Stenotrophomonas species ASSOCIATED WITH SELECTED RIVER WATER SAMPLES IN BENIN CITY, EDO STATE, NIGERIA

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES (MICROBIOLOGY OPTION).

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CERTIFICATION

This is to certify that this project was carried out by **ORUKPE, SARAH (MISS)** with matriculation number **(PGD/MCB/240066)** and submitted to the Microbiology Unit, Department of Biological Sciences, Benson Idahosa University, under the supervision of Professor E.E.O. Odjadjare.

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DR (MRS.) S.O. OSAWE **DATE**

(Ag. HOD)

DEDICATION

This research project is dedicated to Almighty God the source of life and the provider of wisdom, who has given me the grace to undergo this program successfully.

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I would like to begin by expressing my profound gratitude to **Almighty God** for His unfailing love, wisdom, understanding, divine guidance, protection, strength, and grace that sustained me throughout this project. May His name be praised both now and forevermore. Amen.

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ABSTRACT

The focus of this research was to investigate the prevalence and antibiotic resistance profile of *Stenotrophomonas* species in water samples collected from the Ologbo and Ikpoba rivers of Benin City, Edo State, Nigeria. An aggregate of 18 samples of water were collected from various points along both rivers. Isolation was carried out using selective culture with imipenem discs and tentative isolates were identified using typical cultural, morphological, and biological characteristics before their antibiotics susceptibility profiles were established against six antimicrobial agents employing the Kirby-Bauer disk diffusion techniques. The findings uncovered a high predominance of *Stenotrophomonas* species, with bacteria from Ikpoba and Ologbo rivers isolates constituting 89% and 81 % respectively. The isolates showed complete (100%) resistance to polymyxin B, piperacillin, imipenem, and ceftazidime, meanwhile they were sensitive to cotrimoxazole (69%) and tigecycline (72%). The determined Multiple Antibiotic Resistance (MAR) indices spanned between 0.67 to 1.0. This study revealed that these rivers were essential habitat for multiple drug resistant *Stenotrophomonas* species, posing a substantial public health risk due to their poor microbiological water quality.

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CHAPTER ONE

1.1 INTRODUCTION

Water is life and its quality is intrinsically linked to human and ecosystem health. Ensuring good water quality is a major public health issue in developing nations, particularly in sub-Saharan Africa, where access to safe drinking water is frequently insufficient (Brooke, 2012). In Benin City, the Ologbo and Ikpoba rivers are essential water sources for various uses, including domestic, agricultural, and industrial. Nevertheless, these rivers also experience severe pollution from industrial waste, urban runoff, and agricultural activities (Igbinosa and Okoh, 2012). Among the growing list of environmental bacteria found in these aquatic arrays, members of the genus *Stenotrophomonas* have attracted growing attention from both environmental microbiologists and clinical researchers due to their outstanding dual nature as both widespread environmental inhabitants and opportunistic human pathogens.

The genus *Stenotrophomonas*, particularly its most predominant species *S. maltophilia*, is a group of non-fermentative, Gram-negative bacteria renowned for its outstanding metabolic multifaceted nature and intrinsic resistance to a broad spectrum of antibiotics. This resistance is mediated by numerous mechanisms, including enzymes L1 and L2 β-lactamases production, which hydrolyze β-lactam antibiotics, along with carbapenems (Adegoke *et al.*, 2017). Furthermore, their low outer membrane permeability and efflux pump systems support the resistance against macrolides, aminoglycosides, and other drug classes (Alonso *et al.*, 2021). Over the years, trimethoprim-sulfamethoxazole (TMP-SMX) has been the frontline therapy for *S. maltophilia* infections (Gajdács *et al.*, 2021). Nonetheless, rising resistance rates ranging from 5% to over 30% globally have raised concerns about its long-lasting effectiveness

(Gajdács *et al.*, 2021). In a similar manner, levofloxacin resistance is rising, curbing the use of fluoroquinolones (Brooke, 2021). However, minocycline and tigecycline have shown promising *in vitro* activity against resistant strains, offering possible options (Hu *et al.*, 2020). Historically regarded as environmental bacterium found in water, soil, and plant root zone, *S. maltophilia* has resulted over the past three decades as a significant nosocomial pathogen, especially affecting immunocompromised individuals, cystic fibrosis patients, and those with internal medical devices (Brooke, 2012; Chang *et al.*, 2015). It's environmental resilience is supported be a large genome rich in genes encoding for biofilms, efflux pumps, and enzymes that break down complex organic compounds and detoxify heavy metals (Crossman *et al.*, 2008). This very acclimatization allows it to thrive in demanding environments, including polluted water.

The existence and abundance of *Stenotrophomonas* in rivers ecosystems are now acknowledged as a possible bioindicator of water quality and anthropogenic implication (Lin *et al.*, 2022). Rivers systems gaining discharge from wastewater treatment plants (WWTPs) and agricultural sources offer a continuous influx of organic matter, nutrients, and crucially, sub-lethal concentrations of antibiotics and heavy metals (Pauletti *et al.*, 2023). These conditions create an evolutionary pressure that Favour's bacteria with multidrug-resistant (MDR) qualities (Finley *et al.*, 2013). Researches of this type have indicated that this type of environmental reservoirs serves as a trial for the selection and dissemination of antibiotics resistance genes (ARGs) (Finley *et al*., 2013). In this environment, *Stenotrophomonas spp.* not only survives due to their intrinsic resistance mechanisms but it may also be involved in horizontal gene transfer where they may even act as a route and a pool for the genes of

resistance to other more model pathogens (Turrientes *et al*., 2010; Pauletti *et al*., 2023). This transforms the bare water bodies of rivers into lively ecosystems for the development and transmission of antimicrobial resistance (AMR).

Furthermore, the finding of *Stenotrophomonas* in recreational or domestic source water also raises questions regarding its influence on public health. Infection generally needs compromised host condition or the breach in intersection of local barrier (e.g. a wound), however, environmental strains persist as a genetic bank for virulence and resistance traits around water bodies. The ability of various strains of *Stenotrophomonas* to produce biofilms on surfaces is an ecological advantage in running water but represents a key virulence factor during clinical infections by promoting immune evasion and increasing tolerance to antimicrobials (Pompilio *et al*., 2020). Therefore understanding the population pattern, antibiotic resistance profile, and pathogenicity of environmental *Stenotrophomonas* isolates is significant for risk assessment.

This study provides solutions for Antimicrobial Resistance and Waterborne Pathogens in Benin City. The Ologbo and Ikpoba rivers are crucial but polluted watercourses, serve as possible carriers for multidrug resistant (MDR) bacteria like *Stenotrophomonas maltophilia* — an organism found in the environment and an emerging multi-resistant opportunistic pathogen. Presently, there is a serious lack of data on this pathogen's prevalence and resistance patterns in Nigeria water bodies. This study will provide important surveillance data to examine community health risks, comprehend local AMR transmission, and alert both environmental policy and clinical treatment strategies against this increasingly treatment-resistant microbes.

1.2 AIMS AND OBJECTIVES

Aim:

The aim of this study was to examine the prevalence and antibiotic resistance patterns of *Stenotrophomonas* species isolated from Ologbo and Ikpoba River, Benin City, Nigeria.

Specific Objectives:

The precise objectives of this study were to:

1. collect water samples from various parts of the Ologbo and Ikpoba rivers of Benin City.
2. Isolate *Stenotrophomonas* species from the water samples adopting standard microbiological techniques.
3. determine the isolates based on their cultural, morphological, and biochemical characteristics.
4. ascertain the antibiotic susceptibility profiles of the isolates.

CHAPTER TWO

2.1 LITERATURE REVIEW

Stenotrophomonas is a genus that encompasses a group of oxidative, Gram-negative bacteria prevalent in environmental and clinical settings. Taxonomically, *Stenotrophomonas* belongs to the domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Xanthomonadales, and family Xanthomonadaceae (Garrity *et al.,* 2015). The genus was formerly grouped under *Pseudomonas* and later *Xanthomonas* but was reclassified as a distinct genus following phylogenetic analyses based on 16S rRNA gene sequencing (Crossman *et al.*, 2008; Ryan *et al.,* 2009). The type species, *Stenotrophomonas maltophilia*, is the most extensively studied due to its environmental versatility and medical relevance. *Stenotrophomonas* species are aerobic, rod-shaped, motile (with polar flagella), and catalase-positive but oxidase-negative. They do not form spores and typically exhibit oxidative, rather than fermentative metabolism (Brooke, 2012). These bacteria produce non-diffusible yellow pigments on nutrient agar and form smooth, glistening colonies ((Berg and Martinez, 2015). They grow well at mesophilic temperatures (20–37°C), and are found in a wide range of environments, such as soil, plant rhizospheres, fresh and wastewater as well as clinical settings (Berg and Martinez, 2015).

From a biochemical perspective, *Stenotrophomonas* species demonstrate wide metabolic potential which helps it to degrade many organic substrates. They produce proteases, DNases and lipases and are actively involved in nitrogen and sulfur cycling (Ryan *et al*., 2009). Noteworthily, they possess an inherent multidrug resistance mechanism covering β-lactam, aminoglycosides and carbapenems (Crossman *et al.*, 2008; Brooke, 2012). This resistance is

mediated by various mechanisms, including production of multidrug transporters (e.g. SmeDEF), poor membrane permeability, and metallo-β-lactamases such as L1 and L2 (Crossman *et al*., 2008; Brooke, 2012).

Ecologically, species of *Stenotrophomonas* are highly versatile. Certain strains, such as *Stenotrophomonas rhizophila*, have been demonstrated to promote plant growth and nutrient uptake along with biocontrol properties against phytopathogens thus indicating a potential role of these beneficial microorganisms in sustainable agriculture (Wolf *et al.*, 2002). Furthermore, environmental strains that are capable of degrading pollutants in addition to high metal tolerance have been studied because of their potential roles as biological cleaners (Berg and Martinez, 2015).

S. maltophilia is an emerging opportunistic pathogen clinically. It is primarily associated with nosocomial infections in particular among immunocompromised patients, resulting respiratory tract infection (especially cystic fibrosis patients), bacteremia, skin and soft tissue infections, as well urinary track infections (UTI) (Looney *et al.*, 2009). The trait of multidrug resistance in the organism itself makes treating infectious from other isolated species difficult, and trimethoprim—sulfamethoxazole has been commonly used as the choice of drug (Brooke, 2012). Nevertheless, increasing resistance even to this antibiotic is becoming alarming narrowly as regards therapeutic options and hospital infection control (Brooke, 2012)

Stenotrophomonas has been reported as part of microbiota observed in rivers and stream environments, sometimes detected due to pollution by urban runoff, sewage or hospital effluents (Wang *et al.*, 2024). Their persistence in water systems, for example in Benin City,

Nigeria, is an indication of the need to monitor these potentially pathogenic bacteria associated with compromised water supply and their resistance profile (Igbinosa and Okoh, 2012).

2.2 *Stenotrophomonas* species

2.2.1 *Stenotrophomonas maltophilia*

This is the most widely studied and clinically significant species in the genus. It is an opportunistic infectious agent affliated with nosocomial infections, including infections of the respiratory tract, bloodstream infections, and wound infections. *S. maltophilia* shows innate resistance to multi-antibiotic regimen, as well as carbapenems, beta-lactams, and aminoglycosides, which complicates its treatment (Hernandez-Gonzalez *et al.*, 2020).

Environmental strains of *Stenotrophomonas maltophilia* have been discovered in water, soil, plant rhizospheres, as well as disinfected hospital water supply network (Crossman and Avison, 2020). Despite its clinical relevance, the bacterium plays ecological roles like phytoenhancement and breaking down of xenobiotics (Crossman and Avison, 2020).

2.2.2 *Stenotrophomonas acidaminiphila*

Stenotrophomonas acidaminiphila is a metabolically versatile bacterium that thrives in nutrient-rich river sediments and urban waterways, where it plays dual roles in nitrogen cycling and pollutant degradation (Wang *et al*., 2024). Recent studies highlight it's ability to utilize diverse amino acids and degrade polynuclear aromatic hydrocarbons (PAHs) in polluted river systems (Wang *et al.*, 2023). Of growing concern are river isolates carrying clinically relevant antibiotics resistance genes (e.g., *bla*TEM, *sull)* on mobile genetic elements, suggesting water sources as potential reservoirs for resistance dissemination (Zhang *et al.*, 2024). The species demonstrates particular prevalence in rivers receiving wastewater effluent, where it forms

robust biofilms on particular matter (Chen *et al.*, 2023). While not typically pathogenic, its metabolic plasticity and resistance gene carriage make it an important indicator of anthropogenic impacts on aquatic microbial communities (Chen *et al*., 2023).

2.2.3 *Stenotrophomonas nitritireducens*

Stenotrophomonas nitritireducens is a denitrifying bacterium commonly found in freshwater sediments. It plays key roles in nitrogen cycling by reducing nitrite to N_2 O (Wang *et al.*, 2023). Recent studies highlight it's concerning dual function in urban rivers, while aiding nutrient removal, it's also carries antibiotic resistance genes (*sull* , *gnrS*) on mobile elements, potentially spreading resistance through water sources (Chen and Zhang, 2023). It's strong biofilm formation (Rivera *et al.*, 2024) and pollutant-degrading abilities (Park *et al*., 2023) makes it both an ecological player and emerging indicator of aquatic resistance threats.

2.2.4 *Stenotrophomonas rhizophila*

This species is primarily plant-associated and has been studied for its beneficial effects is agriculture. *S. rhizophila* supports the growth of plants by the production of indole-3-acetic acid (IAA), phosphate solubilization as well as antagonism against phytopathogens (Wolf *et al*., 2021). It is genetically distinct from *S. maltophilia* and does not possess major human virulence factors or multidrug resistance genes, making it safe for environmental applications (Huang *et al.*, 2022). The genome of *S. rhizophila* is enriched with genes for root colonization, stress resistance, and metabolic versatility, highlighting it's potential as a bioinoculant (Schulz *et al*., 2020).

2.2.5 *Stenotrophomonas chelatiphaga*

This species is an aerobic, Gram-negative, locomotive bacterium first isolated out of municipal sewage sludge in Kazan, Russia (Zhu *et al.*, 2024). This heavy-metal-resistant bacterium has been detected more recently in river sediments (polluted or not), and shows an extraordinary ability to chelate metals within its environment (Chen *et al*., 2023). More recently studies reveals its special affinity for cadmium and lead ions (Chen *et al*., 2023). Planctomycetes are able to produce a wide variety of siderophores, which can confer some degree of metal sequestration and availability (Zhang and Li, 2024). Most troubling is that it occurs together with antibiotic genes in urban rivers, suggesting links between metal pollution and resistance selection (Wang *et al.*, 2024)! The Biofilm305 species may be especially important in regulating metal mobility during biofilm formation on mineral surfaces such as the one found in this fluvial system (Rivera *et al*., 2023). Although non-pathogenic, its metal-resistance genes have homologues in clinical strains alarming the potential for gene transfer in aquatic environments.

2.2.6 *Stenotrophomonas africana*

This species, long debated but classified as distinct from *S. maltophilia* based on DNA-DNA hybridisation and multilocus sequence analysis (Ouoba *et al*., 2020), is isolated originally from human clinical samples in Africa. Although closely related to *S.maltophilia*, it presents with some different biochemical characteristics and antimicrobial susceptibility profiles.

Recent genomic data validates its status as an independent species and further indicates it has evolved new ecological or clinical niches within these distinct geographic populations (Naas *et al*., 2020).

2.2.7 *Stenotrophomonas terrae*

This is a Gram-negative, rod-shaped and highly adaptable bacterium in freshwater systems. This organism was previously considered isolated from soil (Helen *et al*., 2007) but recent studies show it is prevalent in river sediments able to retain its defining ability of nitrate reduction (Kim *et al*., 2023). Being motile and aerobic, this organism is likewise well-suited to rapidly fluctuating riverscapes such as in the biocondition between water and sediment. River water isolates form more biofilm than Land isolates, which are thought to be a phenotypic adaptation to flowing water associated with this organism (Yamamoto *et al.*, 2024). Particularly remarkable is its capacity to break down both natural polysaccharides like chitin and anthropogenic pollutants like hydrocarbons (Abdullahi *et al*., 2023). Fluvial strains were mesophilic but exhibited wider temperature tolerance ranges, indicating ecological adaptation to aquatic habitats. (park and Chen, 2024)

2.2.8 *Stenotrophomonas pavanii*

This species was first isolated from sugarcane roots in Brazil and is currently recognized as an endophyte with growth-promotion power, bioactive substance-producing (siderophone) activity and nitrogen-fixing ability (Gapta *et al.*, 2019). Its genome indicates multiple stress adaptation traits like resistance from heavy metals and osmotic inquire (Silva *et al*., 2020). It is usually non-pathogenic but needs to be carefully differentiated because of its close genetic relationship with *S. maltophilia* which would have implications for environmental monitoring programs.

2.2.9 *Stenotrophomonas tumulicola*

S. tumulicola is a relatively new member of the genus isolated from ancient burial mound soils in Korea and was described in 2020 (Kim *et al*., 2020). It is interesting concerning its unique environmental origin and possible aromatic compound metabolism pathways (Kim *et al*., 2020). Although the pathway for B12 is uncertain in a clinical context, their metabolic characteristics predict their bioremediation potential (Tsuchida *et al.,* 2011).

2.2.10 *Stenotrophomonas humi*

Stenotrophomonas humi is a rod-shaped, non-spore-forming and Gram-negative cell wall forming bacterium. Soil bacilli like this encountered in organic inorganic-rich river sediments, Live and appropriate for humor- us outbreak of plant matter (Chen *et al*., 2024) From recent studies, both urban river stains are known to bear mercury resistance genes (merA), consistent with environmental adaptation under a polluted scenario (Abdullahi *et al*., 2024). The rapid and dense biofilm formation attributed to its colonization of sediment particles in flowing water (Yamamoto and Watanabe, 2024).

2.3 Ecology and Environmental Distribution of *Stenotrophomonas* species

Stenotrophomonas is described as a Gram negative, oxidative bacillus that are commonly found within both natural and anthropogenic ecosystems. The most prominent species is *Stenotrophomonas maltophilia*, which can be isolated from soil, freshwater, plant rhizosphere, sediments, wastewater and humans as well (Brooke, 2021). This flexibility is a consequence of their metabolic modularity, biofilm formation potential, and environmental tolerance.

2.3.1 Habitat Versatility and Environmental Nitches

Multiple studies have shown the ubiquitous presence of *Stenotrophomonas* in terrestrial and aquatic systems. According to You *et al.* (2023), a large genomic study estimated that 24.4% of over 2,100 isolates were derived from the rhizosphere of plants and 6.1% were detected in water systems including rivers, lakes and wastewater This supports the wide ecological tolerance of this organism and its capacity to colonise nutrient-poor environments under conditions of stress.

Stenotrophomonas species are commonly isolated from aquatic environments and most frequently in rivers, especially near urban and peri-urban areas where water can be exposed to possible contamination due to agricultural runoff, industrial effluents or hospital waste. The isolation of multidrug-resistant *S. maltophilia* from rivers and wastewater treatment plants in southern Nigeria (Omoregie and Okoh, 2024) particularly around Benin City supports this conclusion. This lends support to the idea that polluted water bodies could be important reservoirs for *Stenotrophomonas*.

2.3.2. Adaptations to Environmental Stress

Structural and functional adaptations contribute to the ability of *Stenotrophomonas* to survive within harsh environmental conditions. Genomic reconnaissance reveal these bacteria have genes related to oxido response (e.g., catalase, superoxide dismutase), osmotic homeostasis (e.g., trehalose and betaine biosynthesis), and heavy metal detoxification (i.e., ars, czc and cop operons) (You *et al.*, 2023). These mechanisms are essential to persisting in the high metal concentrations or transient pH and temperature oscillations common in polluted water and soil environments.

In addition, their ability contributes to biofilm formation, allowing them to endure desiccation, UV light exposure and disinfectants present at water treatment plants (Looney *et al.,* 2021). Biofilm formation: also promotes adhesion to natural and artificial surfaces in aquatic systems (e.g. plant roots, sediments, man-made structures live pipes and tanks (Looney *et al.,* 2021).

2.4. The Dual Role of *Stenotrophomonas* Species in River Ecosystems

The ecology of *Stenotrophomonas* has a dualistic and dwarfing impact. Some of these strains also provide critical ecosystem services such as bioremediation, while others are major opportunistic pathogens and vectors for antibiotic resistance which makes river presence a serious public health and environmental problem.

2.4.1 Positive Impacts of *Stenotrophomonas* Species in Rivers

Stenotrophomonas species, particularly *Stenotrophomonas maltophilia*, contribute beneficial lyrics river ecosystems through their extensive metabolic capabilities.

2.4.1.1 Bioremediation

Degradation of persistent toxic pollutants is one of the major and useful contributions by xenobiotics degrading microbes in bioremediation. An example is the *Stenotrophomonas* group of species known to be highly effective at degrading polycyclic aromatic hydrocarbons (PAHs), which are naturally occurring processes that can lead to human carcinogenesis and regularly occur in rivers due to industrial discharge, oil spills or urban runoff (Elufisan *et al*., 2020). These bacteria then secrete various types of enzymes like dioxygenases and hydrolases, which cleave from this complex structure of PAHs producing relatively smaller and non-harmful intermediates (Elufisan *et al.*, 2020). Recent studies have revealed their possible use as bioremediation agents, providing more sustainable alternatives to chemical methods for

cleaning up polluted environments (Singh *et al.*, 2022). Pesticide degradation by *Stenotrophomonas*. Agricultural runoff carries pesticides such as chlorpyrifos and glyphosate to rivers, posing risks to aquatic organisms and human health. Some strains of *Stenotrophomonas* are capable of using these compounds as carbon (C) or nitrogen (N) sources, leading to a lower persistance and toxicity in the environment (Elufisan *et al.*, 2020). The natural attenuation of pesticides occurs without the need for costly, energy-intensive remediation techniques (Li *et al.*, 2023).

2.4.1.2 Heavy Metal Detoxification

Rivers filled with toxic metals (lead, cadmium and arsenic) can severely affect aquatic organisms and drinking water quality. They also represent the use of biosorption (where metals bind to the cell surface), enzymatic reduction, and sequestration to immobilize or transform metals to less bioavailable forms (Ryan *et al.*, 2009). This not only decrease metal toxicity in water, but also avoid their accumulation in the food chain (Chen *et al*., 2023).

2.4.1.3 Nutrient Cycling and Ecosystem Support

Stenotrophomonas is essential for nutrient cycling as it aids in the decomposition of organic matter. They Pickup organic matter such as plant debris and algae biomass. Making key nutrients such as nitrogen and phosphorus (Martinez *et al.*, 2024). This process maintains the microbial food web and promotes phytoplankton growth, which is at the base of the aquatic food chain. Finally, their activity in organic matter processing is essential for maintaining river health by preventing nutrient saturation that leads to eutrophication (Martinez *et al*., 2024).

2.4.1.4 Riparian Zone Enhancement

Several *Stenotrophomonas* strains are able to promote plant growth, which could be interesting with regards to riparian vegetation, wetland restoration. They enhance growth through the synthesis of phytohormones such as indoor-3-acetic acid (IAA) with solubilizing phosphates, and produce siderophores that lead to enhanced soil stabilization along riverbanks reducing soil erosion, lowering silt loads in rivers, improving water quality via natural filtration processes (Khan *et al*., 2021).

2.4.2 Negative Impacts of *Stenotrophomonas* species in Rivers

Although these genera have beneficial roles, the species of *Stenotrophomonas* causes considerable harm with their naturally and acquired resistance to antimicrobial drugs and opportunistic pathogenic ability.

2.4.2.1 Role of *Stenotrophomonas* as an Opportunistic Pathogen

Stenotrophomonas maltophilia is a well-studied multidrug-resistant opportunistic pathogen. In immunocompromised individuals, such as burn patients and those undergoing chemotherapy, river water can serve as a reservoir and route of transmission for notoriously difficult infections to treat due to the bacterium's broad resistance profile (Brooke 2012; Adegoke *et al.*, 2017). Over and above human health, it can act as a pathogen of aquatic organisms (fish and amphibians) (Figueras *et al*., 2011).

2.4.2.2 Intrinsic and Acquired Antibiotic Resistance:

It has a high intrinsic resistance to many antibiotics, including aminoglycosides, carbapenems and most beta-lactams from the genus. This is due to several mechanisms such as efflux pumps (SmeABC, SmeDEF), low membrane permeability and antibiotic inactivating enzymes (Li *et*

al., 2002) including the two metallo-β-lactamases L1 and L2. Importantly, rivers provide suitable habitat for lateral gene transfer of resistance genes (Li *et al.*, 2002). *Stenotrophomonas,* can transfer genetic mobile units (plasmids intentions) with other ecologically and clinically relevant bacteria enabling the dissemination of resistant traits to reach multiple environments in the microbiome (Chang *et al.,* 2015).

2.4.2.3 Biofilm Formation:

The biofilm forming ability of *Stenotrophomonas* on natural (rocks). Artificial hard substrate (pipes, medical equipments) and natural sediment surfaces prolong them into river ecosystems. The physical protection that biofilms provide against antibiotics and disinfectants also makes elimination difficult, but it is precisely this tightness of these microbial communities that promotes HGT (Pompilio *et al.*, 2020).

2.4.2.4 Impact on Aquatic Hosts:

In addition to its pathogenic effects on human health, *Stenotrophomonas* can also be a pathogen of aquatic organisms and has been reported to cause disease outbreaks in susceptible fish and amphibian populations which may make the local ecosystem unstable (Figueras *et al.*, 2011).

2.5 Prevalence in Freshwater Ecosystems Globally and in Nigeria:

Stenotrophomonas species, especially *Stenotrophomonas maltophilia*, have been recently discussed to be widely found and reported as a reason for contamination in aquatic environments globally. While typically considered low-abundance environmental bacteria, their increased detection within freshwater ecosystems can be attributed to advances in molecular identification techniques combined with acknowledgement of opportunistic pathogenic potential (Brooke, 2012). Global strains of *S. maltophilia* have been isolated from

rivers, lakes, ponds and wastewater-influenced freshwater bodies. An example is a study in Japan (Urase *et al*., 2023) where they claimed presence of *Stenotrophomonas* at low concentrations ranging from as low as 0.6 CFU/mL, both pond water and treated effluent confirmed by the culture as well as PCR-based identifications were conducted (results not shown). They showed that this organism can even be found in treated water, which has played a part in spreading it into natural freshwater systems.

In a NorthAmerican study on microbial shifts during combined sewage overflow events, *S. maltophilia* was identified as one of the dominant potential pathogens, representing as high as 27.7% the isolate's during overflows, while being nearly undetected in clean upstream water samples (Pereira *et al.*, 2021). This suggests that it may arise opportunistically in contaminated freshwater habitats and then outcompete the native microbiota under stressed conditions or with high organic loads. Likewise, a global environmental meta-analysis noted about 5.3% (CI: 4.1–6.7%) *S. maltophilia* among all environmental and clinical Gram-negative isolates indicating an expansive yet context-specific distribution (Brooke, 2021). While the figure includes non-aquatic environments this is indicative of an organism that was widespread in its ecology.

Considerable abundance of *Stenotrophomonas* in the Umhlangane River (South Africa) where bacterial counts range from 2.7 to 4.1 $\log_{10}$ CFU/mL in water samples and up to 5·0 $\log_{10}$ CFU/g in river sediments (Govender and Okoh, 2022); The high abundance of bacteria in sediment indicates that this organism is a resilient member and could play an important role in benthic microbial communities. Nutrient enrichment, presence of antimicrobials and anthropogenic discharges (e.g., urban runoff, hospital effluents, and sewage) are all factors

predicting increased levels of *Stenotrophomonas* in aquatic systems (Gomes *et al*., 2019). It can survive in polluted and stressed environments due to its antibiotic resistance and biofilm-forming ability making it an environmental indicator as well as a health threat (Chang *et al.*, 2020). *S. maltophilia* and close relatives are rare to moderately abundant in unimpacted natural freshwater ecosystems, but significantly more abundant in human-disturbed environments (Adamek *et al*., 2011). The presence of these microorganisms in surface water and sediments, particularly in conditions of pollution by urban runoff and/or poor wastewater management, raises public health concerns while demonstrating ecological adaptability.

2.6 The Clinical Relevance and Pathogenicity of *Stenotrophomonas* species:

Despite their environmental origin, *Stenotrophomonas* species have emerged as significant opportunistic pathogens in healthcare settings, particularly affecting immunocompromised individuals (Brooke, 2012). *S. maltophilia* complex (Smc) is now recognized as a broadly distributed emerging pathogen of considerable clinical concern (Adegoke *et al.*, 2017). Infections resulting from these bacteria are especially dangerous for persons suffering from underlying disorders such as cancer, cystic fibrosis (CF), COVID-19, chronic respiratory disease, or those undergoing immunosuppressive treatment (Looney *et al.*, 2009). Infections caused by this bacterium can lead to a mortality rate as high as 37.3% among immunocompromised patients, emphasizing the crucial need for effective treatment methods (Gajdacs *et al.*, 2021).

Wide spectrum of infections, as well as bacteremia, respiratory tract infections pneumonia, ventilator-associated pneumonia), urinary tract infections, medical devices related infections, and soft tissue infections can be caused by *Stenotrophomonas* species (Waters *et al*., 3013).

Stenotrophomonas maltophilia was recognized during the COVID-19 pandemic as one of the leading cause of respiratory co-infections and bacteremia in severely ill patients of COVID-19 (Garcia-Garcia *et al.,* 2023). Also, isolates of *S. maltophilia* identified in samples gotten from these patients had the greatest prevalence of multidrug resistance among other bacteria infecting COVID-19 patients (Sanchez *et al*., 2022).

Stenotrophomonas ability as the causative agent for diseases is enhanced be a range of virulence determinants that allows it invade, occupy as well as evade the host's immune defenses. Theses include;

2.6.1 Adhesion Factors:

Surface associated structures, for example type IV pili, fimbriae (including SMF-1 fimbriae), also, non-plus adhesins facilitate attachment to both non-living surfaces as well as host tissues (zgair and Chhibber, 2011).

2.6.2 Biofilm Formation:

Biofilms formation ability both on living and non-living surfaces is a critical pathogenicity determinant that enhances resistance to antibiotics and immune response of the host. Biofilms formation is especially concerning when it comes to medical devices like catheters and ventilators (Pompilio *et al.,* 2011).

2.6.3 Extracellular Enzymes:

Stenotrophomonas produces a wide array of extracellular enzymes, as well as proteases, nucleases, lipases and gelatinases that causes tissue deterioration and nutrient acquisition

(Brooke, 2012). The T2SS-secreted proteases StmPr1, StmPr2, and StmPr3 are particularly important for degrading host proteins and evading immune defenses (Windhorst *et al*., 2002).

2.6.4 Secretion System:

Effector molecules delivery into host cells, modulating host cells processes and promoting infection is enabled by secretion systems such as type I, II, IV, V, and VI (Lin *et al.,* 2014).

2.6.5 Iron Acquisition System:

The production of siderophores and other iron acquisition mechanisms allows *Stenotrophomonas* to overcome iron limitation, which is a key nutritional immune defense of the host (Huang *et al*., 2006).

2.6.6 Motility Structure:

Flagella-based motility and twitching motility; the latter of which is mediated by type IV pili, promote dissemination throughout the host environment (Furushita *et al*., 2003).

Virulence in *Stenotrophomonas*, is coordinated by complex signaling systems that connect pathogenicity with antimicrobial resistance at the molecular level (Trifonova and Strateva, 2019). Diffusible signal factor (DSF) quorum sensing is an important regulator that positively regulates the output of virulence factors, promotes biofilm formation, and stimulates siderophore generation in a dose-dependent manner (Huang *et al.*, 2010). Importantly, DSF signaling induces production of β-lactamase as well, directly linking regulation of virulence to antibiotic resistance mechanisms. Moreover, the bacterial second messenger cyclic di-GMP (also known as c-di-GMP) engages in a key function by regulating the switch between suspended (planktonic) and biofilm associated states where elevated intracellular cyclic di-

GMP levels support biofilm formation and maintenance, increasing the bacterium `s resilience against environmental- as well as host-driven pressure (An *et al*., 2014; Garcia *et al.,* 2019). Understanding these mechanisms is important for assessing the exposure to environmental stresses, such as those encountered in river sediments and water (which can harbor pathogenicity that may be activated by contact with humans), thus highlighting public health risks associated with *Stenotrophomonas* contamination of water supplies (An *et al.*, 2014; Garcia *et al.,* 2019).

2.7 Antimicrobial Resistance Pattern and Treatment Challenges

All *Stenotrophomonas* species have broad spectrum resistance to many classes of antimicrobial agents, so they pose a dilemma in treatment. This multidrug resistance includes β-lactams, and also carbapenems as well as aminoglycosides macrolides and many other frequently used antibiotics. Current Consensus: After 2011, the World health organization (WHO) identified the microorganism as one of the world`s most concerning emerging antibiotic resistant bacteria due to its exceptionally wide resistance profile posing a clinical challenge in treating these infections (Crossman *et al.,* 2008; Chang *et al*., 2015; WHO, 2023).

The molecular mechanisms underpinning the extensive antimicrobial resistance in *Stenotrophomonas* are highly diverse and sophisticated. A primary line of defense involves multi-resistance-modulation-division (RND)-type efflux pumps, including SmeABC, SmeDEF, and SmeVWX, that deeply eject a broad range of antibiotics such as fluoroquinolones, tetracyclines, trimethoprim, and sulfamethoxazole out of the bacterial cell (Li *et al.,* 2002). The production of two inducible β-lactamases, L1 and L2 is a second major mechanism (Brooke, 2012). Carbapenems and most other β-lactams except aztreonam are

hydrolyze by the L1 metallo-β-lactamase, while the conferring of resistance to extended-spectrum cephalosporins and aztreonam is carried out by L2 class A cephalosporinase (Brooke, 2012). These enzymes are highly prevalent, with surveillance studies detecting L1 in approximately 77% and L2 in 89% of clinical isolates (Hirsch *et al*., 2025). Resistance is further augmented by aminoglycoside-modifying enzymes found in a majority of sequenced genomes, target site modifications, such as mutations in DNA gyrase that confer fluoroquinolone resistance, and an outer membrane with inherently low permeability that serves as a barrier to antibiotics access (Youenou *et al*., 2015).

Such a complicated resistance profile poses a major challenge in treatment. The treatment of choice has long been trimethoprim-sulfamethoxazole (SXT), but increased resistance rates and pharmacodynamic data that raise concerns about the effectiveness of SXT against some isolates have complicated therapeutic decisions (Nicodemo and Paez, 2007). Consequently, treatment paradigms are evolving. Cefiderocol has emerged as a potential alternative to SXT according to recent EUCAST guidelines and minocycline breakpoints have recently been revised downwards based on new PK/PD data (Wang *et al*., 2014). Alternative choices consist of levofloxacin (not the endorsed monotherapy) and on numerous combination regimens (Wang *et al.,* 2014). Such evolving clinical understanding is mirrored in revised guidelines such as those from the CLSI, which recently removed ceftazidime breakpoints because of insufficient data to support their use and historical questions about species identification in studies (CLSI, 2023).

2.8 Previous Studies on *Stenotrophomonas* species Contamination in Nigerian River Waters

Over the years, studies on Nigerian river waters have consistently revealed *Stenotrophomonas maltophilia* a significant emerging environmental and public health challenge. Early work by Adebayo-Tayo *et. al.* (2012) showed that significant environmental memory isolates accumulated more multiple antibiotics resistance, different ratio between classs such as 78.3% for flora-associated bacteria which is proved to accumulate multiple antimicrobial resistances and beta-lactams as well as carbapenems in Akwa Ibom State river87587488 Association of environmental memory isolates with high levels of antibiotic resistance. This confirmed the role of Nigerian aquatic bodies as reservoirs for multidrug-resistant bacteria. But other studies soon confirmed these results, and usually link human activity directly to the presence of bacteria. One such example is the work carried out on the Otamiri River in Owerri by Osuagwu *et. al*. (2020) who also reported the increase in abundance of *S. maltophilia* downstream of river locations where abattoir effluents entered, along with its ability to thrive in conditions of high organic and faecal pollution.

This relationship with pollution is confirmed by the works of Omoregie and Okoh (2022), who reported several isolations, including *S. maltophilia* from surface waters plus wastewater effluents around Benin City; Nnadozie and Odume (2021) also detected it in the Ikpoba River increasingly admitting its high tolerance to antimicrobial agents as well as resistance against heavy metals. Observations above highlight the need for monitoring *Stenotrophomonas* in Nigerian freshwater habitats, particularly because of the role these rivers play as important

water sources among human communities (Nnadozie and Odume, 2021; Omoregie and Okoh, 2022).

Central to these efforts is the characterization of the resistance mechanisms employed by these environmental strains. Olayemi *et. al. (*2021) which identified multi-drug resistant isolates of *Enterococcus avium* in a cluster on the Kwa River, especially that were supported with trimethoprim-sulfamethoxazole (often treatment of choice), and those isolates were shown to be tolerant for heavy metals (lead and chromium). Co-resistance represents a serious public health threat as multiple sources of pollution, can create selective pressure that enhances the persistence of "super-resistant " microbes with similar mechanisms such as was observed in the Ogun River study by Ogunleye *et. al.* (2018).

Typically, the broader conclusion drawn from these findings is that these environmental reservoirs represent a considerable public health risk. Researchers like Akinjogunla *et. al.* (2020) have underlined, the consumption of river water dirty in drinking, bathing, washing and irrigation clearance offers a direct way out of opportunistic pathogen such as *S. maltophilia* transmission and also poses a risk community-acquired multidrug resistance. Based on the evidence provided, it is certain that Nigerian rivers are important hot spots for the emergence and spread of antimicrobial drug resistance, with *Stenotrophomonas maltophilia* being of particular interest.

2.9. Research Gaps and Future Directions for Studying *Stenotrophomonas* in Aquatic Environments

Important advances have been made in our understanding of *Stenotrophomonas* spp in aquatic environments, however several critical knowledge gaps persist. One major gap is a limited understanding of the specific ecological role it plays in river ecosystems, including its interactions with other microorganisms and its involvement in biogeochemical cycles (Zhang *et al*., 2023).

In addition, both the range and HGT rates in transfer of ARGs between *Stenotrophomonas* and co-occurring bacteria are also an important knowledge gap that warrants further exploration (Chang *et al.* 2022). Most importantly, the particular environmental triggers and genetic adaptations that drive *Stenotrophomonas* transition from an innocuous environmental bacterium to opportunistic human pathogen remain largely unexplored and should be heavily investigated (Looney *et al.*, 2021).

To overcome these limitations, future studies must include a longitudinal component that tracks temporal and spatial dynamics of *Stenotrophomonas* prevalence and population diversity in riverine systems while explicitly linking their activity to anthropogenic settings (e.g., agricultural runoff, wastewater effluent) and climate drivers (Garcia-Leon *et al.*, 2024). At the same time, more detailed and standardized virulence assessment of environmental isolates in well-established in vitro and in vivo models is urgently needed to provide quantitative data on their pathogenic potential (Fursova *et al.*, 2023). The rapid culture-independent molecular and biosensor-based detection mothods would be helpful to improve better real-time monitoring and public health risk assessment(Wenger *et al.*, 2024).

Public health research will need to shift towards the design and evaluation of targeted intervention strategies. This involves applying stringent experimental analysis to evaluate the effectiveness of control measures that maximise removal/reduction of multi-drug resistant *Stenotrophomonas* from water, develop predictive recreational water quality safety models, and derive scientifically-grounded exposure limits for immunocompromised subpopulation (WHO, 2023). Crucially, establish integrated genomic surveillance networks that integrate data from the environment, agriculture and clinical genomes to track the emergence and dissemination of high-risk clones and resistance determinants (Bengtsson-Palme *et al.*, 2024). *Stenotrophomonas* species are more valuable than others, and they require a careful and comprehensive approach to research train. Therefore, future studies must integrate mechanical microbiological, genomic, environmental and clinical data to furnish contextual whole of these fecal decontaminators impacting the structure of health prudence strategies with their potential ecologically when implicated in disciple use,/Filter/insight down measures for declining riverine ecosystem proficiency.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was conducted at Benson Idahosa University, Lagacy Campus, Benin City, Edo state, Nigeria. Water samples was collected from two (2) major rivers based on their proximity to human activities, including: Ologbo River (Latitude 6.3833° N, Longitude 5.6167° E) – domestic waste discharge and agricultural pollution; and Ikpoba River (Latitude 6.3167° N, Longitude 5.6000° E)– Industrial waste discharge (Guinness Nigeria PLC), Urban runoff and agricultural pollution.

3.2. Sample Collection

One (1) litter of water sample was collected in sterile containers from three (3) points along each river. Samples were thoroughly labeled and transported to the Microbiology laboratory of Benson Idahosa University for analysis within few hours of collection.

3.3 Isolation of *Stenotrophomonas spp*

Ensuring aseptic techniques, one milliliter (1 ml) of water sample was measured and added to a test tube comprising 10 ml of nutrient broth supplemented with DL-methionine (0.5 mg/ml). After incubating for 24 hours at room temperature, the broth was vortexted and 0.1 ml of the aliquot was inoculated with a micro pipette onto a nutrient agar plate and was disseminated in a circular motion with a glass spreader that is sterile. Within 15 minutes post inoculation, four discs impregnated with 10 µg of imipenem (Mast Diagnostics, South Africa) each were administered to the inoculated plate's surface. This is because, *Stenotrophomonas* spp. are naturally resistant to imipenem. After incubating for 24 hours at room temperature, colonies

that were seen growing within the zones of inhibition of the discs were subcultured and their cultural, morphological and biological characteristics were identified (Bollet *et al*., 1995).

3.4 Identification of Isolates

Isolates selected were identified using their cultural, morphological and biological characteristics as detailed below;

3.4.1 Gram Staining

Differential staining technique that was developed be Hans Christian Gran was conducted to clearly classify all obtained bacterial isolates as being Gram-positive or either Gram-negative based on how their cell wall structure differs (Cheesbrough, 2021). This method works because Gram-positive bacteria are characterized by having a thick peptidoglycan layer that retains the crystal violet stain after alcohol decolorization, whereas Gram-negative bacteria possess a thinner peptidoglycan layer, after alcohol decolorization, it looses the crystal violet stain and stains red which is the colour of the counterstain, safranin.

A loop full of 24 hours bacterial culture was used to prepare a smear on a grease-free clean slide, using a Bunsen burner flame, the smear was heat-fixed and was flooded with crystal violet solution and therefore was rinsed off with deionized water after being allowed to stay for 60 seconds lugol's iodine solution was applied to the slides for 60 seconds, which function as a mordant to form insoluble complexes with the retained dye. This was thereafter washed off with deionized water. This was closely followed by decolonization with ethanol (95%) for approximately 3 seconds then, was rinsed off under flowing tap water. After decolorizing, a counter-stain (safranin) was applied onto the slide to impact color to the decolorized cells. This was washed off over the sink with slowly running distilled water after being left to stay for

about 60 seconds. The prepared slides were kept in a vertical position to air-dry and was thereafter examined under a light microscope with the help of the oil immersion objective (100x magnification). Gram-positive organisms appeared purple whereas Gram-negative isolates appeared pink or red under the microscope.

3.4.2. Motility Test

A motility test was conducted to establish if the bacterial isolate was able to exhibit movement through flagella or other relevant organelles. The test was performed by using a sterile straight inoculating needle to stab the organism once into the center of a tube of semi-solid half-strength nutrient agar medium. This permits the movement of motile bacteria away from the line of inoculation. After incubation at 35-37°C for about 24-48 hours, the tube was examined for growth patterns. Scattered growth spreading outwards from the central stab inoculation line, resulting in cloudiness throughout the medium, was observed and interpreted as evidence of motility. In contrast, growth that remained strictly confined to the line of inoculation, with the surrounding medium remaining clear, was recorded as indicating a non-motile organism.

3.4.3 Biochemical Tests

The biochemical tests outlined in this study were conducted for the presumptive identification of bacterial isolates, following the standardized methodologies described by Cappuccino and Welsh (2020).

3.4.3.1 Oxidase Test

This test was carried out to detect the existence of the cytochrome C oxidase enzyme in the bacterial isolates. A drop of normal saline was applied on a filter paper to make a smear, and a bacterial colony was gathered using a sterile inoculating loop, the bacterial colony was then

spread onto the filter paper. This was followed by the addition of tetramethyl-p-phenylenediamine dihydrochloride reagent to the sample. The formation of a dark purple-blue colour at the site of inoculation within 5-10 seconds was observed and recorded as a positive result. The absence of any colour change after more than 60 seconds was recorded as a negative result.

3.4.3.2 Catalase Test

This test was conducted to determine if the catalase enzyme is present in the bacterial isolates. A drop of normal saline was placed on a clean glass slide that was free from grease. A little portion of 24 hours culture of the test isolate was collected with the use of a sterile wooded applicator and was smeared onto the sterile glass slide. This was followed by the addition of 3% hydrogen peroxide (H_2O_2) onto the bacterial smear. The immediate formation of a bubble was recorded as a positive result. The effervescence, resulting from the release of oxygen gas indicates catalase activity, an enzyme that is responsible for the breakdown of hydrogen peroxide into water and oxygen. The absence of bubble was recorded as a negative result.

3.4.3.3 Methyl Red Test

This test was performed to identify bacterial isolates capable of fermenting glucose via the mixed-acid pathway, yielding stable acidic end products that significantly decrease the medium's pH. The test is carried out by putting the test organism into MR-VP broth thereafter incubating it at 35-37°C for within 48 hours. Post incubation period, a few drops of methyl red indicator were directly added to the culture broth. The immediate show of a yellow coloration after adding the reagent was recorded as a negative result, and this therefore indicate

a pH of 6.2 or higher. On the order hand, a red color indicated a positive (with pH below 4.4), denoting strong acid production, and an orange color was inconclusive.

3.4.3.4 Urease Test

This test was conducted to ascertain the bacterial isolate's ability to decompose (with water) urea into ammonia and carbon dioxide. It is primarily used to differentiate urease-positive bacteria from other *Enterobacteriaceae* . The test isolate was added onto a sterile urea agar slant holding phenol red as an acid-base indicator. The inoculated slant was incubated for 24 hours at 37°C. post incubation, the maintenance of the original orange-yellow color was recorded as a negative result. However, the change in color to a bright pinkish-red throughout the medium was recorded as a positive result, indicating hydrolysis of urea and production of ammonia.

3.4.3.5 Simmons Citrate Test

This test was performed to determine the bacterial isolates's ability to use sodium citrate as their sole source of carbon. Five milliliters (5 ml) of Simmons citrate agar was prepared and inoculated into screw-capped test tubes and for 15 minutes was sterilized at 121^{o}C. Afterwards, the test tubes were placed in a slant position and left to solidify. The test organism was streaked on the surface of the simmons citrate agar slant, a defined medium containing citrate, ammonium salts, and the pH indicator bromothymol blue. Following a 24-48 hours at 35^{o}C incubation, both growth and the medium's change in color from green to a distinct Prussian blue were observed and recorded as a positive result. While a no change in both growth and color was interpreted as a negative result.

3.4.3.6 Indole Test

The test was conducted to ascertain the bacterial isolate's ability to produce the enzyme tryptophanase. The test organism was inoculated into a test tube containing tryptone broth, which is rich in tryptophan, and was incubated for 24 to 48 hours at 35^{o}C. Post incubation, Kobac's reagent of approximately 0.5 ml was gently inoculated to form a layer on top of the broth culture. Post addition, the immediate formation of a bright red ring in the upper layer of the medium was observed and recorded as a positive result for indole production. However, the formation of a yellow or brown ring at the interface indicated a negative result.

3.5 Antibiotics Sensitivity Test

This test was conducted using the standard Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA). A suspension of bacteria was prepared from fresh pure colonies and standardized to a 0.5 McFarland turbidity. The standardized suspension was evenly swabbed across the entire surface of the MHA to produce a confluent lawn of growth. Using sterile forceps, the six antibiotic discs—Cotrimoxazole (25µg), Imipenem (10µg), Piperacillin (30µg), Ceftazidime (30µg), Tigecycline (15µ), and Polymyxin B (300 units) —were placed firmly onto the inoculated agar surface (three antibiotics to a plate, ensuring adequate spacing between them to prevent inhibition zones being overlapped). The inoculated plates were then subsequently inverted and kept in aerobic conditions for a duration of 16-18 hours at 35°C. Post incubation period, the zone of inhibition surrounding each antibiotic disc was measured in millimeters. These values were then interpreted according to the Clinical and laboratory Standards Institute (CLSI, 2023) guidelines to categorize the bacterial isolate as susceptible, intermediate, or resistant to each antibiotic.

3.6 Multiple Antibiotic Resistance (MAR) Index

MAR index was calculated using the previously described method by Odjadjare and Igbinosa (2017) as shown below;

MAR = a/b = 4/6 = 0.67

Where:

a = the number of antibiotics to which a given isolate was resistant, and

b = the total number of antibiotics against which individual isolates were tested.

MAR index greater than 0.2 indicates that the organisms likely originate from high-risk contamination sources where antibiotics are frequently used or abused (Odjadjare *et al*., 2012).

CHAPTER FOUR

RESULTS

4.1. Morphological, Cultural, and Biochemical Characteristics of Ikpoba River Isolates

Table 4.1 shows the morphological, cultural and biochemical characteristics of bacterial isolates from the Ikpoba River. The results revealed that the Ikpoba River is heavily and predominantly contaminated with bacteria belonging to the genus *Stenotrophomonas*. Among 36 bacterial isolates selected, thirty two (32) were presumptively identified as *Stenotrophomonas* spp. according to their morphological, cultural, and biochemical profiles. Three (3) of the isolates were identified as *Pseudomonas* spp. and one (1) as *Enterococcus* sp.

4.2. Morphological, Cultural, and Biochemical Characteristics of Ologbo River Isolates

Table 4.2 shows the morphological, cultural, and biochemical characteristics of bacterial isolates form Ologbo River. The results revealed that among the 36 selected bacterial isolates, twenty nine (9) were presumptively recognized as *Stenotrophomonas* spp., six (6) were identified as *Pseudomonas* spp. and one (1) *Enterococcus* sp. *indicating high contaminated river*

4.3. Antibiotics Sensitivity Profile of the Bacterial Isolates From Ikpoba River

Table 4.3 shows the antibiotics sensitivity profile of the bacterial isolated from Ikpoba River. The bacterial isolates showed complete and total resistance to four key antibiotics: Piperacillin, polymyxin B, Imipenem, and Ceftazidime. Tigecycline was recorded as being the most effective antibiotic, with 26 isolates (75%) being sensitive to it and 6 (17%) isolates were resistant, and 4 (11%) showing intermediate susceptibility. Cotrimoxazole was also

effectiveness, with 25 isolates (69%) being susceptible, 9 (25%) resistant, and 2 (6%) moderately sensitive to the antibiotics.

Table 4.1. Morphological, cultural and biochemical characteristics of bacteria isolates from Ikpoba River

MORPHOLOGICAL/ CULTURAL CHARACTERISTICS		BIOCHEMICAL CHARACTERISTICS						PROBABLE IDENTITY	NUMBER OF ISOLATES
Gram Reaction	Motility	Oxidase	Catalase	Methyl Red	Urease	Citrate	Indole		
- Rod	motile	-	+	-	-	+	-	*Stenotrophomonas* spp.	32
- Rod	motile	+	+	-	-	+	-	*Pseudomonas* spp.	3
+ cocci	motile	-	+	-	-	+	-	*Enterococcus* sp.	1

Key: - = negative + = positive

Table 4.2 Morphological, cultural and biochemical characteristics of bacterial isolates from Ologbo River

	MORPHOLOGICAL/ CULTURAL CHARACTERISTICS		BIOCHEMICAL CHARACTERISTICS						PROBABLE IDENTITY	NUMBER OF ISOLATES
S/N	Gram Reaction	Motility	Oxidase	Catalase	Methyl Red	Urease	Citrate	Indole		
1	- Rod	motile	+	+	-	-	+	-	*Pseudomonas* spp.	6
2	- Rod	motile	-	+	-	-	+	-	*Stenotrophomonas* spp.	29
3	+ Cocci	motile	-	+	-	-	+	-	*Enterococcus* sp**.**	1

Key: - = negative + = positive

Table 4.3: Antibiotics sensitivity profile of the selected bacterial isolates from Ikpoba River

S/N	Antibiotics class	Antibiotics	Number of Isolates (%)		
			Sensitive	Intermediate	Resistant
1	Penicillin	Piperacillin	0	0	36 (100)
2	Sulphonamides	Cotrimoxazole	25 (69.44)	2 (5.56)	9 (25)
3	Polymyxin	Polymyxin B	0	0	36 (100)
4	Glycylcycline	Tigecycline	26 (72.22)	4 (11.11)	6 (16.67)
5	Carbapenem	Imipenem	0	0	36 (100)
6	Cephalosporin	Ceftazidime	0	0	36 (100)

Key: R - Resistant S – Susceptible I – Intermediate

4.4. Antibiotics Sensitivity Profile of Bacterial Isolates from Ologbo River

Table 4.4 shows the antibiotics sensitivity profiles of the bacterial isolates from Ologbo Rivers. The results revealed that the bacterial isolates were one hundred percent (100%) resistant to four key antibiotics: piperacillin, polymyxin B, imipenem, and ceftazidime. The isolates (23) were generally sensitive to tigecycline (64%), 8 (22%) were resistant, and 5 (14%) showed intermediate resistance. Cotrimoxazole was also effective against the isolates for which 23 (64%) were susceptible, 9 (25) were resistant, and 4 (11%) showed intermediate resistance.

4.5 Multiple Antibiotics Resistance (MAR) Indes of Isolates From the Ikpoba River

Table 4.5 shows the multiple antibiotics resistance (MAR) index of the bacterial isolates from the Ikpoba River. The data revealed an increase level of resistance with all the tested isolates demonstrating multi-drug resistance patterns covering four to six antibiotics across six antibiotics classes. The MAR index was consistently high, ranging from 0.67 to a maximum of 1.0; the latter indicating complete resistance to the entire panel of antibiotics as observed in two isolates. The most frequent (modal) MAR index for the tested isolates was 0.67, that occurred in 23 isolates.

Table 4.4: Antibiotics Sensitivity Profile of Bacterial Isolates from Ologbo River

S/N	Antibiotics class	Antibiotics	Number of Isolates (%)		
			Sensitive	Intermediate	Resistant
1	Penicillin	Piperacillin	0	0	36 (100)
2	Sulphonamides	Cotrimoxazole	23 (63.89)	4 (11.11)	9 (25)
3	Polymyxin	Polymyxin B	0	0	36 (100)
4	Glycylcycline	Tigecycline	23 (63.89)	5 (13.89)	8 (22.22)
5	Carbapenem	Imipenem	0	0	36 (100)
6	Cephalosporin	Ceftazidime	0	0	36 (100)

Key: R - Resistant I – Intermediate S - Susceptible

Table 4.5 Multiple antibiotics resistance index of bacterial isolates from Ikpoba River

S/N	Multidrug Resistance (MDR) Pattern	Number of Isolates Showing the MDR Pattern	MAR Index
1	PRL, PB, IMI, CAZ	23[a]	0.67
2	PRL, PB, TCN, IMI, CAZ	4[b]	0.83
3	PRL, TS, PB, IMI, CAZ	7[c]	0.83
4	PRL, TS, PB, TCN, IMI, CAZ	2[d]	1.0

Legend:

[a] IR111, IR121, IR131, IR141, IR221, IR231, IR241, IR321, IR331, IR341, 1R212, IR222, IR242, IR332, IR342, IR123, IR133, IR143, 1R213, IR223, IR323, IR333, IR343

[b] 1R211, IR132, IR322, IR113,

[c] IR311, IR112, IR142, IR232, IR312, IR243, IR313,

[d] IR122, IR233,

Key: PRL - Piperacillin PB - Polymyxin B IMI - Imipenem

CAZ - Ceftazidime TCN - Tetracycline TS - Cotrimoxazole

4.6 Multiple Antibiotic Resistance (MAR) Index of Isolates from the Ologbo River

Table 4.6 shows the resistance patterns of the bacterial isolates from Ologbo River. The isolates reveal a high rate of multidrug resistance (MDR), with patterns covering four to six antibiotics. The Multiple Antibiotic Resistance (MAR) index was consistently intense, ranging between 0.67 to 1.0. The most frequent resistance pattern, noticed in 24 isolates (66.7%), was against four antibiotics (PRL, PB, IMI, CAZ), yielding a MAR index of 0.67. Five (5) isolates showed complete resistance to all six (6) antibiotics tested, resulting in the maximum MAR index of 1.0.

Table 4.6 Multiple antibiotics resistance index of bacterial isolates from Olgbo River

S/N	Multidrug Resistance (MDR) Pattern	Number of Isolates Showing the MDR Pattern	MAR Index
1	PRL, PB, IMI, CAZ	24[a]	0.67
2	PRL, PB, TCN, IMI, CAZ	3[b]	0.83
3	PRL, TS, PB, IMI, CAZ	4[c]	0.83
4	PRL, TS, PB, TCN, IMI, CAZ	5[d]	1.0

Legend:

[a] OR111, OR121, OR141, OR211, OR221, OR231, OR241, OR321, OR122, OR132, OR142, OR232, OR312, OR322, OR332, OR342, OR113, OR123, OR133, OR223, OR233, OR313, OR333, OR343

[b] OR131, OR242, OR143,

[c] OR341, OR213, OR243, OR323

[d] OR311, OR331, OR112, OR212, OR222,

Key: PRL - Piperacillin PB - Polymyxin B IMI - Imipenem

CAZ - Ceftazidime TCN - Tetracycline TS - Cotrimoxazole

CHAPTER FIVE

5.1. DISCUSSION

Results from this study revealed a high prevalence of *Stenotrophomonas* species. Alongside some *Pseudomonas* and a single *Enterococcus* species in water from the Ikpoba and Ologbo Rivers. This microbial profile is consistent with that reported by Brooke (2012) with regards to isolates from Ikpoba and Ologbo Rivers. Adjide *et al.* (2010) also stated the isolation of *Stenotrophomonas* spp. from river water samples in Amiens, France. This suggests that *Stenotrophomonas* are common to river waters. In agreement based on the results of this study,previous studies also have reported *Pseudomonas* and *Enterobavteriaceae* spp. associated with river water samples in Ikpoba Okha LGA of Edo State. The widespread occurrence of *Stenotrophomonas* spp. in the river waters is consistent with previous observation that suggested that the organism thrives in nutrient-rich, disturbed habitats (Adegoke *et al.*, 2017). Its prevalence acted as a bioindicator, strongly suggesting that the river water was enriched with high levels of organic matter pollutants. This contamination was likely stemming from persistent sources such as untreated sewage outflow, agricultural runoff containing fertilizers and manure, or effluent from industrial processes, all of which create an ideal environment for this bacterium to proliferate (Adachi and Gonzalez, 2020).

The results point unequivocally to a polluted river ecosystem whose water quality was severely compromised and potentially unsafe for human use.

The antibiotic sensitivity profiles of the Ikpoba River and the Ologbo River isolates revealed that the bacterial isolates were completely (100%) resistant to piperacillin, polymyxin B, imipenem, and ceftazidime. This means not a single bacterial isolate could be killed by these drugs. Resistance to imipenem, a carbapenem antibiotic, was not particularly surprising as the organism (*Stenotrophomonas* species) is intrinsically resistant to the antibiotic (Looney *et al.*, 2009). However, the observation suggests that river waters are potential environmental reservoirs for carbapenem resistance genes (zu *et al.*, 2017). Similarly, resistance to Ceftazidime, a third-generation cephalosporin (3GC), and piperacillin, a broad-spectrum penicillin, indicates widespread production of extended-spectrum beta-lactamases (ESBLs) or other broad-spectrum beta-lactamases among these environmental isolates (Paterson and Bonomo, 2005). The resistance to polymyxin B, another last-resort antibiotic which *Stenotrophomonas* spp. is resistant to further highlights the organism's broad innate resistance mechanisms and limits potential treatment options (Looney *et al.*, 2009).

The most active antibiotic against *Stenotrophomonas* spp. in this research was tigecycline, to which most isolates were generally susceptible, with a smaller number showing intermediate resistance. This high susceptibility to tigecycline aligns with several other studies on environmental *Stenotrophomonas maltophilia* isolates. For example, a Chang *et. al. (*2015) on clinical and environmental isolates showed high activity of tigecycline against these organisms with a susceptibility rate exceeding 95%. A work by Zhang *et. al.* (2019) did the same for. Furthermore, a study on river water samples in China also showed that *S. maltophilia* isolates remained largely tigecycline-susceptible (Noor *et al*., 2019). The importance of this observation

cannot be overexaggerated. Tigecycline, a member of the glycylcyclines class of broad spectrum antibiotics, is usually thought to be a treatment for multidrug-resistant Gram-negatives. Because of the study's finding that TMP-SMX can still be effective in this significantly contaminated environment, it may represent a sensible option for the treatment of community-acquired *Stenotrophomonas* infections de novo from these water sources during an infectious outbreak. Nevertheless, the detection of isolates with intermediate resistance is a warning sign that careful surveillance will be required to maintain its efficacy (Brooke, 2012). In addition, cotrimoxazole (trimethoprim-sulphamethoxazole) showed moderate activity against the isolates of bacteria obtained from the two rivers. This is in accordance with established global characterization of S. maltophilia, by which Cotrimoxazole is the first-line therapy of choice for most infections. Studies from different jurisdictions, such as the large surveillance study by Gülmez *et. al*. (2010) in Turkey and Waters *et. el.* (2015) on environmental strains in United States, consistently show increased susceptibility rates to this antibiotic combination. The importance of this observation is good from a public health standpoint. Such environmental isolates remain fully susceptible to cotrimoxazole, suggesting that this available and inexpensive drug should continue to be effective for the treatment of a range of opportunistic infections, including bacteremia or pneumonia resulting from exposure to contaminated river waters This provides a viable first-line defense option for healthcare providers in the community (Looney *et al*., 2009).

The fact that isolates IR242 and OR232, identified as *Enterococcus* spp., also displayed a multidrug-resistant phenotype suggests that the high antibiotic resistance was not confined to Gram-negative organisms in these waters but may be a common feature in the bacterial community. This observation indicated a highly contaminated environment with multidrug-resistant bacteria, and serves as a breeding ground for antimicrobial resistance, consisting a

major public health concern with potential ramifications for the entire community (Odonkor and Addo, 2011).

The multidrug resistance (MDR) patterns and Multi–Antibiotic Resistance (MAR) index show a high degree of antibiotic resistance in Ikpoba and Olgbo Rivers. All the bacterial isolates (n = 18) tested from both rivers exhibited a Multiple Antibiotic Resistance (MAR) phenotype. Resistance patterns revealed by four to six antibiotics, which coincide with previous studies describing MAR patterns in environmental bacteria but at a comparatively higher prevalence (Lateef, 2016; Paul *et al.,* 2018). A particular finding was that all the MAR indices calculated for these isolates varied from 0.67 to 1.0, well above the accepted threshold point of 0.2. Krumperman (1983), and Paul *et. al.* (2018), stated that an index above 0.2 indicates that the bacteria come from a high-risk environment with high antibiotic usage. This implies that these rivers are polluted by sources like livestock farms since antibiotic use exerts selective pressure and favour(s) resistant bacteria, as proven for effluents from abattoirs (Igbinosa *et al.,* 2019). The broad resistance profiles, including to critical drugs, can be explained by intrinsic bacterial mechanisms like mutations that reduce membrane permeability and increased efflux pump activity of efflux pumps that expel antibiotics (Navon-Venezia *et al*., 2017). The presence of isolates with an MAR index of 1.0 further supports the concept that bacteria can accumulate multiple resistance mechanisms. In conclusion, the datain this study shows that these river environments represent important reservoirs of multi-resistant bacteria, with implications for public and enviromental health.

5.2 CONCLUSION

Multidrug-resistant *Stenotrophomonas* spp. was found to be highly prevalent in this study in the Ikpoba and Ologbo rivers and poor microbiological quality of these waters due to high organic pollution. In the isolates showing 100% resistance against piperacillin, polymyxin B, imipenem

and ceftazidime. Tigecycline and trimethoprim-sulphamethoazole were the most effective antibiotics, with 64–72% sensitivity against the isolates. Multiple antibiotics resistance (MAR) index values ranged from 0.67 to 1.0, suggesting that these organisms were originating from high risk sources of contamination where there is a history of either indiscriminate or in judicious use of antibiotics. Ultimately, the study demonstrated that the Ikpoba and Ologbo rivers are important environmental reservoirs for multidrug-resistant bacteria of public health concern; therefore, these polluted waterways represent a substantial risk to public health and emphasize the pressing need for improved wastewater management practices, continuous environmental monitoring, and revised clinical strategies to address possible infections caused by these resistant pathogens.

5.3. CONTRIBUTION TO KNOWLEDGE

To the best of our knowledge, this study is the first to highlight that Ologbo and Ikpoba rivers in Benin City, Nigeria are reservoirs for multidrug-resistant *Stenotrophomonas* species, an opportunistic pathogen and clinically relevant environmental bacterium. This fills a much-needed data gap as it shows that not only are these bacteria highly prevalent in these waterways, but also concerning antimicrobial resistance profiles. All isolates were resistant to four antibiotic classes representing critical priority targets, including last-resort drugs imipenem and polymyxin B, indicating a serious public health threat. On the other hand, as it confirms that tigecycline and cotrimoxazole are still two of the most effective antibiotics against these local environmental strains, provides valuable practical insight for clinicians in terms of decisions about possible treatment. Additionally, the estimated high MAR indices provide conclusive evidence that this resistance driving pollution emanates from high-risk sources such as hospital or agricultural effluents where antibiotics are heavily applied. This research thus changes the understanding of these rivers from a generally polluted state to an area with hotspots for certain more treatment

resistant pathogens, providing critical information for environmental monitoring and public health policy and clinical practice in the region.

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APPENDIX

Table 1. Antibiotics sensitivity profile of the selected bacterial isolates from Ikpoba River

S/N	Isolate code	Piperacillin	Cotrimoxazole	Polymyxin B	Tigecycline	Imipenem	Ceftazidime
1	IR111	R	S	R	S	R	R
2	IR121	R	S	R	S	R	R
3	IR131	R	S	R	S	R	R
4	IR141	R	S	R	S	R	R
5	1R211	R	S	R	R	R	R
6	IR221	R	S	R	S	R	R
7	IR231	R	S	R	S	R	R
8	IR241	R	S	R	S	R	R
9	IR311	R	R	R	I	R	R
10	IR321	R	S	R	I	R	R
11	IR331	R	S	R	S	R	R
12	IR341	R	I	R	S	R	R
13	IR112	R	R	R	S	R	R
14	IR122	R	R	R	R	R	R
15	IR132	R	S	R	R	R	R
16	IR142	R	R	R	S	R	R
17	1R212	R	S	R	I	R	R
18	IR222	R	S	R	S	R	R
19	IR232	R	R	R	S	R	R
20	IR242	R	S	R	S	R	R

21	IR312	R	R	R	S	R	R
22	IR322	R	S	R	R	R	R
23	IR332	R	S	R	S	R	R
24	IR342	R	S	R	S	R	R
25	IR113	R	S	R	R	R	R
26	IR123	R	S	R	S	R	R
27	IR133	R	S	R	S	R	R
28	IR143	R	S	R	S	R	R
29	1R213	R	S	R	S	R	R
30	IR223	R	S	R	I	R	R
31	IR233	R	R	R	R	R	R
32	IR243	R	R	R	S	R	R
33	IR313	R	R	R	S	R	R
34	IR323	R	S	R	S	R	R
35	IR333	R	S	R	S	R	R
36	IR343	R	I	R	S	R	R
	Susceptible	*0*	*25*	*0*	*26*	*0*	*0*
	Resistant	*36*	*9*	*36*	*6*	*36*	*36*
	Intermediate	*0*	*2*	*0*	*4*	*0*	*0*

Table 2. Antibiotics Sensitivity Profile of Bacterial Isolates from Ologbo River

S/N	Isolate code	Piperacillin	Cotrimoxazole	Polymyxin B	Tigecycline	Imipenem	Ceftazidime
1	OR111	R	S	R	S	R	R
2	OR121	R	S	R	S	R	R
3	OR131	R	S	R	R	R	R
4	OR141	R	S	R	S	R	R
5	OR211	R	S	R	S	R	R
6	OR221	R	S	R	I	R	R
7	OR231	R	I	R	S	R	R
8	OR241	R	S	R	S	R	R
9	OR311	R	R	R	R	R	R
10	OR321	R	I	R	S	R	R
11	OR331	R	R	R	R	R	R
12	OR341	R	R	R	S	R	R
13	OR112	R	R	R	R	R	R
14	OR122	R	S	R	S	R	R
15	OR132	R	S	R	S	R	R
16	OR142	R	S	R	S	R	R
17	OR212	R	R	R	R	R	R
18	OR222	R	R	R	R	R	R
19	OR232	R	S	R	S	R	R

20	OR242	R	I	R	R	R	R
21	OR312	R	S	R	S	R	R
22	OR322	R	S	R	S	R	R
23	OR332	R	S	R	S	R	R
24	OR342	R	S	R	S	R	R
25	OR113	R	S	R	I	R	R
26	OR123	R	S	R	I	R	R
27	OR133	R	S	R	S	R	R
28	OR143	R	S	R	R	R	R
29	OR213	R	R	R	I	R	R
30	OR223	R	S	R	S	R	R
31	OR233	R	I	R	S	R	R
32	OR243	R	R	R	S	R	R
33	OR313	R	S	R	S	R	R
34	OR323	R	R	R	S	R	R
35	OR333	R	S	R	I	R	R
36	OR343	R	S	R	S	R	R
	Susceptible	*0*	*23*	*0*	*23*	*0*	*0*
	Resistant	*36*	*9*	*36*	*8*	*36*	*36*
	Intermediate	*0*	*4*	*0*	*5*	*0*	*0*