl_2$ and NaCl with r=889.

Keywords: Biofilms, Isolates, Metals, Salts

1. Introduction

A biofilm is an assemblage of microbial cells in which cells adhere to each other on a surface. These adherent cells are enclosed in a self-produced matrix of extracellular polymeric substances (EPS) primarily composed of polysaccharide. An established biofilm structure comprises of microbial cells and EPS, has a defined architecture, and provides an optimal environment for the exchange of genetic materials between the cells. EPS may account for 50% to 90% of the total organic carbon of biofilms [7] and can be considered the primary matrix material of the biofilm. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case of the EPS of Gram negative bacteria [6].

The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates confers the anionic property [15]. This property is important because it allows association of divalent cations such as

calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm [7]. In the case of some gram-positive bacteria, such as the staphylococci, the chemical composition of EPS may be quite different and may be primarily cationic. Hussain *et al*., 1993 found that the slime of coagulase-negative bacteria consists of a teichoic acid mixed with small quantities of proteins [10].

EPS is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. EPS may be hydrophobic, although most types of EPS are both hydrophilic and hydrophobic [15] EPS may also vary in its solubility. Sutherland 2000 noted two important properties of EPS that may have a marked effect on the biofilm. First, the composition and structure of the polysaccharides determine their primary conformation. For example, many bacterial EPS possess backbone structures that contain 1, 3- or 1, 4-β-linked hexose residues and tend to be more rigid, less deformable, and in certain cases poorly soluble or insoluble. Other EPS molecules may be readily soluble in water. Second, the EPS of biofilms is not generally uniform but may vary spatially and temporally [15]. Leriche *et al*., 2000 used the binding specificity of lectins to simple sugars to evaluate bacterial biofilm development by different organisms. These researchers' results showed that different organisms produce differing amounts of EPS and that the amount of EPS increases with age of the biofilm [12]. EPS may associate with metal ions, divalent cations, other macromolecules (such as proteins, DNA, lipids, and even humic substances) [7]. EPS production is known to be affected by nutrient status of the growth medium; excess available carbon and limitation of nitrogen, potassium, or phosphate promote EPS synthesis. Slow bacterial growth will also enhance EPS production [15].

Microbes form biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on surfaces, favorable environmental conditions, nutritional clues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentration of antibiotics. When a cell switches to biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated [11]. The attachment of microorganisms to surfaces is a very complex process, with many variables affecting the outcome. In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface "conditioning" films [5]. The characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength, and temperature, may play a role in the rate of microbial attachment to a substratum. Several studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems [4]. This effect may be due to water temperature or to other unmeasured, seasonally affected parameters. Fletcher and Loeb, found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the attachment of *Pseudomonas fluorescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively charged bacterial cells and the glass surfaces [8]. Cowan, *et a*l., showed in a laboratory study that an increase in nutrient concentration correlated with an increase in the number of attached bacterial cells [3].

2. Materials and Methods

2.1. Sample Collection

The surface water samples were collected from Echem stream in Ogwuari village Nsugbe, Ori stream in Amanuke town, Ofifia stream Awka, Nnamdi Azikiwe University, Awka, Nkisi River in Onitsha and Nkisi spring water in Onitsha. The borehole water samples were collected from a borehole located at No. 3A Ogwugwu Lane, inland town, Onitsha; borehole located at Amagu village, Amanuke; a borehole at female hostel, Unizik Awka; borehole at No 5 Aduma Street, Obosi, and a borehole at Amaku Road, Awka, all in Anambra state, Nigeria.

2.2. Generation of Biofilm

Biofilm was generated from both the borehole water and surface water samples by allowing the water samples to stands for 4weeks at room temperature. The waters were then discarded and the resulting slimy layer formed on the containers collected using swab sticks.

2.3. Isolation of Biofilm Bacteria

The biofilm generated from the water samples were inoculated on plates containing Reasoner's 2 agar (R2A medium contains 0.5g of yeast extract powder, 0.5g of protease peptone, 0.5g of casimino acid, 0.5g of glucose, 0.5g of soluble starch, 0.3g of sodium pyruvate, 0.3g of K_2HPO_4, 0.05g of $MgSO_4$, 15g of agar) and on nutrient agar plates for comparison. The inoculated plates were incubated at 37°C for 2-5 days.

After incubation, colonies with visually distinguishable morphologies were randomly selected and isolated by directly streaking on nutrient agar plates and on R2A plates and incubated for 24-hours. The isolated colonies were sub-cultured on to nutrient agar plates to obtain pure cultures.

2.4. Molecular Characterization of the Isolated Bacteria

N- BLAST search of the 16S rRNA sequence of the isolated bacteria was reported by Bhat Biotech private Company India.

2.5. Preparation of Inoculums

Pure cultures of the isolates were transferred into 100 ml Erlenmeyer flasks containing nutrient broth and incubated at 37°C for 24-hour. The 24 hours old cultures were used as inocula in all the experiment.

2.6. Effect of Temperature on the Growth of Isolates

For determination of optimum temperature, 0.1 ml inoculum each was provided into 10ml of nutrient broth medium and incubated overnight at different temperature ranging from 30 to 50°C. Growth was measured in terms of

optical density at 660 nm using spectrophotometer.

2.7. Effect of pH on the Growth of Isolates

pH is a limiting factor which governs bacterial growth. To determine pH optima, nutrient broth medium meant for the growth of the isolates was adjusted to different pH ranges 5.0 to 11.0 and seeded with 0.1 ml of the inoculum. The culture was incubated overnight at 37°C. Growth was measured as OD at 660 nm using spectrophotometer.

2.8. Effect of Metals on Growth of Bacterial Isolates

To determine the metal tolerance of the isolates, different concentration of four metal salts; Iron chloride ($FeCl_2$), Zinc chloride ($ZnCl_2$), Calcium chloride ($CaCl_2$), and Magnesium oxide (MgO) were used in nutrient broth. Only 0.1ml of the inocula were seeded into tubes containing different concentrations of the metals and incubated at 37°C for 24hours. Growth was measured in terms of OD at 660 nm using spectrophotometer

2.9. Effect of Salt (NaCl) Concentration on the Growth of Isolates

To study the optimum salt concentration on bacterial growth, different concentrations of NaCl ranging from 2.0% to 10.0% were used in nutrient broth. Growth was measured in terms of OD at 660 nm using spectrophotometer

3. Results

3.1. Effect of Temperature and pH on the Growth of Isolates

Below is the result of the effects of temperature and *pH* on the growth of the bacterial isolates. Growth was observed in all the isolates at the various temperature and *pH* values. Gradual decrease in growth of the isolates was observed with a further increase in the temperature and *pH*.

Table 1. *Effect of Temperature on the growth of isolates.*

	Temperature (°C)				
	30	**35**	**37**	**40**	**50**
Stenotrophomonas pavanii	1.230	1.490	1.650	1.278	0.143
Stenotrophomonas maltophilia	1.120	1.356	1.520	1.194	0.160
Chromobacterium violaceum	0.567	1.584	1.305	1.152	0.170
Bacillus cereus	1.052	1.125	1.204	1.013	0.002
Bacillus subtilis	1.345	1.560	1.120	1.020	0.064
E. coli	0.430	1.500	1.245	0.230	0.120
P. aeruginosa	0.214	1.420	1.302	1.250	0.200

Table 2. *Effect of different pH values on growth of isolates.*

	pH 5	**pH 6**	**pH 7**	**pH 8**	**pH 9**	**pH 10**	**pH 11**
S. pavanni	0.044	0.162	0.327	0.559	0.236	0.246	0.257
S. maltophilia	0.346	0.432	0.500	0.657	0.387	0.331	0.030
C. violaceum	0.431	0.520	0.368	0.256	0.200	0.156	0.091
Bacillus cereus	0.121	0.185	0.240	0.311	0.307	0.137	0.255
Bacillus subtillis	0.320	0.200	0.156	0.735	0.580	0.125	0.045
E. coli	0.018	0.125	0.602	0.346	0.250	0.300	0.145
P. aeruginosa	0.050	0.305	0.120	0.305	0.470	0.650	0.045

3.2. Effect of Different Metal Concentrations on the Growth of Bacterial Isolates from Biofilms

Effect of different metal concentrations on the growth of bacterial isolates showed that growth was observed at the different concentrations of the heavy metals as shown on the graphs below.

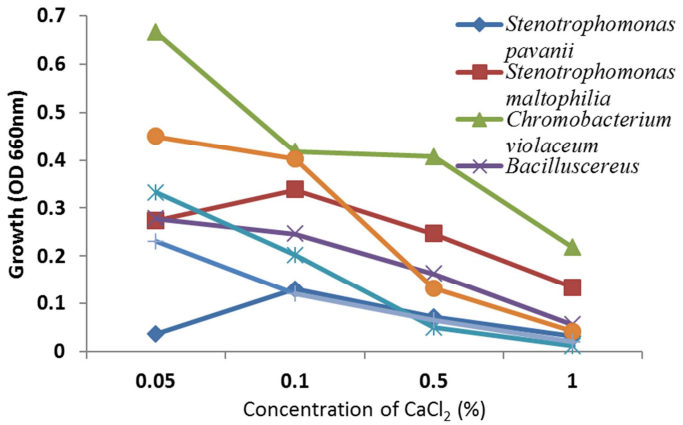

Figure 1. *Effect of different concentrations of $CaCl_2$ on growth of bacterial isolates.*

Effect of different concentrations of $CaCl_2$ on the growth of the isolates showed a gradual decrease in growth of all the strains. However *Stenotrophomonas maltophilia* showed optimum growth at 0.1% $CaCl_2$ concentration, whereas *Bacillus subtilis, E. coli* and *P. aeruginosa* grew best at 0.05% $CaCl_2$ concentration with the least growth observed in *Bacillus subtilis at* 1% $CaCl_2$ as shown in Figure 1.

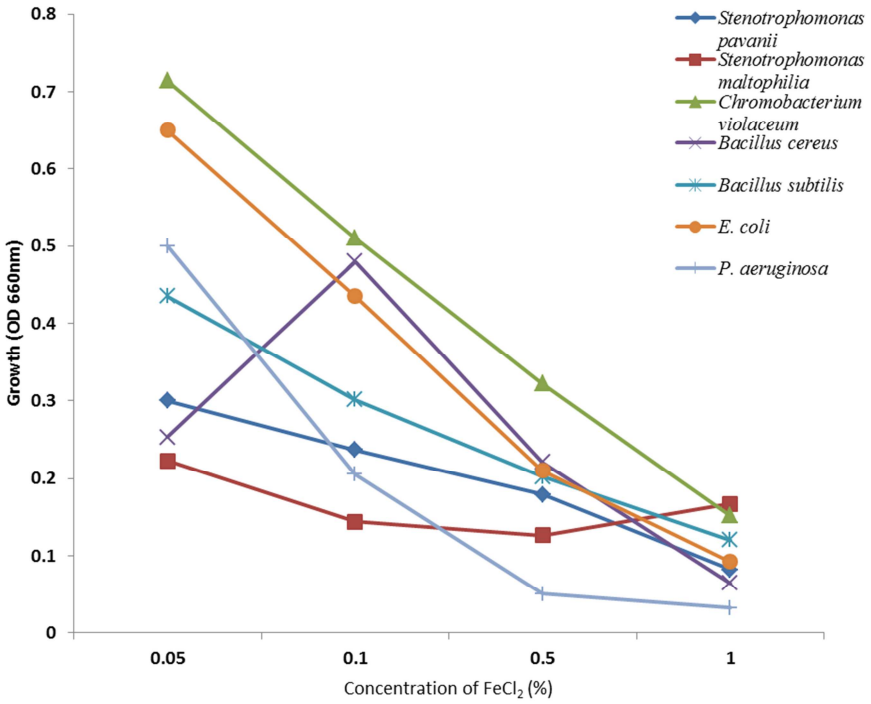

Figure 2. *Effect of different concentration of $FeCl_2$ on the growth of bacterial isolates.*

A decline in the growth of the isolates with percentage increase in $FeCl_2$ concentration is shown in Figure 2. *Chromobacterium violaceum* and *E. coli* showed best growth at 0.05% $FeCl_2$ concentration. Optimum growth was also observed in *S. maltophilia, S. pavanii,* and *Bacillus subtilis* at 0.05% $FeCl_2$ concentration while *Bacillus cereus* had its optimum growth at 0.1% concentration of $FeCl_2$.

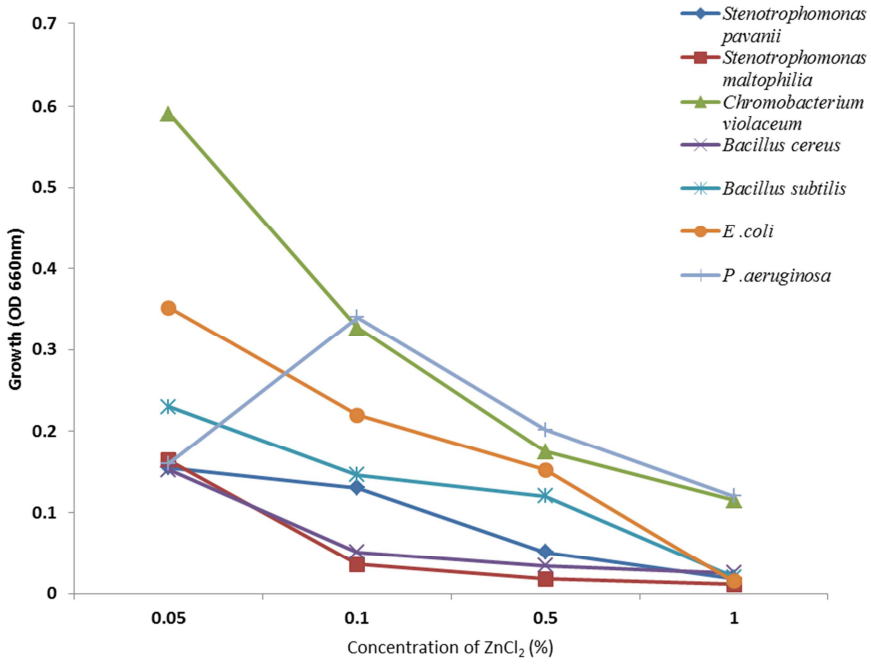

Figure 3. *Effect of different concentrations of $ZnCl_2$ on the growth of the isolates.*

The effect of different concentration of $ZnCl_2$ showed a decrease in growth with an increase in concentration of $ZnCl_2$ in all the organisms as shown in Figure 3. *Chromobacterium violaceum* gave the highest reading of 0.590 at 0.05% while *S. maltophilia* had the least growth with reading of 0.011 at 1% $ZnCl_2$ concentration. Optimum growth was at 0.05% concentration for *S. maltophilia, S. pavanii, Bacillus cereus* and *Bacillus subtilis.*

The effect of Magnesium Oxide on the growth of the isolates is shown in Figure 4. A decrease in growth was observed in all the isolates with percentage increase in MgO concentration. *Stenotrophomonas pavanii* showed optimum growth at concentration of 0.1%, whereas optimum was at 0.5% concentration of Magnesium oxide for *Stenotrophomonas maltophilia*, *Bacillus cereus* and *Chromobacterium violaceum.* 0.05% concentration of Magnesium oxide was best for the growth of *Bacillus subtilis, P. aeruginosa* and *E. coli.*

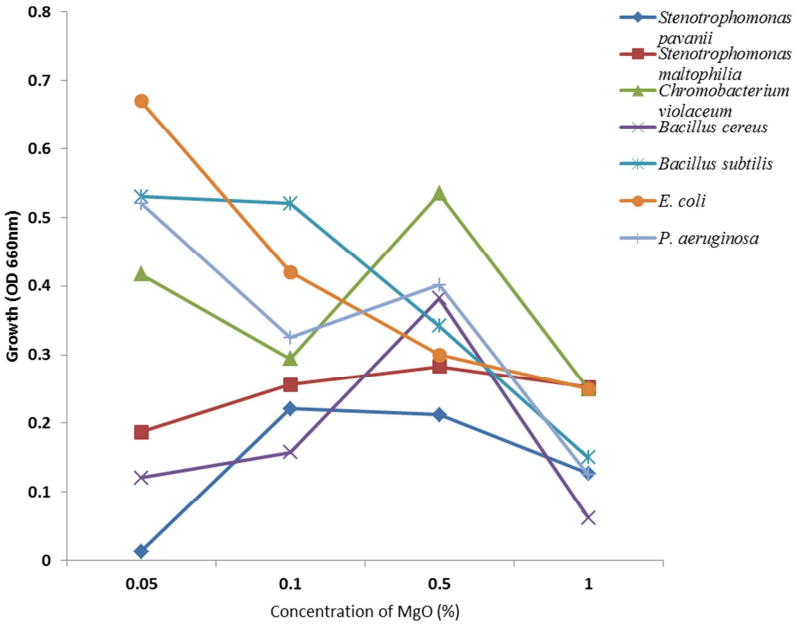

Figure 4. *Effect of different concentrations of MgO on the growth of the isolates.*

3.3. Effect of Different Salt (NaCl) Concentrations on Growth of Isolates

The effect of different salt concentrations on the growth of the bacterial isolates is shown in the figure below. Gradual decrease in growth was observed in all the isolates with a percentage increase in NaCl concentration.

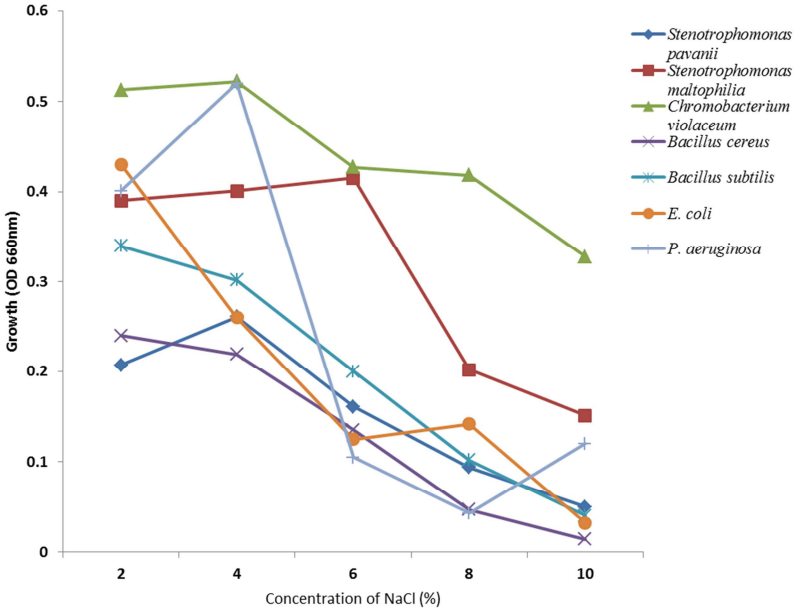

Figure 5. *Effect of different Salt (NaCl) concentrations on growth of isolates.*

The effect of increase in concentration of Sodium chloride (NaCl) on the growth of the isolates is shown in Figure 5. The optimum growth for *Chromobacterium viloaceum Stenotrophomonas pavanii* and *P. aeruginosa* was found at 4% NaCl concentration and at 6% NaCl concentration for *Stenotrophomonas maltophilia*. Two percent NaCl concentration was best for the growth of *Bacillus cereus, Bacillus subtilis* and *E. coli.*

4. Discussion

The work investigated the effect of different salts and heavy metal concentrations on bacterial biofilm from surface water and borehole water samples. The investigation highlights the presence of microorganisms that are able to come together and form biofilm.

Seven strains were selected based on their biochemical reaction and molecular characterization. The bacterial isolates were identified as *Stenotrophomonas pavanii, Stenotrophonmonas maltophilia Chromobacterium violaceum, Bacillus cereus, Bacillus subtillis, Escherichia coli,* and *Pseudomonas aeruginosa.* Since salt (NaCl) concentration, temperature and pH have roles in enzymatic function as well as overall metabolic efficiency, these factors do have an effect on survivability and growth of microorganisms.

The effect of pH on the growth was evaluated and table 2 shows the pH profile of the strains indicating that the bacterial strains isolated were found to grow within the pH ranges of 5.0 -11.0. Optimum growth for *Stenotrophomonas pavanii* and *stenotrophonmonas maltophilia* was at pH of 8.0 and lesser growth were observed at pH 5.0 and 11.0 for *Stenotrophomonas pavanii* and *Stenotrophonmonas maltophilia* respectively. *Chromobacterium violaceum* had its growth optimum at pH 6 while *Bacillus cereus* and *Bacillus subtilis* grew best at pH 8.0. Similar result was recorded at pH ranges of 7.0 – 10.0 for *Bacillus cereus* [13; 1]. Optimum growth was at pH of 7 and 10 for *E. coli* and *P. aeruginosa* respectively. Sivendra *et al.,* reported similar result in pH value for growth of *Chromobacterium violaceum* at 5 to 9 and some at pH value of 4, 10 and 11[14]. *Stenotrophomonas maltophilia* showed the highest growth of OD 0.657 at pH 8.0 and least growth of 0.030 OD at pH 11.0.

In this study, the optimum temperature for growth of *Stenotrophomonas pavanii* (OD 1.650) *Stenotrophonmonas maltophilia* (OD 1.520) and *Bacillus cereus* (OD 1.204) was at 37°C as shown in table 1, Confirming the work of Okanlawon [13] who reported that the optimum temperature for *B. cereus* is between 30°C and 37°C but some strains can grow at temperature as low as 4.5°C and up to 55°C. Optimum temperature was at 35°C for *Chromobacterium violacem, E. coli* and *P. aeruginosa.* Further increase in temperature resulted in decrease in growth in all isolates.

The result figure 5 of the effect of NaCl salt on growth showed a decrease in growth with an increase in NaCl concentration from 2% to 10%. *Stenotrophomonas pavanii* and *P. aeruginosa* had their optimum growth at 4% concentration and at 6% NaCl concentration, *Stenotrophonmonas maltophilia* gave highest growth. Optimum growth was at 4% for *Chromobacterium violaceum*. This confirms the report that *Chromobacterium violaceum* grew at 4% and 5% w/v concentration of NaCl by Sivendra *et al.,* [14]. *Bacillus cereus, Bacillus subtilis* and *E. coli* gave optimum growth at 2% w/v of NaCl concentration.

By affecting the growth, morphology and activities, heavy metal influence the microbial population and resulting in decreased biomass as well as diversity. Therefore microbes have developed mechanisms to tolerate the metal either by presence of heavy metals through efflux, complexation or reduction of metal ions or to use them as terminal election acceptors in anaerobic respiration [9]. Most mechanism reported involved the efflux of metal mechanisms has been found on both chromosomes and plasmids. Bacteria that are resistant to and grow on metals play an important role in the biogeochemical cycling of the metal ions [1]. Metal contamination is widespread. Heavy metals are defined as a group of metals whose atomic density is greater than $5g/cm^2$ in nature; there are about 50 heavy metals of special concern because of their toxicological effect to human beings and other living organisms. Many of them like Zn, Cu, Co, Mg, Ni, Mn and Fe have nutritional characteristics known as essential trace element are necessary for living organisms [2] because at certain concentration levels, these elements participate in some enzyme activities. When in excess concentration, the toxic effects of these dual functional ions are revealed.

Result of the effect of different concentration of $CaCl_2$ on growth of the isolates showed a gradual decrease in growth of all the strains with an increase in metal concentration. However, *Stenotrophonmonas maltophilia* showed highest growth of 0.338 OD at 1% concentration of $CaCl_2$, whereas *Bacillus subtilis, E. coli* and *P. aeruginosa* grew best at 0.05% $CaCl_2$ concentration as shown in Figure 1. Zinc chloride affected growth of the isolates in a similar manner. It was observed that *C. violaceum* had optimum growth (0.590 OD) at 0.05% of $ZnCl_2$ concentration.

A decline in growth of the isolates was observed with a percentage increase in $FeCl_2$ concentration figure 2. *Chromobacterium violaceum* and *E. coli* showed best growth at 0.05% concentration. Increase in concentration of Magnesium oxide caused decrease in growth of the organisms. *Stenotrophonmonas pavanii* showed optimum growth at concentration of 0.1% whereas optimum for *Stenotrophonmonas maltophilia* was at 0.5% (0.283, OD) concentration of MgO. Growth optimum was at 0.5% concentration of MgO for *Bacillus cereus* and *Chromobacterium violaceum.* 0.05% concentration of Magnesium oxide was best for the growth of *Bacillus subtilis, P. aeruginosa* and *E. coli,* with *E. coli* giving the highest growth (0.670 OD) as shown in figure 4.

5. Conclusion

Microorganisms in water are able to attach to surfaces and

form biofilms. All the borehole water and surface water sampled generated bacterial biofilms, which could be pathogenic to humans. The bacterial isolates include: *Stenotrophomonas pavanii, Stenotrophomonas maltophilia, Chromobacterium violaceum, Bacillus cereus, Bacillus subtilis, Escherichia. coli, and Pseudomonas aeruginosa.*

This work evaluated the effect of high concentrations of salts and heavy metals on the microbial population of the biofilm from water and has shown that despite the decline observed in growth of the isolates with an increase in the concentration of salt (NaCl) as well as the heavy metal salts, the biofilms bacteria were able to thrive in adverse environmental conditions.

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