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Enumeration and identification of gram negative bacteria present in soil underlying urban waste-sites in southwestern Nigeria

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Abstract: *Samples of soils underlying wastes were collected from four sites representing four demographic regions of a medium sized town in southwestern Nigeria. Standard methods and reference strains of isolated bacteria were employed for identification. Evaluation of the enzymatic and biochemical reactions showed that all isolated and identified microbes were non-fermenting heterotrophic (HTB). For example, Klebsiella pnemuniae may be involved in wound infections, particularly following bowel surgery. Similarly, Pseudomonas aeruginosa can produce serious nosocomial infections if it gains access to the body through wounds or intravenous lines. From the 15 culure plates, 88 colonies with various characteristics were enumerated. They differed in aspect of viscosity and color. The bacterial species were identified by percent positive reactions while oxidative and sugar fermentation tests revealed various characteristics among the isolated strains. All of the isolates were negative for citrate utilization, gelatin liquefaction, nitrate reduction, methyl red and Voges Proskaur, motility and hydrogen sulphate production. The quantity of HTB present in an area serves as an index of the general sanitary conditions of that area. The presence of a large number of HTB, in an ecological area may be considered a liability, as it can enhance the spread of diseases and on a larger scale may enable epidemics to arise. Therefore, there is need for control of waste sites by recovery and regular germicidal sanitation.*

Key words: *Enumeration, Urban wastes, Non-fermenting, Heterotrophic bacteria*
PDF of full length paper is available online

Introduction

Solid waste generation is a function of land use and its composition is inversely proportional to the potential soil damage and bacterial contamination of the environment and public health (Lober, 1996 and Omuta, 1999; Shakibaie *et al.,* 2009; Achudume and Olawale, 2009). Thus, a healthy environment is dependent upon the maintenance of proper wastes disposal practices (Boateng, 1990). The increased population density in modern Nigeria is generating a massive amount of domestic wastes, to the extent that failure of local authorities to cope adequately with waste collection has resulted in indiscriminate dumping (Olaore, 1983). Waste sites have become sources of hazardous complex mixtures that are entering the natural environment {Nigeria Environmental Study Action Group Team (NEST, 1991)}.

There have been reports suggesting association of coliform and heterotrophs in water in the distribution system and illness in the consumer water (Hargreaves *et al*., 2001; Denn, 2004). Because of the use of coliform as pollution indicators and their common association with infectious diseases, there is considerable information concerning bacteria of fecal origin. There is less information about heterotrophic bacteria (HTB), however, as it has only been established recently that HTB can cause gastrointestinal illness and pneumonia in people exposed to multiple point-of-use/ point- of-entry (POU/POE) equipment (Denn, 2004). For example, *Klebsiella pneumoniae* so called virulence factors such as pili, serum resistance and siderophore production, is ubiquitous in nature and a commmensal organism of the gastrointestinal track, where it does not cause disease but may be involved in urinary track infection particularly in females, and may be involved in wound infections, particularly following bowel surgery. Similarly, *Pseudomonas aeruginosa* is the most important species for public health considerations, although it does not cause any effects if it is ingested. It is resistant to many antibiotics and can produce serious nosocomial infections if it gains access to the body through wounds or intravenous lines (Nandalal and Somashekar, 2007). HTB historically had been assumed to be mainly non- pathogenic (Kelly *et al*., 1999), however the growing evidence for HTB-associated illnesses indicates that the HTB levels associated with human health, risk should be determined.

The magnitude of the solid waste problem is worsening by the day (Achudume *et al*., 2007). While present data reporting cannot assess the overall prevalence of chronic exposures in the vicinity of wastes sites, studies can provide a focused environmental sampling. The major aim of such studies is to provide a reliable investigative approach that may be translated into a preventive public health approach. Therefore, the present study was undertaken to enumerate and identify bacteria in soil underlying a refuse site in wet and dry seasons that is typical of a moderate

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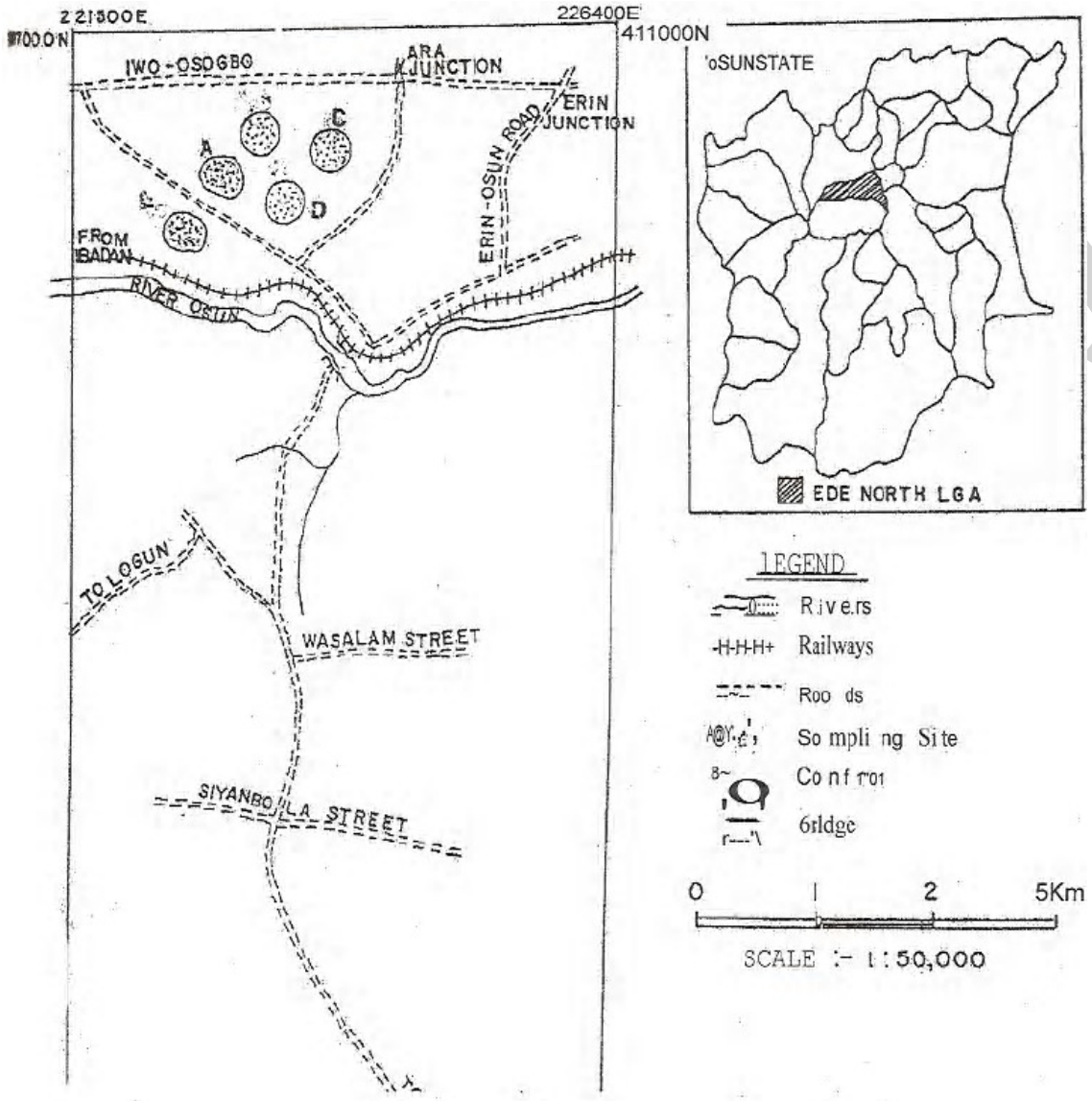

Fig. 1: Locations and sampled areas in Ede north local government of Usun State, Nigeria

urban city in Southwestern Nigeria and to discuss its possible implications.

Materials and Methods

Study area: The study area was Ede (Fig. 1) southwestern Nigeria. Ede is located on longitude 4°31 east of the Greenwich meridian, latitude 7°44 north of the equator (Ede Local Government, 1994). The topography of the region is gently undulating, dotted by low hills. This area was chosen because of its population of about 145,000 people is appropriate for our study. This population figure has a direct relationship with the volume of solid waste generated (NEST, 1991), the location is lined with shops and pretty traders and more so, there are direct telluric or anthropogenic inputs from the traders.

Sampling procedure: Single samples of soil sediment underlying solid waste were collected from the common dumping site located along the "Ara Town Junction", from four different sites during two seasons. These four sites were chosen to represent four major demographic regions of Ede (Ayolagba, 2000). Three of the sites were located along a transecting region of the dumping sites and the fourth was located in the south central region. All samples (A,B,C and D) were taken from a depth of (0.9-30cm). The samples were transferred to polythene bags approved by Nigeria's Federal Environmental Protection Agency (FEPA). These were sealed and stored in a deep freezer (-20°C). A steep hill largely prevents the dumping of waste adjacent the common site and was considered as a central point from which the first pair of reference samples was taken.

Table - 1: Prevalence (CFU g^{-1}) of heterotrophic bacteria at 37^0C in open waste site

Sampling	GMSW	THB wet	THB dry	Total/Site
A	1.00	$0.0033x10^{11}$	$1.4x10^{11}$	$1.4033x10^{11}$
B	1.00	$0.0021x10^{11}$	$1.5x10^{11}$	$1.5021x10^{11}$
C	1.00	$0.00072x10^{12}$	$6.3x10^{12}$	$6.3007X10^{12}$
D	1.00	$0.0047x10^{11}$	$7.6x10^{11}$	$7.6047x10^{11}$
*Control	1.00	$0.0015x10^{8}$	$4.5x10^{8}$	$4.5015x10^{8}$

* Significantly different ($p< 0.05$) from all open sites, GMSW = General municipal solid waste, THB = Heterotrophic bacteria

Table - 2: Identified gram negative bacteria species identified by % positive reactions

Probable microorganism(s)	Strains	% identification
Pseudomonas aeruginosa	25	32
Pseudomonas pickettii	14	23
Actinomycete erikson	10	12
Actinomycete Israeli	18	10
Neisseria pneumoniae	6	8
Klebsiela pneumoniae	8	8
Micrococcus luteus	4	6
Klebsiela oxytosa	2	2
Bacillus cereus	2	2

Filtration of samples: Ten-gram portions of soil samples were suspended in 90 ml of deionized water in a 250 ml conical flask. The samples were shaken at 150 rpm (Gallenhamp, serial no 40) at $_{3\ 7}$ ^{o}C and subjected to the filtration method by 0.45 μm membranes in a glass-100 laminated flow chamber. Filtered samples were incubated (Gallenhamp compenstat, England) aerobically at room temperature for 48 hr, thus expanding the HTB populations present in the samples (Kelly *et al.*, 1999).

Isolation and identification: Each sample was subjected to triplicate serial dilutions (10^{-1} to 10^{-3}) and then plated on heterotrophic plate count agar (MHPC) developed especially for isolation of heterotrophic bacteria from soil (Bridson, 1995). The culture plates were incubated aerobically at 37° C for 48 hr. The developed colonies were submitted to Gram stain, followed by biochemical genera and species identification tests. These tests include gelatin liquefaction, nitrate reduction, oxidative fermentation, catalase, sugar fermentation, methyl red and Voges-proskaur triple sugar iron (TSI), citrate utilization and sulphide-indole motility. All developed colonies in each plate were isolated for identification. The criteria used were colony morphology and colony colour. The total numbers of colonies found at each sampling count (CFU g^{-1}) were picked for identification (Table 1). The basic principles of the subsequent reactions, were in accordance with the biochemical and enzymatic standards described for aerobic, heterotrophic bacteria by Schreckenberger *et al.* (1999). The test microorganisms *Pseudomonas aeruginosa* NCIB 950, *Micrococcus luteus* NCIB 196, *Bacillus cereus* NCIB 6349 and *Klebsiella pneumonia* NCIB 418. In this study were provided from the culture collections of the Department of Microbiology, Obafemi Awolowo University, ILE-IFE. The evaluation of the enzymatic and biochemical reactions performed in the species level identification of the isolated colonies were carried out according to standard methods of Collins *et al.*, 1989 and Bergey's Manual of Systematic Bacteriology (Buchana and Gibbons, 1974). The biochemical tests employed in the identification system and the respective reactions are summarized below:

a. Citrate utilization test to determine citrate use ability.
b. Gelatin liquefaction test to determine growth and presence or absence of liquefaction, *i.e.* protease.
c. Potassium nitrate reduction test to determine whether aerobic conversion to nitrite is occurring.
d. Oxidative fermentation test (10% carbohydrate solution) to assess gas production.
e. Sugar fermentation test (glucose, lactose, sucrose, maltose and mannitol as substrate) to determine gas and acid production.
f. Methyl Red and Voges-Proskauer (MRVP) to test for mixed acid fermentation.
g. Triple Sugar Iron (TSI) medium to determine starch utilization.
h. Sulphide-Indole Motility (SIM) medium to determine motility, hydrogen sulphate and indole production.

Statistical analysis were made in three replicates. The analysis was based on SPSS (version10.0). In order, a one-way ANOVA test was carried out to detect the significance of differences ($p<0.05$) of variations.

Results and Discussion

More bacteria were observed in the wet season than the dry season and the samples in site C were more densely populated than were the other sites (Table 1). From the 15 cultured plates, 88 colonies with various characteristics were enumerated. They differed in aspects of: circular viscosity, with some being sharp pointed and brilliant, as well as in opacity and color, with colonies ranging from light brown to dark brown and others being-pink, white or cream colored. Table 2 summarizes the isolated bacterial species as identified by percent of the positive reactions from the isolated colonies. Table 3 summarizes the biochemical tests employed to identify gram-negative bacteria. While species in the *Pseudomonas* and *Micrococcus* genera were positive for oxidase, those in Actinomyces, *Neisseria* and *Bacillus* genera were negative. *Klesiella* bacteria showed alkalinity.

All identified bacteria were negative for citrate utilization, gelatin liquefaction (indicative of protease production), nitrate reduction (methyl red and Voges-Proskaur), motility and hydrogen sulphate production. Only Actinomyces, *Klebsilla* and *Micrococcus* bacteria were found to change sugars, as assessed by indicators of alkalinity. Actinomyces changed glucose and mannitol, while *Klebsiella* changed glucose and *Micrococcus* showed alkaline production to all the sugars.

Table - 3: Summary of the biochemical tests employed in the identification of gram- negative, non–fermenting, heterotrophic bacteria

Isolate code	Cell shape	Grams utilisation	Citrate utilisation	Gelatin liquefaction	Nitrage reducation	Oxidative fermentation (O-F)	Glucose	Maltose	Mannitol	Sucrose	Lactose	Methyl red (MR)	Voges-proskaur (VP)	Butt	Slant	Hydrogen sulphide production	Indole production	Motility	Hydrogen sulphate production	Probable organism (s)
R^1	SR	-	-	-	-	OX	NC	NC	NC	NC	NC	-	-	NC	NC	NC	-	-	-	*Pseudomonas aeruginosa*
R^2	SR	-	-	-	-	OX	NC	NC	NC	NC	NC	-	-	NC	NC	NC	-	-	-	*Pseudomonas pickettii*
R^3	LR	-	-	-	-	NONE	A	NC	A	NC	NC	-	-	A	NC	NC	-	-	-	*Actinomyces erikson*
R^4	LR	-	-	-	-	NONE	A	NC	A	NC	NC	-	-	A	NC	NC	-	-	-	*Actinomyces israeli*
R^5	COCCL	-	-	-	-	NONE	NC	NC	NC	NC	NC	-	-	NC	NC	NC	-	-	-	*Neisseria branhamella*
R^6	LR	-	-	-	-	Alk	A	NC	NC	NC	NC	-	-	A	NC	NC	-	-	-	*Klebsiela pnemoniae*
R^7	COCCL	-	-	-	-	OX	A	A	A	A	A	-	-	A	A	NC	-	-	-	*Micrococcus luteus*
H^1	LR	-	-	-	-	Alk	A	NC	NC	NC	NC	-	-	A	NC	NC	-	-	-	*Klebsiela oxytosa*
H^2	LR	-	-	-	-	NONE	A	A	NC	NC	NC	-	-	NC	A	NC	-	-	-	*Bacillus cereus*

SR: Short Rod, LR: Long Rod, A: Acid production, NC: No change (*i.e.* no acid/gas production), - : Negative, + : Positive, Alk: Alkaline COCOL = ..., OX: .....

The present analysis of microorganisms found in urban soil waste sites in Ede revealed an unexpected finding. This finding is a judgment bias in which two organisms that matches on several dimensions but are very different as perceived as more similar to each other. The description of the open waste sites can be likened to an overgrown site because the types of organisms provide a count of the general bacterial population not including the usual coliforms. Most identified strains were gram-negative, and others gram-positive, non-fermenting HTB populations. The HTB populations detected in the municipal soil samples were less dense in the dry season than in the rainy season (Table 1).

Although many of the bacterial species isolated in this study are not themselves pathogenic or directly indicative of the presence of other disease carrying organisms, the amounts of HTB at a particular distribution-sampling site may indicate deterioration in the microbiological quality in that environment. Therefore, these data can be used as an index of the general sanitary conditions of that portion of the system and may be used to determine whether and where corrective action may be needed.

The HTB strains revealed in this study are consistent with previous reports (Norton and Lechevallier, 2000; Hargreaves *et al.*, 2001; Colford *et al.*, 2002). The predominant genera were the *Pseudomonas*, *Neisseria*, *Klebsiella* and *Bacillus*. These are considered ubiquitous and, opportunistic pathogens. They can survive adverse conditions by forming spores and, becoming dehydrated. Dehydrated spores can then be packaged and sold as viable cultures (Denn, 2004). Waste, becomes a limited liability in an ecological area, augmenting the spread of ticks, mosquitoes and various flies. While most HTB are thought to be harmless in and of themselves, mounting evidence suggests that some are a health threat. The degree of the danger however is poorly characterized (Denn, 2004). Even bacterial contamination of POU/ POE treatment devices occurs because municipal water plants remove most bacteria, but do not produce sterile water. Once a carbon filter removes chlorine, bacteria can multiply. Although these bacteria are not hazardous to healthy individuals, it is possible that some of them- in addition to Legionellae and nonturbaculosis micobacteria- are opportunistic pathogens and could be hazardous to those body defences that are compromised. A new study shows that not enough is known about heterotrophic bacteria and the levels that may be associated with human health risk (Denn, 2004).

The populations at greatest potential risk of exposure are infants and toddlers (ages 0.5-5) who may ingest soil through mouthing of hands, toys and other hapten objects. Infact, it has been reported that infants and toddlers ingest 10mg to 10 g day^{-1} of soil (Lewis *et al.*, 1994). According to the present data a child who ingests 0.1 g^{-1} day of contaminated soil, would potentially be consuming an average of $1.8x10^6$ Cfu g^{-1} day^{-1} (*i.e.,* at site D with the minimum bacterial count). These ingested HTB are a real risk for small children and immuno-compromised persons who can develop pyrogenic reactions and peritonitis.

As there is no consistent pattern or manner of regulating the types and characteristics of wastes materials, prior to their incidental disposal at dumping sites, the presence of substantial numbers of HTB. Other pathogens like the prevalence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in dried materials during bedding, handling hankerchiefs etc, sweeping floor account for the entry of microorganisms in human population (Nandalal and Somashekar, 2007) and represent a significant microbial disease health risk. Epidemics may occur periodically as a result. Our data suggest that there is need for controlled dumping of wastes into the commons by source recovery and/or intermediate point sources where wastes could be held for treatment or combined for neutralization and treatment before being brought to common refuse areas. The general population faces a potential dangerous situation created by inadequate control measures and the seemingly guiltless behaviors of many individuals acting alone. The present findings emphasize that in the interest of enhancing sustainable public health; there are urgent needs for disinfection program at Ede dumping sites and for the quick removal of wastes.

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