Investigation of Bacterial Load and Their Antimicrobial Susceptibility in an Artisanal Refining Environment

ABSTRACT

Background: Bacteria present in the atmosphere often show predicable patterns across space and time. and these patterns and properties of the bacteria can be affected by presence of soot which is generated by artisanal refining and excessive burning of fossil fuel. These bacteria are being inhaled by humans on daily basis and this can have detrimental effects on human health and the environment..

Aim: This work was carried out to investigate the microbial load and antimicrobial susceptibility of an environment associated with artisanal refining activities.

Methodology: **The** **eight** samples were taken randomly from four different locations in a high artisanal refining state Rivers State (Ojoto Roundabout, Nembe Waterside, Rumuokalagbor Village, Rivers State University Teaching Hospital (RSUTH), Rivers State University Microbiology laboratory and Mile 1 Park) all in Port Harcourt, Rivers State and compared to two locations from another state Kano state (No. 33 Lamido Crescent and God is Good Motors Park, Kano State) without artisanal refining activities all in Nigeria and tested for viable bacteria load. The six test and two control samples were collected on prepared dry nutrient agar exposed to free air for a period of five (5) minutes and were covered properly and transferred to the laboratory and incubated at 37^{O}C for 24 hours. The isolates were morphologically and biochemically determined and identified.

Results: The Total Heterotrophic count indicates that samples from Rumuokalagbor village have a high number of bacteria growth colonies with a colony forming unit of 1.43×10^{6} while sample from Rivers State University Teaching Hospital had lesser colony forming unit of 7.5×10^{5},. However, the Total Heterotrophic Bacteria Count from our control is seen to be very low with 3.2×10^{5} and 2.8×10^{5} respectively. Microorganisms such as *Staphylococcus aureus*, *Bacillus species* and *Staphylococcus species.* were identified from the various locations. Few isolates were gotten from the entire laboratory with a total of 22 isolates, 18 *Bacillus species* (77), 3 *Staphylococcus species* (18%) and *1 Staphylococcus aureus* (5%). The antimicrobial sensitivity results revealed Ciprofloxacin (77%) having higher sensitivity followed by Levofloxacin (66.6%). Norfloxacin (0%), Rifampicin (0%) and Ampiclox (0%) were seen to be highly resistant to the bacteria isolated.

Conclusion: This work was able to identify *Bacillus species*,*Staphylococcus species and Staphylococcus aerues*. as bacteria associated with artisanal refining at the different sampled sites. Strict implementation on stopping artisanal refining in our communities is recommended to reduce the public health risk posed by soot inhalation.

Key words: Bacteria, soot, artisanal, refining, fossil fuel.

1. INTRODUCTION

The process of procuring stolen crude oil and further refining them in the so-called bush refineries with the use of local resources and skills (drawing on the indigenous technology used to distil locally made gin – popularly described as ogogoro or kaikai) is known as artisanal refining. Illegal skills are usually employed with crude oil being boiled in welded metal pipes and drums and the resultant fumes collected, cooled and condensed in tanks to be used locally for lighting, energy or transport [1].

Illegal bunkering as it is popularly called in Nigeria is an organized 'theft of crude oil from product pipelines through the use of improvised passage and direct pumping from oil well heads into barges by criminal syndicates [2]. Crude oil is transported

around the creeks with the help of boat yards, fire wood and other neccessities are also supplied to the camp by local women in the community Many of the people involved in illegal bunkering work for local markets, poorly refining products for communities desperate for affordable sources of energy are provided [2]. Although the economic impact of oil theft associated with artisanal refineries has been widely reported [3], the impact of the operations of these refineries on the highly sensitive environment of the Niger Delta is scarcely reported [4].

According to Ogbuagu *et al.,* [5], it has been reported that petroleum refining contributes wastes into the environment some of which could contain toxic components such as the polynuclear aromatic hydrocarbons (PAHs), which have been reported to be the real contaminants of oil and most abundant of the main hydrocarbons found in the crude oil mixture [6]. Once introduced into the environment, PAHs could be stable for as short as 48 hours (naphthalene) or as long as 400 days (fluoranthene) in soils [7]. They thus, resist degradation and, remain persistent in sediments and when in organisms, could accumulate in adipose tissues and further transferred up the trophic chain or web. Different studies [8, 9] agrees that acute exposures to aromatic hydrocarbons, which are common constituents of oil, are known to cause respiratory symptoms and high molecular weight PAHs are of significant concern because of the mutagenicity, carcinogenicity and bioaccumulation in organic tissues due to their lipophilic character [10].

Gasoline is a complex mixture of hydrocarbons and other chemical compounds used as fuel for spark-ignition internal combustion engines, primarily in light duty transportation vehicles. As of an increase of an increase in population, with a resultant increase in vehicular and industrial activities, it is in high demand in developing countries. Moreover, refineries are producing at below installed capacities or are not functioning at all, which has resulted in the inability to refine enough gasoline to meet local consumption. In artisanal refining, crude oil is boiled at atmospheric temperature; the resultant fumes are condensed and collected in tanks and used locally as automotive fuel. This local refining skill is believed to have been drawn from indigenous technology and this activity in the Niger Delta is increasing [11, 12]. The artisanal refineries operating in the creeks of the Niger Delta, though illegal, provide employment to the locals as well as bridge the gaps in the availability and supply of refined petroleum products in the oil-bearing communities of the region [12, 13]. Nigerian National Petroleum Company (NNPC) in its report stated that Nigeria is not currently refining crude oil and therefore the corporation distributes only imported petroleum products in the country.

Though gasoline produced by artisan refiners is not tested well enough to certify its compliance to any local or international set parameters; it still relieves the effect of gasoline scarcity. Makeshift techniques are used by artisan refiners in processing the raw crude oil, via thermal cracking, into useful products. These procedures could be unsophisticated and not very safe however, it could be effective.

The petroleum fractions obtained by local refiners are skeptically referred to as “bunkering oil” or adulterated products. Indigenous innovation and creativity in harnessing our natural resources should be appreciated, regulated, and the products assessed if they meet local and international specifications. Also, there is a need to assess the level of quality compliance of the gasoline samples distributed in the area to guard against environmental pollution and engine malfunctioning.

According to Vempatapu & Kanaujia [14], physicochemical properties like distillation profile, research octane number (RON), motor octane number (MON), and Reid vapor pressure are frequently used to detect the adulteration and quality of gasoline. It is on this basis that this research was designed to compare the physicochemical properties of regular automotive gasoline and locally refined gasoline,identify the bacteria present in the atmosphere as a result of these actions and the effect it will have on human health as well as antimicrobial susceptibility of these bacteria. The aim of this study is to investigate the microbial load and antimicrobial susceptibility of an environment associated with artisanal refining activities.

2. MATERIALS AND METHODS

2.1 Study Location

The study area is in Port Harcourt metropolis of Rivers State. It is a coastal city located in the Niger Delta region of Nigeria. It lies on latitudes $04^{o}45^{o}$ and 55^{o} North and Longitude 06^{o} and $70^{o}50^{o}$ East, with a land mass of 109,966 square kilometers which is about 0.95% of Rivers States land mass of 10,432,281 square kilometers [15]. The air samples were collected from four different points that were identified using the Geographical Positioning System (GPS) in a State(Rivers State) with high incidence of artisanal refining activities in Nigeria [16]. The four samples were collected from four locations identified as Ojoto Roundabout (Lat:4.78^{O}, Lng:6.99^{O}), Nembe Waterside (Lat:4.75^{O}, Lng:7.03^{O}), Rivers State University Teaching Hospital (Lat: 4.77^{O}, Lng:7.05^{O}) and Rumuokalagbor Village (Lat:4.81^{O}, Lng:7.01^{O}) all in Port Harcourt, Rivers State and compared to two areas without such activities in Kano State, No. 33 Lamido Crescent and God is Good Motors Park, Kano State, all in Nigeria. The choice of these two states is as a result of the high incidence of artisanal refiningin Rivers State and Kano State not undergoing such processes and their maps are shown in **figure 1** and **2** below.

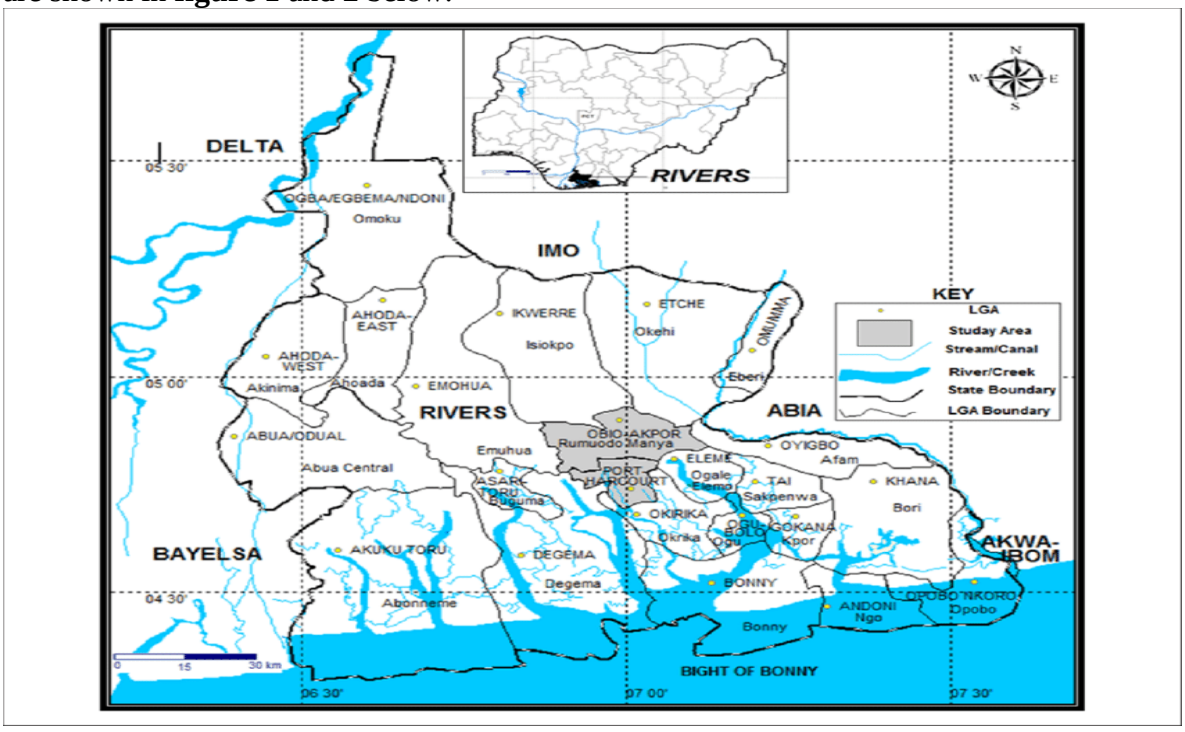

Figure: 1 Map of Rivers State showing the position of Port Harcourt City.

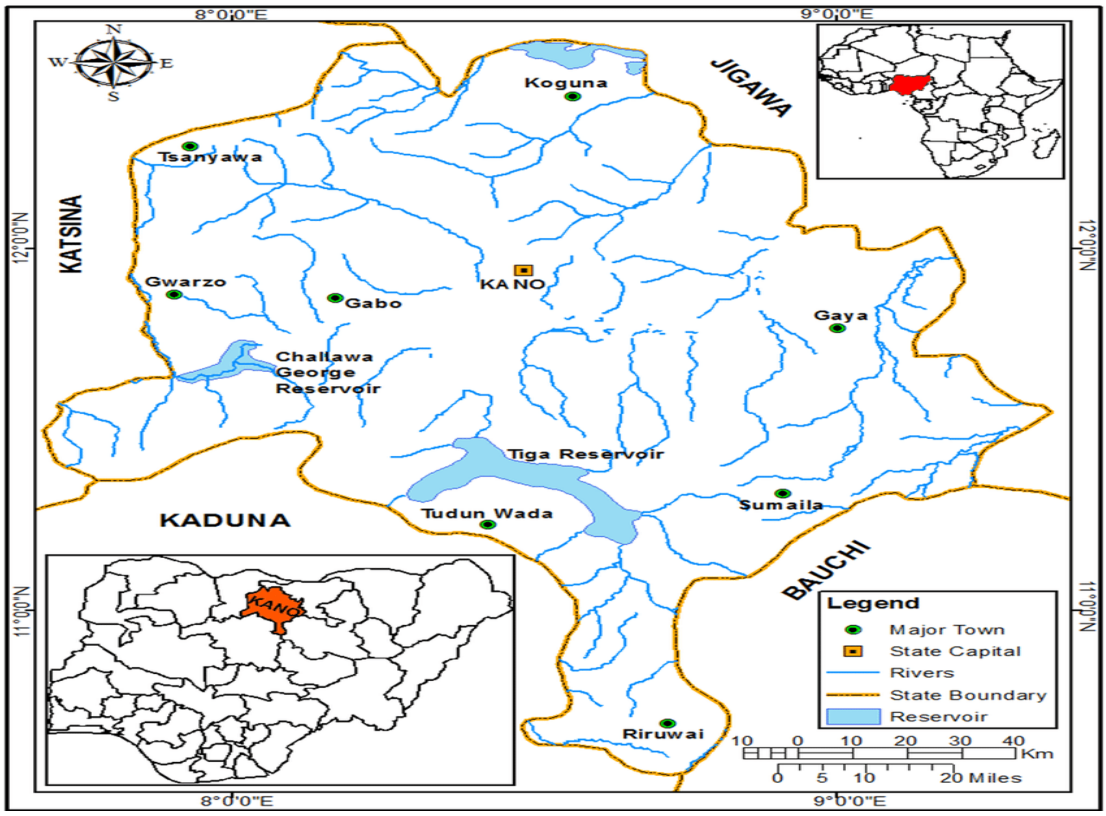

Figure: 2 Showing the Map of Kano State.

2.2 Sample Collection and Processing

The experimental sample used in this research was air. Air samples were collected at Ojoto round about, Nembe Waterside, Rivers State University Teaching Hospital (RSUTH), Rumuokalagbor Village, Rivers State University Microbiology Laboratory, Mile 1 Park all in Port Harcourt, Rivers State and No. 33 Lamido Crescent Kano State and GiG Park, Kano State, all in Nigeria. The different locations were chosen to ascertain the microbial content in the atmosphere.

2.3 Media Preparation

2.3.1 Nutrient Agar Preparation

The nutrient agar plate was prepared according to the manufacturer's specification by weighing out the required grams of the powder and dissolving it in the required volume of water. All components of the mixture were fully dissolved by heating in an autoclaved at 121^{O}C for 15 minutes, this was poured into petri dishes after cooling and allowed to solidify[17].

2.4 Sample Inoculation

During the dry season, one sterile nutrient agar plate each was taken to the four identified locations and also to the two control areas and exposed for five minutes at a distance of one kilometre from artisanal activities; they were carefully closed and

transported in an air tight cold chain bag to avoid contamination before incubation in an incubator for 24 hours at 37^{O}C. The plates were checked for bacteria growth the next day and they were counted and recorded. The isolates were sub-cultured on sterile Nutrient agar plates and were used for biochemical tests and bacteria identification.

2.5 Biochemical Test

2.5.1 Gram Stain

The most important procedure in microbiology is the gram-staining technique. The different procedure separates most bacteria into groups of basic cell wall composition. Gram positive bacteria with thick layer of peptidoglycan, 90% of cell wall stains purple while gram negative bacterial with thin layer of peptidoglycan, 10% of cell wall and high lipid content stains red/pink.

Procedure: Gram stain was done for each of the bacteria isolated. A drop of normal saline was placed on a grease free glass slide. With the aid of a wire loop, a small amount of the colony was picked and emulsified in normal saline. The smear was allowed to air dry. The slides were placed on the staining rack and flooded with the primary stain which is crystal violet, allowed for 60 seconds and rinsed in water. Lugol's Iodine was added and left for 60 seconds then rinsed in water. Rapid decolourization was done using ethanol and rinsed in water. It was counter stained with Safranin and allowed for 60 seconds then rinsed with water. The slides were allowed to air dry and examined microscopically using the immersion oil[17].

2.5.2 Catalase Test

Hydrogen peroxide is broken down into oxygen and water by the enzyme catalase. The presence of the enzyme in a bacteria isolate is evident when an inoculum is introduced into hydrogen peroxide and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.

Procedure: With the aid of a wire loop a small amount of colony growth was transferred onto the surface of a clean, dry glass slide. A drop of 3% H_2O_2 was dropped on the glass slide it was then observed for bubbles production[17].

2.5.3 Coagulase Test

Coagulase is an enzyme-like protein and causes plasma to clot by converting fibrin to fibrinogen. *Staphylococcus aureus* produces two forms of coagulase: bound or free. Bound coagulase (Clumping factor) is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in an alternation of fibrinogen so that it precipitates on the staphylococcal cell, causing the cell to clump when a bacterial suspension is mixed with plasma. This does not require coagulase reacting factor. Free coagulase involves use of plasma coagulase reacting factor (CRP), which is modified or derived from thrombin molecule to form a coagulase CRP complex. This complex in turn reacts with fibrinogen to produce the fibrin clot.

Procedure: The slide method was used. A drop of normal saline was placed on a clean grease free glass slide. Sterile wire loop was used to pick a colony and emulsify. A drop of fresh rabbit plasma was added to it and then mixed thoroughly. The mixture

was observed for agglutination microscopically. To confirm the presence or absence of agglutination, the mixture is covered with cover slip and examined under the microscope[17].

2.5.4 Motility Testing

Hanging drop method was used to show organisms that are motile. A ring of plasticine was applied on a clean slide. A loopful of overnight broth culture of the organism was placed in the centre of a coverslip. The ring of plasticine was carefully pressed on the cover-slip with the drip of culture in the center of the ring and not touching the slide. With a quick movement, the slide was inverted so that the cover-slip is uppermost. The preparation was examined microscopically using x10 and x100 objective[17].

2.6 Statistical Analysis

Data obtained from the study were analyzed using Microsoft Excel Package, 2019.

2.7 Antimicrobial Sensitivity Test (Kirby-Bauer disk diffusion method)

A bacterial suspension equivalent to 0.5 Mcfarland standard was prepared by picking 2 colonies from the pure culture and dissolving in physiological saline solution. The suspension was spread platted using a sterilized spreader on nutrient agar. Antimicrobial impregnated disk CPX, NB, CN, LEV, S, E, RD, CH, APX were placed on the culture medium surface using a sterilized forceps. The plates were incubated at 37^{O}C for 24 hours. After incubation, the antimicrobial's efficacy was determined by measuring the diameter of zones of inhibition and interpreted. The bacterial strains were classified as Susceptible (S) or Resistance (R) using the Clinical and Laboratory Standard Institute (CLSI) technique. t**he different antibiotics used and their and Classes are shown in** table 1

Table:1 Showing the Various Antibiotics used and their and Classes

Antibiotics	Class of antibiotics
Ciprofloxacin (10**µg)**	Fluoroquinolones
Norfloxacin(10**µg)**	Quinolones
Gentamycin(10**µg)**	Aminoglycosides
Streptomycin(30**µg)**	Aminoglycosides
Rifampicin(30**µg)**	Macrolides
Erythromycin(30**µg)**	Macrolides
Chloramphenicol(30**µg)**	Macrolides
Ampiclox(20**µg)**	Penicillin
Levofloxacin(20**µg)**	Fluoroquinolones

3. RESULTS

The total Heterotrophic Bacteria Count for the different locations where soot is present and also absent is shown in table 2. The Total Heterotrophic Count shown in table 3**,** indicates that samples from Rumuokalagbor village have a high number of bacteria growth colonies with a colony forming unit of 1.43×10^{6} while sample from Rivers State University Teaching Hospital had lesser colony forming unit of 7.5×10^{5}, even as soot particles were usually found on the benches indoor and the free air we

breathe. However, the Total Heterotrophic Bacteria Count from our control is seen to be very low with 3.2 x 10^5 and 2.8 x 10^5 respectively.

Table: 2 Showing Total Heterotrophic Bacteria Count at the different Locations

Location of isolates	Number of Colonies	Colony Forming Unit (cfu/ml)
Ojoto round about	88	8.8 x 10^5
Nembe Waterside	80	8.0 x 10^5
Rumuokalagbor Village	143	1.43 x 10^6
R.S.U.T.H	75	7.5 x 10^5
Mile 1	84	8.4 x 10^6
R.S.UMicrobiology Laboratory	52	5.2 x 10^5
Control No 33 Lamido Crescent, Kano State	32	3.2 x 10^5
GiG Park, Kano State	28	2.8 x 10^5

Key:
RSUTH= Rivers State University Teaching Hospital.
RSU= Rivers State University.
GiG= God is Good Motors.

The table:3 shows the identified bacteria from the different locations and the Gram staining results. Microorganisms such as *Staphylococcus aureus, Bacillus spp*. and *Staphylococcus spp.* were identified from various locations. Few isolates were gotten from the entire laboratory with a total of 22 isolates, 18 *Bacillus spp.* (77), 3 *Staphylococcus spp*. (18%) and *1 Staphylococcus aureus* (5%) as also seen in figure 3 whereas table 4 shows the catalase, coagulase and motility tests results carried out on the isolates. And figure 3 Shows the Percentage distribution of the Total Bacteria isolated.

The antimicrobial sensitivity results are seen in table 5 with Ciprofloxacin (77%) having higher sensitivity followed by Levofloxacin (66.6%). Norfloxacin (0%),

Rifampicin (0%) and Ampiclox (0%) were seen to be highly resistant to the microorganisms.

Table 3: Shows identified Bacteria from the different locations

Location	Isolates	Shapes	Grams Reaction	Suspected Organism
RSUTH	1st Isolate	Rod (pairs)	+	*Bacillus spp.*
	2nd Isolate	Cocci (Cluster)	+	*Staphylococcus spp.*
	3rd Isolate	Rod (pairs)	+	*Bacillus spp.*
	4th Isolate	Rod (pairs)	+	*Bacillus spp.*
	5th Isolate	Rod (pairs)	+	*Bacillus spp.*
	6th Isolate	Rod (pairs)	+	*Bacillus spp.*
RSU Microbiology LAB	7th Isolate	Rod (pairs)	+	*Bacillus spp.*
	8th Isolate	Cocci (Clusters)	+	*Staphylococcus spp.*
	9th Isolate	Cocci (Clusters)	+	*Staphylococcus spp.*
	10th Isolate	Rod (pairs)	+	*Bacillus Specie*
MILE 1 Park	11th Isolate	Rod (pairs)	+	*Bacillus spp.*
	12th Isolate	Rod (pairs)	+	*Bacillus spp.*
Rumuokalagbor Village	13th Isolate	Cocci (Clusters)	+	*Staphylococcus spp.*
Nembe Waterside	14th Isolate	Rod (pairs)	+	*Bacillus spp.*
	15th Isolate	Rod (pairs)	+	*Bacillus spp.*
Ojoto Junction	16th Isolate	Rod (pairs)	+	*Bacillus spp.*

	17th Isolate	Rod (pairs)	+	*Bacillus spp.*
	18th Isolate	Rod (pairs)	+	*Bacillus spp.*
CONTROL				
No 33 Lamido Crescent, Kano State	19th Isolate	Rod (pairs)	+	*Bacillus spp.*
	20th Isolate	Rod (pairs)	+	*Bacillus spp.*
GiG park, Kano State	21st Isolate	Rod (pairs)	+	*Bacillus spp.*
	22nd Isolate	Rod (pairs)	+	*Bacillus spp.*

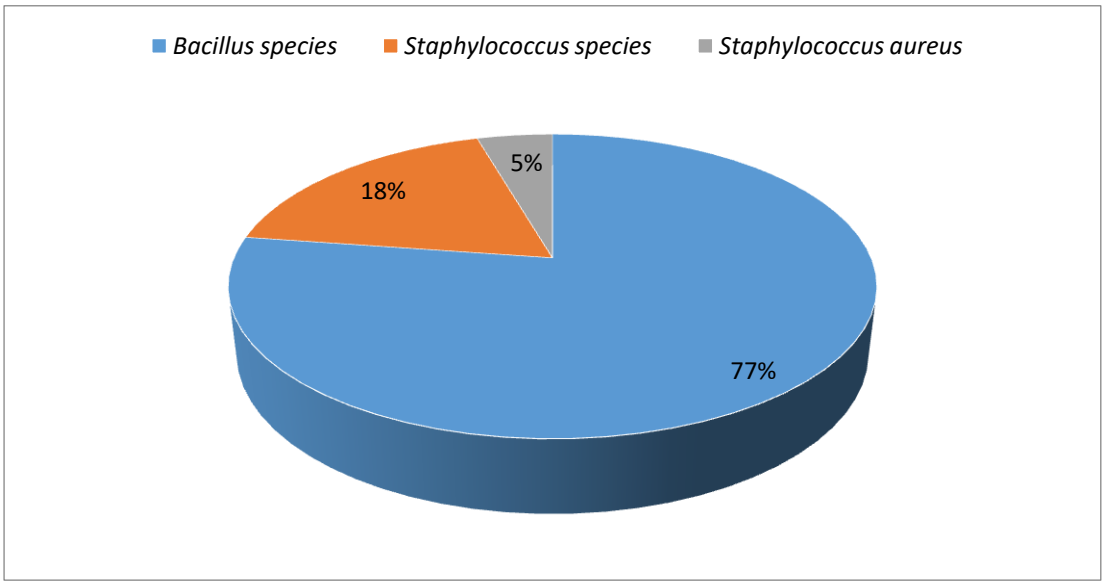

Figure 3: Shows the Percentage distribution of the Total Bacteria isolated

Table 5: Shows the Reaction Percentage of the Isolates to the Antimicrobial Agents

Isolate	Ciprofloxacin (CPX)	Norfloxacin(N)	Gentamycin (CN)	Streptomycin (S)	Rifampicin (RD)	Erythromycin (E)	Chloramphenicol (CH)	Amplicox (APX)	Levofloxacin(LEV)

1st	26mm	R	R	28mm	18mm	16mm	20mm	R	20mm
2nd	26mm	R	18mm	22mm	14mm	20mm	R	12mm	18mm
9th	16mm	R	R	R	16mm	R	R	R	18mm
10th	20mm	R	14mm	16mm	16mm	18mm	18mm	R	22mm
11th	20mm	R	R	R	18mm	20mm	14mm	R	18mm
13th	22mm	12mm	20mm	R	18mm	20mm	20mm	16mm	20mm
14th	22mm	R	R	22mm	16mm	22mm	22mm	14mm	22mm
17th	18mm	R	12mm	24mm	16mm	16mm	14mm	R	22mm
18th	24mm	R	20mm	26mm	16mm	20mm	16mm	14mm	24mm

4. DISCUSSION

Microorganisms such as bacteria are ubiquitous in nature and as such are common contaminants in our environment. These microorganisms are affected by the condition of the environment. If the environment is harsh or difficult for their survival, they will have to adapt to survive. In this study, it was observed that more bacillus species were found in the atmosphere in which artisanal refining took place and they develop spores, mostly at the terminal end and this could have adverse effects on humans in this geographical location due to the inhalation of such organisms. If the menace of soot is not controlled in the nearest future it might lead to a spike in certain new diseases which could be cardiovascular related. Studies by Mezie [18] opines that black soot released from artisanal refineries can penetrate deep into the lungs and has been associated with a variety of catastrophic health impacts in children, including premature death, heart attacks, and strokes, as well as acute bronchitis, and worsened asthma.Other researches have shown that a number of fatal diseases result from exposure to soot and its associated volatile organic compounds and prominent among these diseases are respiratory problems such as Chronic Obstructive Pulmonary Disease (COPD) and asthma while others are bronchiolitis, lung cancer and cardiovascular event[19, 20, 21]. New researches suggests that air pollution may have an effect on human health by altering the bacteria genetic makeup; it shows that hydrocarbon, a major component of crude oil that causes air pollution, dramatically changes the genetic and phenotypic composition of bacteria, which can affect their survival in the lining of airways and their resistance to antibiotics [22]. This study has

revealed the high presence of microorganisms in the soot impacted adjoining environment from artisanal refineries in the Niger Delta region.

Results from this present study have shown that from the total number of eight samples consisting of six for artisanal refining and two from areas without artisanal refining, a total of 22 isolates were obtained, 18 (77%) were *Bacillus species*, 3 (18%) were *Staphylococcus species* and 1 (5%) was *Staphylococcus aureus* (figure 3). However, it was observed that air samples collected from areas with artisanal refining activities had more microbial growth and developed terminal spores which aid their survival in air as seen in table 3.

Staphylococcus aureus are usually found in the nose, skin or throat of all healthy individuals, therefore the presence of the bacterium in air could be because of transfer of human aerosol probably from coughing, sneezing, talking and other similar mechanism. The presence of soot seen in the identified areas seems to have helped the development of these bacteria in air.

Morrissey *et al*., [23] opined that the effects of hydrocarbon on bacteria, "organisms central to ecosystems in humans and in the natural environment, are poorly investigated or studied". It is therefore important for scientist to do more ground breaking studies on how soot affects the microbial world.

The reaction of the isolates exposed to some antimicrobials have also shown that these bacteria can still react to some commonly available antimicrobials if they become infective in humans. Although the presence of spores by the organisms could bring about the problem of resistance. Similar studies by Amala et al [24] reveals that the presence of the soot induces resistance with increase in exposure time and

soot concentration.

5. Conclusion

The presence of soot caused by artisanal refining in Port Harcourt, Rivers State, Nigeria causes an increase in bacterial growth and development of spores that causes an increase in the lifespan of the bacteria and is considered not to be safe for humans. These bacteria isolates include *Bacillus sp.*, and *Staphylococcus aureus* and other *Staphylococcus sp.* Therefore, strict implementation on stopping artisanal refining in our communities is recommended. This could go a long way in reducing public health risk posed by such exposures to the air we breathe. Further prolonged study should be conducted using molecular based techniques for further identification of the organisms present in air samples. The Government should put policies in place to stop artisanal refining activities which increases the presence of soot.

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