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Antibiotics Susceptibility Pattern of *Pseudomonas aeruginosa* isolated from Obafemi Awolowo University Ile-Ife Oxidation Pond

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Abstract

Oxidation ponds offer a cost-effective, natural method for treating municipal wastewater, preventing environmental hazards associated with direct disposal into the environment. This study aimed to identify, characterize, and determine the antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolates found in Obafemi Awolowo University (OAU) oxidation pond. Five (5) wastewater samples were aseptically obtained from the OAU for physicochemical and microbiological analyses. The samples were processed using standard bacteriological methods. The samples collected for DO and BOD were also analyzed using Winkler's titration method. Sixteen (16) isolates of the total forty-two (42) isolates were confirmed as *P. aeruginosa*. The mean microbial loads ranged from 2.1 x 10^4 to 1.15 x 10^7 CFU/ml respectively. As regards each pH, temperature, and total dissolved solids (TDS), the null hypothesis was not rejected as the p-values were greater than 0.05. P-values for pH, temperature, and TDS were 0.990, 0.996 and 0.984 respectively. This implies that there was no significant difference in the mean values of each physicochemical parameter obtained during each sampling period of this study. The dissolved oxygen was zero for all samples and the BOD value was between 1.2±0.00 mg/L and 6.4±0.00 mg/L. 87.5% of the *P. aeruginosa* were resistant to Imipenem, 100% to Amoxicillin-Clavunate and 94% to both Cefuroxime and Ampiclox. None of the isolates were resistant to Gentamicin, Ofloxacin, and Levofloxacin (69%, 100%, and 100% susceptibility respectively). *P. aeruginosa* thrives in anoxic conditions, making it unlikely to be extensively or pan-drug resistant. However, Gentamicin and Ofloxacin can be effective treatments for community-acquired infections

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Introduction

Water is essential for life and plays a significant role in numerous chemical and biological activities (Aung, Jiang, and He, 2018). It makes up 71% of the earth's surface, with 96.5% of it being ocean-bound, according to the United States Geological Survey (USGS, 2019). Different places use water for different purposes. It serves as a home for a vast array of living things of various shapes and sizes, offering the ideal conditions for their survival and growth (Wingender and Flemming, 2011).

Water is used in Homes, schools, industries, and farmlands. For instance, water is regarded as the most important resource for the global development of sustainable agriculture (Chartzoulakis and Bertaki, 2015). In homes, water is used for both indoor and outdoor household purposes, some of which are drinking, food preparation, bathing, washing of clothes and dishes, brushing of teeth, and watering of the garden (USGS, 2018). Manufacturing industries and other businesses use water during the production process for either creating their products or chilling equipment used to create their products, according to the Centers for Disease Control (CDC, 2016).

A product may be manufactured, processed, cleaned, diluted, cooled, or transported using industrial water. Smelting operations, oil refineries, and businesses that make chemical, food, and paper products all utilize water. Most of the time, producing food, paper, and chemicals requires a lot of water (USGS, 2019). All of these activities generate water whose quality has been compromised.

The term "wastewater" describes the contaminated water produced by many sources (Amoatey and Bani, 2011). To make the water suitable for human use or other purposes, it must be treated because its original composition and quality have been changed. Although there are other ways to clean wastewater, oxidation ponds work best in tropical regions and small populations like towns or schools (Butler *et al*., 2017).

To remediate or treat wastewater, oxidation ponds are an inexpensive, simple, and self-sufficient treatment unit that uses the combined effects of sunlight, temperature, wind, pond geometry, and oxygen concentration in addition to the action of general microbial communities of bacteria, viruses, fungi, and protozoa (Olutiola *et al*., 2010; Butler *et al*., 2017).

A lot of wastewaters are now treated before being released into the environment, wastewater treatment plant effluents, such as those from oxidation ponds, when released into rivers and streams nevertheless pose a risk to public health because they contain water contaminants and some free-living microorganisms and pathogens (Mansfeldt *et al*., 2020). One of the major pathogens discovered in wastewater, *P. aeruginosa*, is a facultative aerobe that has evolved to withstand harsh environments and some antibiotics (Arai, 2011).

Huang *et al.* (2018) reported that the pathogen survived ultra-violet disinfection better than many free-living and pathogens found in a wastewater treatment plant. With the inherent capacity to colonize various niches, including vertebrate and invertebrate hosts, the gram-negative gamma proteobacterium (*P. aeruginosa)* has been implicated in many opportunistic infections (Cornelis & Dingemas, 2013), both in clinical settings and in the community. *Pseudomonas*, is one of the multidrug-resistant genera of bacteria declared by the World Health Organization (WHO) in 2017 to be resistant to a large number of antibiotics, including Carbapenems and third generation Cephalosporins(World Health Organization, 2017).

Resistance to antibiotics has led to increase in morbidity and mortality rate globally and will most likely become the worst enemy of man by 2050 (Pulingam *et al*., 2022). Several bacterial pathogens, including *P. aeruginosa*, have been found to exhibit resistance to a variety of antibiotics used to cure several nosocomial or community-acquired infections (Restrepo *et al*.,2018; El-Mahdy and El-Kannishy, 2019). *P. aeruginosa* is regarded mostly as an opportunistic pathogen responsible for morbidity and mortality in humans and infects other hosts including plants, nematodes, insects and other mammals (Diggle and Whiteley, 2020).

It is implicated in many respiratory infections, urinary tract infections, eye infections etc. (Chandra, 2017), and has been found to contain strains that are strongly resistant to actions of common antibiotics such as Cotrimoxazole, Tetracycline, Amoxicillin and Augmentin, Streptomycin, Septrin and Chloramphenicol (Alabi *et al*., 2021). Importantly, it has been described as a bacterium found in locations associated with human activity (Crone *et al*., 2020). Hence, there is a need for urgent attention to avert any imminent health havoc associated with the use of untreated or poorly wastewater in the human population.

This study seeks to enumerate and determine the antibiotic susceptibility pattern of *P. aeruginosa* isolates, a common pathogenic bacterium found in wastewater from wastewater samples obtained from Obafemi Awolowo University oxidation pond. As a way of contributing to the global fight against water-borne diseases and community-acquired infections arising from direct or indirect use of untreated recklessly disposed into the environment, or receiving water bodies, the Obafemi Awolowo University, Ile-Ife has two (2) oxidation pond systems, where the wastewater generated in all departmental rooms, Staff quarters, health centres, the seven (7) undergraduates hostels and a Postgraduate hall of residence are treated before being released into the environment or water bodies. Although studies have been conducted to evaluate the efficiency of the school's oxidation ponds in remediating various metallic pollutants, and the effect that the generated effluent has on the receiving stream, no robust study has been done to investigate the antibiogram of *P. aeruginosa* isolates in the pond and the physicochemical parameters influencing the survival of the pathogen. Judging from the ubiquitous nature of the *P. aeruginosa*, its ability to form biofilm using the metallic element Iron (Fe) and the presence of iron in the influents and effluents section of the pond (Ogunfowokan *et al*., 2009), there is a huge chance that antibiotics-resistant *P. aeruginosa* isolates exist in the ponds.

This study stands to maintain public health by preventing water-borne diseases, food-borne infections and the emergence of resistant strains of *P. aeruginosa*. It therefore plays a substantial role in achieving the third and sixth goals of the United Nations Sustainable Development Goals (SDGs), which are to maintain good health and wellbeing, and to ensure clean water and sanitation respectively. This study seeks to enumerate and determine the antibiotic susceptibility pattern

Materials and Methods

Study area

The samples analysed in this research study were obtained from the Obafemi Awolowo University Oxidation Pond. The sampling site was constructed in 1967 and started operation two years later (1968). It is located between the Southwestern part of the University and drains water from the students' hostel, academic area and staff residence (Olutiola *et al.*, 2010)

Sample Collection

Samples were taken from the oxidation pond for microbial analysis and determination of dissolved oxygen and biochemical oxygen demand. Sample were collected aseptically and were transported immediately to the laboratory for analysis.

Determination of wastewater physicochemical parameters

Total dissolved solid (TDS)

Total dissolved solid (TDS) is measured in water samples using a TDS metre. The metre stirs the sample to remove air bubbles, then readings are taken three times for each sample, with averages recorded in ppm.

Temperature

The temperature was measured to determine the degree of hotness or coldness of the wastewater sample. Mercury-in-glass thermometer was used for the measurement. The averages of all readings taken were recorded in degrees Celsius (℃) (Corwin and Yemota, 2020).

pH

The pH was measured to determine the acidity and alkalinity of the wastewater samples collected. A pH metre (calibrated with buffer solution of pH 7) was used in this study. The probe of the pH was inserted in water. Readings were taken and it was done thrice for each sample. The average of the readings was taken and recorded (Corwin and Yemota, 2020).

Dissolved oxygen (DO)

Winkler’s method of dissolved oxygen determination was used. In the laboratory, a sample collected in a dissolved oxygen bottle (300 ml) was fixed by adding 2 ml of manganese sulphate with the aid of a pipette. Thereafter, 2ml of alkali-iodide-azide reagent was added to the sample using a pipette. The sample was left for 8 hours before titration against sodium thiosulphate using 2 ml starch solution as the indicator. After the addition of starch to 50ml of the sample, a blue colouration was observed. A clear colour was taken as the end point of titration (Moyel, 2014).

Biochemical oxygen demand (BOD)

Sample collected in a biochemical oxygen demand sampling bottle was sealed and kept in a dark place for 5-8 days before the fixing and titrating using the Winkler’s method of titrimetric analysis (Moyel, 2014).

Microbiological Analysis of Collected Wastewater

Sample enrichment

The water sample was enriched using peptone water. 8ml of the sample was mixed with 2 ml of prepared peptone water and mixed. The sample-peptone water mixture was then incubated at 37 ℃ for 18-24 hours (Barret *et al*., 1980).

Isolation of bacteria isolates

Samples were homogenized, diluted, and poured onto agar plates for uniform colonization. Ceramide agar was inverted at 37 ℃ and 42 ºC for 24 hours (Al-Saffar & Jarallah, 2019; Okanlawon *et al.*, 2023).

Identification and Characterization

Colonial structure, morphological, and biochemical characteristics were used to determine the identity of bacterial isolates, which were then classified using Bergey's Manual of Determinative Bacteriology and the Compendium of Soil Fungi. (Okafor *et al*., 2023).

Antibiotic Susceptibility Testing (AST)

The Kirby Bauer disk diffusion method of antibiotic susceptibility was used in this study. Few colonies of 18-24h old culture were aseptically transferred into the sterile normal saline solution. After standardizing the bacterial suspension to 0.5 McFarland standard, a carpet culture was made on Mueller Hinton Agar by evenly spreading the standardized inoculum using a sterile swab stick (Willey *et al*., 2020). The plates were incubated at 37 ℃ for 18-24h, after which the plates were examined for zones of growth inhibition around the disc. The diameter of the Zone of inhibition was measured in millimetres (9 mm) using a calibrated metre rule. The Clinical and Laboratory Standard Institute (CLSI, 2020) was used to interpret the zone of inhibition measured as resistant, susceptible or intermediate.

Statistical Analysis

The data generated from the study was compiled and processed using excel spread sheet and SPSS version 25 software for the p-value at 0.05.

Results

The mean temperature of water samples collected from O.A.U oxidation ponds ranged from 24.2 ± 0.52 ℃ to 28.7 ± 0.57 ℃, with the highest temperature recorded during the fourth sampling and the lowest during the first. The mean acidity or alkalinity values were highest during the fifth sampling and lowest during the first (4±0.23 and 6.7±0.17). The mean dissolved oxygen value was 0.0±0.00, due to undetermined volumes of Sodium thiosulphate titrated during DO analysis or no color reaction after titration. The biochemical oxygen demand values ranged between 1.2±0.00 mg/l and 3.5±0.00 mg/ml, with the latter obtained during the first and fourth samplings. The mean total dissolved solids value ranged between 10.7±0.57 ppm and 16.7±1.52 ppm.

The null hypothesis which states that there is no significant difference in the mean temperature values obtained during the sampling period was not rejected as the p-value obtained was 0.996 ($p\text{-value} > 0.05$). The null hypothesis was not rejected as the p-value obtained was 0.990 ($p\text{-value} > 0.05$) for the pH. The actual p-value of 0.984 obtained showed that there is no significant difference in the mean values of the measured TDS value of the water samples.

Table 1: Result of the physicochemical analysis for wastewater samples

Sample Period	Temperature (°C)	pH	TDS (ppm)	DO (mg/L)	BOD (mg/L)
S1	24.6 ± 0.55	6.6 ± 0.17	12.7 ± 0.57	0.0±0.00	3.5 ± 0.00
S2	24.2 ± 0.51	6.8 ± 0.07	12.3 ± 0.57	0.0±0.00	6.4 ± 0.00
S3	26.0 ± 0.00	6.9 ± 0.00	16.7 ± 1.52	0.0±0.00	0.0±0.00
S4	28.7 ± 0.57	6.7 ± 0.11	10.5 ± 0.57	0.0±0.00	1.2 ± 0.00
S5	26.7 ± 0.57	7.4 ± 0.23	12.7 ± 0.57	0.0±0.00	0.0±0.00
P-VALUE	0.996	0.990	0.984	-	-

Legend: S1: Sample 1; S2: Sample 2; S3: Sample 3; S4: Sample 4; S5: Sample 5; P-value: Probability value at 0.05

Table 2: Result for Heterotrophic Bacterial count of *Pseudomonas aeruginosa* from waste water collected from O.A.U Oxidation Pond

Sampling	Microbial Load (CFU/ml)	Mean Microbial Load (CFU/ml)
S1	$1.0\text{x}10^{3}$ $6.0 \text{ x } 10^{2}$	$8 \text{ x } 10^{2}$
S2	$1.1 \text{ x } 10^{7}$ $1.2 \text{ x } 10^{7}$	$1.15 \text{ x } 10^{7}$
S3	$6.0 \text{ x } 10^{5}$ $4.0 \text{ x } 10^{5}$ $1.0 \text{ x } 10^{7}$	$3.7 \text{ x } 10^{6}$
S4	$3.7 \text{ x } 10^{4}$ $2.1 \text{ X } 10^{6}$	$1.1 \text{ x } 10^{6}$
S5	$2.1 \text{ x}10^{4}$	$2.1 \text{ x}10^{4}$

Table 3: The Biochemical Characteristics of *Pseudomonas aeuginosa*

Gram reaction	**Cellular morphology**	**Catalase**	**Oxidase**	**Methyl red**	**Voges Proskauer**	**Indole**	**Nitrate Reduction**	**H_2S Prodction**	**Gelatin hydrolysis**	**Citrate utilization**	**Glucose**	**Fructose**	**Maltose**	**Lactose**	**Sucrose**	**Probable identity**
-	R	+	+	+	-	-	+	-	-	+	+	+	+	+	+	***Pseudomonas aeruginosa.***

Table 4: The Colony Morphology of *Pseudomonas aeruginosa* from the wastewater samples

Colour	Shape	Edge	Surface	Elevation	Size	Opacity
Yellow	Circular	Entire	Smooth	Effused	Medium	Opaque
Yellow	Spindle	Entire	Smooth	Effused	medium	Opaque
Yellow	Circular	Entire	Smooth	Effused	medium	Opaque
Yellow	Punctiform	Entire	Smooth	Flat	medium	Opaque
Yellow	Circular	Entire	Smooth	F4lat	medium	Opaque

Table 5: Results of Antibiotics Susceptibility Pattern of *Pseudomonas aeuginosa*

AUG	CXM	IMP	OFX	GEN	NA	LBC	ACX
R	R	R	S	S	S	I	R
R	R	R	S	S	R	S	R
R	S	R	S	S	R	S	R
R	R	R	S	S	R	S	R
R	R	R	S	S	R	I	R
R	R	R	S	S	R	I	R
R	R	R	S	S	R	S	S
R	R	R	S	S	I	S	R
R	I	R	S	S	R	S	R
R	R	R	S	S	R	S	R

R	R	R	S	S	I	I	R
R	R	R	S	S	R	I	R
R	R	R	S	S	I	S	R
R	R	S	S	S	R	S	R
R	R	R	S	S	I	S	R
R	R	S	S	S	R	S	R

Legend: IMI: Imipenem; OFX: Ofloxacin; AUG: Augmentin; NA: Nalidixic; LBC: Levofloxacin; CXM: Cefuroxime; ACX: Ampiclox
R: Resistance; S: Susceptible I: Intermediate

Table 6: Percentage susceptibility of *Pseudomonas aeruginosa* to the tested Antibiotics

Antibiotics	Susceptible (%)	Intermediate (%)	Resistance (%)
Imipenem (IMI)	12.5	0	87.5
Ofloxacin (OFX)	100	0	0
Gentamicin (GN)	100	0	0
Amoxicilin Clanunate (AUG)	0	0	100
Nalidixic Acid (NA)	6	25	69
Levofloxacin (LBC)	69	31	0
Cefuroxime (CXM)	0	6	94
Ampiclox (ACX)	6	0	94

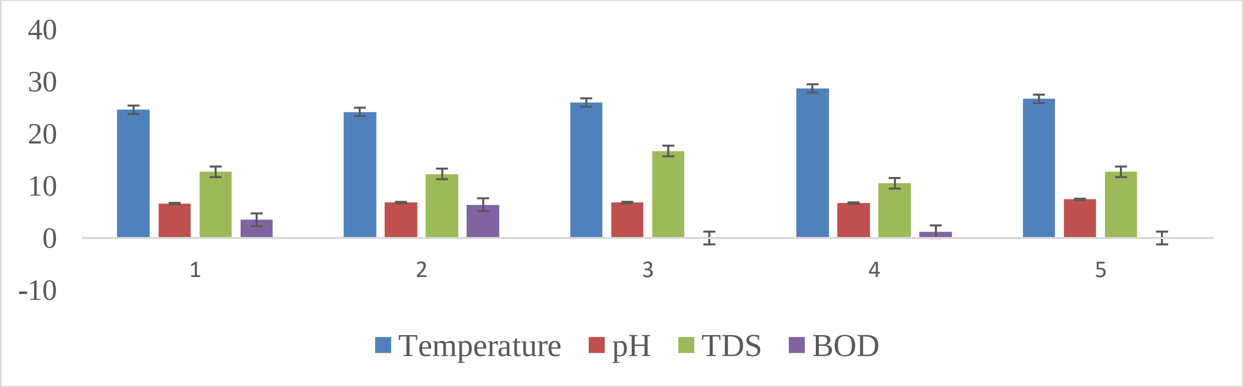

Figure 1: The Physicochemical parameters of the wastewater samples from OAU Oxidation Pond

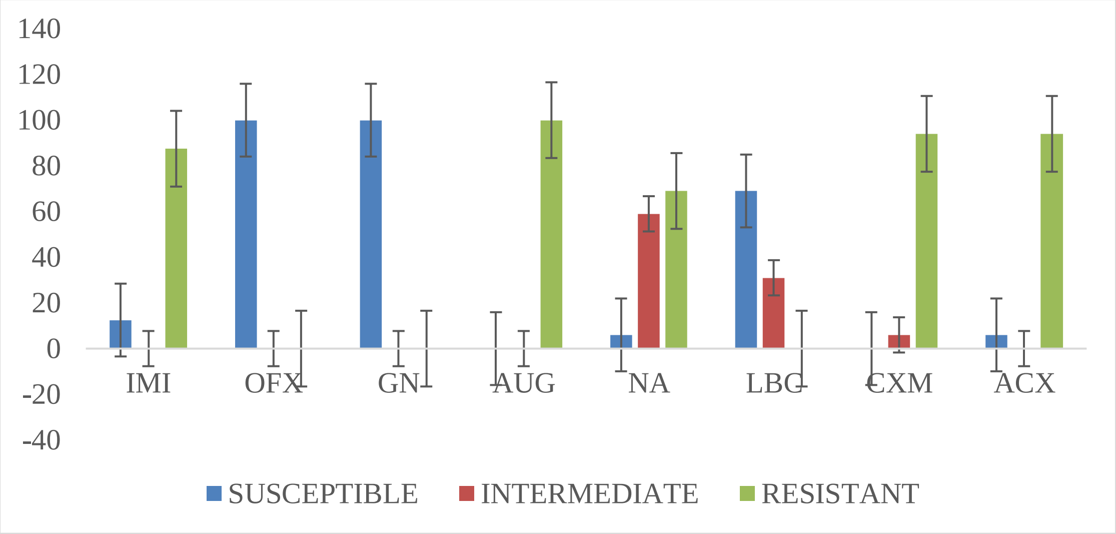

Figure 2: The Percentage of antibiotic susceptibility of *Pseudomonas aeruginosa* from wastewater samples from OAU Oxidation Pond

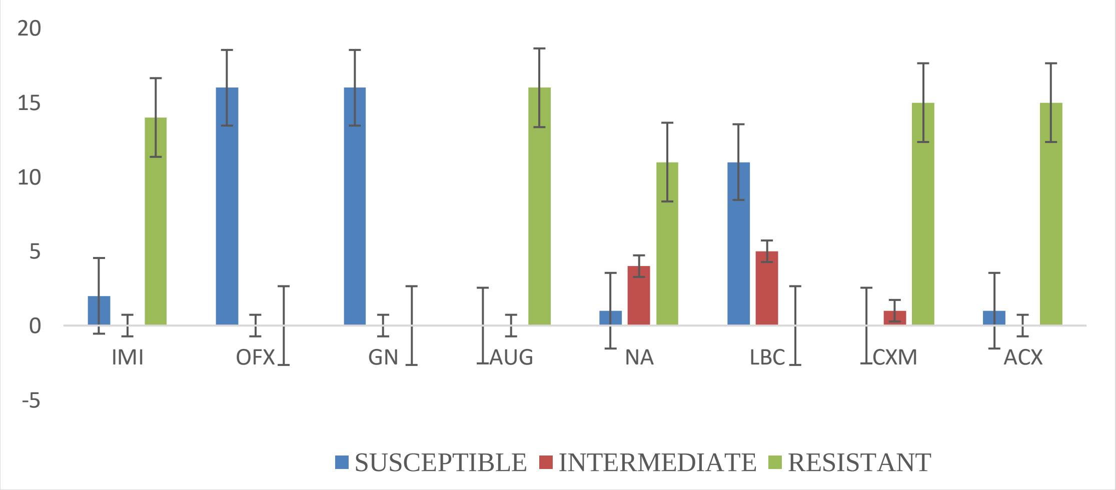

Figure 3: Antibiotic susceptibility pattern of the isolated *Pseudomonas aeruginosa*

Key: IMI = Imipenem; OFX = Ofloxacin; GN = Gentamicin; AUG = Amoxicillin-Clavunate; NA = Nalidixic acid; LBC = Levofloxacin; CXM = Cefuroxime; ACX = Ampiclox

Discussion

Stormwater runoff is a major source of wastewater that replenishes the OAU oxidation pond during the period when this study was conducted. Like grey water and black water, stormwater runoff also contains various substances that can alter several physicochemical parameters of the wastewater found in the oxidation pond. It also contains various microorganisms displaced from their various habitats (Warwick *et al.*, 2013).

The analysis of temperature and pH implies that the same categories of microorganisms that have adapted to living and functioning in a particular ecosystem may persist for a long time until when there is a reasonable change in the environmental conditions that influence the physicochemical parameters. This was corroborated by the results from the analysis of the physicochemical parameters of the wastewater samples. The p-value obtained for temperature, pH and TDS were greater than 0.05 (P Value > 0.05), hence it could be inferred that there was no significant difference in the mean values of each temperature, pH and TDS obtained during the sampling period). The mean values and their standard deviations are represented in Table 1

The zero value of the dissolved oxygen implies that the oxidation pond will support the existence of anaerobes and facultative anaerobes. Arai (2011) has identified *P. aeruginosa* as an adapted facultative anaerobe. The presence of *P. aeruginosa* is therefore not surprising. Ogunfowokan *et al.* (2009) already established the ability of *P. aeruginosa* to form biofilm using Iron. The authors also stated that 15.7mg/l of Iron enters the OAU oxidation pond. In addition to the general adaptive nature of *P. aeruginosa* to anoxic conditions, the likely presence of remnant iron in the oxidation pond and perhaps, occasional or continuous entry of Iron, enhances the survival of *P. aeruginosa* in the wastewater treatment. The high BOD of the wastewater sample at a period when the oxidation pond was not actively receiving influent from the halls of residence and other locations on campus indicates that there are residual organic wastes in the water sample. It also suggests that the spontaneous microbial decomposition of the wastewater is a slow process and may not be sufficient enough to successfully remediate the wastewater sufficiently. This has a public health significance since the oxidation pond connects to other water bodies used by people in Ile-Ife city for agriculture and in slaughter slabs.

Cetrimide Agar is meant to select only *P. aeruginosa* because it screens out many other organisms that cannot make use of cetrimide for their growth, although other microbes grew on Cetrimide agar as well in the course of this study. Thus, in order screen out the other microorganisms that survive the presence of *P. aeruginosa*, incubation was done at 42°C during the fourth and fifth sampling as rightly done by Al-Saffar and Jarallah (2019)

The mean *P. aeruginosa* load for the second sampling, fourth and fifth sampling are 1.15×10^7, 1.1×10^6 and 2.1×10^4 CFU/ml respectively. The abundance of *P. aeruginosa* may not be unconnected to the anoxic nature of the oxidation pond. Crone *et al.* (2020) established that the prevalence of *P. aeruginosa* in uncontaminated niches is low while their sequences abound mostly in human and animal samples. The oxidation pond is an example of such a niches that is contaminated with human wastes.

Different antibiotics which fall under different categories were used in performing antibiotics susceptibility tests for the organism. Imipenem (10µg), Ofloxacin (5µg), Levofloxacin (5µg) and Gentamicin (10 µg) are examples of antibiotics that the presumptively isolated *P. aeruginosa* showed susceptibility to. In contrast to the findings of Igbinosa *et al* (2017) where 66.67% of the *P. aeruginosa isolates* were resistant to Gentamicin, an aminoglycoside, 100% susceptibility to Gentamicin (10 µg) was recorded in this study. In the same vein, all the isolates are susceptible to Ofloxacin, a fluoroquinolone antibiotic. 87.5% of the isolates were resistant to Imipenem, 100% were resistant to Amoxicillin-Clavunate (30 µg) and 94% of the isolates were resistant to Cefuroxime (30 µg).

The resistance rate to Cefuroxime discovered in this study is greater than that found by Igbinosa *et al* (2017). Cefuroxime is a second-generation cephalosporin antibiotic whose action is similar to that of first-generation cephalosporin but exhibits a broader spectrum of activity, working against both gram-positive and gram-negative organisms. It works specifically to inhibit cell wall synthesis of bacterial cell wall by binding to and inhibiting the activity of penicillin-binding proteins (PBPs) (Willey *et al.*, 2020). Although it is effective against gram-negative cells, *P. aeruginosa* is one of such gram-negative bacteria that has a limited effectiveness. *Pseudomonas aeruginosa* is found to produce beta-lactamases and efflux-pumps. These reduce its susceptibility towards Cefuroxime.

Imipenem, a carbapenem antibiotic is a potent cell wall synthesis inhibitor. Like all beta-lactam antibiotics, it produces its therapeutic effects by crossing the cell wall through porins and binding to penicillin-inding proteins (PBP) in the cell membrane (Katzung, 2012). Ameen *et al.* (2015) reported that resistance to Imipenem in *P. aeruginosa* may be due to the porin OprD mutation in conjunction with the production of AmpC6 or the acquisition of MBL genes. Out of 230 strains of *P. aeruginosa* studied by Ameen *et al.* (2015), 49.5% were imipenem resistant; Metallo-β- lactamase (MBL) production was confirmed in 64.9% of the resistant isolates. The 87.5% resistant *P. aeruginosa* could have adopted the same mechanism for their resistance towards Imipenem.

P. aeruginosa also possess innate resistance to Amoxicillin clavunate, hence the 100% resistance that was recorded was not surprising. The built-up of Amoxicillin-clavulanate enables it work against beta-lactamases, giving it an advantage over conventional Amoxicillin, a penicillin antibiotic, especially in its action against gram-positive cells (Willey *et al.*, 2020) Ofloxacin and Levofloxacin are fluoroquinolone antibiotics and belong to the class of antibiotics called quinolones. They act by inhibiting the bacterial enzymes DNA gyrase and topoisomerase IV, which are essential for DNA replication, repair and transcription in a microorganism (Willey *et al.*, 2020). The 100% susceptibility towards Ofloxacin and 69% susceptibility towards Levofloxacin (the remaining 31% being in the intermediate category) showed that the Quinolones are still good antibiotics that can be used against infections caused by *P. aeruginosa*. Other antibiotics tested in this study include Ampiclox (94% resistance rate) and Nalidixic acid (69% resistance rate). The *P. aeruginosa* isolates tested in this study are not pan-drug resistant (PDR) nor are they extensively resistant to the drugs (XDR).

Conclusion and Recommendation

This study has established that the relatively constant nature of the values of temperature, pH and TDS measured during this study has a probability of influencing the kinds of bacteria that could be found in the oxidation pond. The relatively low DO also proves that the indigenous microbes found in the oxidation pond must be anaerobes or facultative anaerobes. Interestingly, *P. aeruginosa* has been established as a facultative anaerobe.

This study also established that *P. aeruginosa* isolates from the OAU oxidation still retain innate resistance to antibiotics like Amoxicillin-Clavunate and Ampiclox. Also, Gentamicin and Ofloxacin could still be used in treating community-acquired infections due to *P. aeruginosa* based on the findings of this study. A molecular surveillance of the pathogens present in the oxidation pond should be done. This will reveal the genetic determinants of virulence and resistance to antibiotics in most pathogens present in the wastewater treatment plant.

Conflict of Interest

Authors declare that there is no conflict of interest

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