ASSOCIATION OF HEAVY METAL TOLERANCE WITH MULTIPLE ANTIBIOTIC RESISTANCE IN BACTERIA ISOLATED FROM WETLANDS OF LAKE VICTORIA BASIN KENYA

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

Dedicated to my daughter Janis and my wife Gladys.

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ABBREVIATIONS AND ACRONYMS

AAM	American Academy of Microbiology
F-AAS	Flame Atomic Absorption Spectrometer
ANOVA	Analysis of Variance
APHA	American Public Health Association
ATCC	American Type Culture Collection
BOD	Biochemical Oxygen Demand
CCAR	Canadian Committee on Antibiotic Resistance
CFU	Colony Forming Unit
DNA	Deoxyribose Nucleic Acid

KEBS Kenya Bureau of Standards

LBDA Lake Basin Development Authority

MDa Mega Daltons

MDR Multiple Drug Resistance

MPN Most Probable Number

NCCLS National Committee for Clinical Laboratory Standards

NPHL National Public Health Laboratories

pH Potential Hydrogen

TBA Tris Boric Acid

TCBS Thiosulphate Citrate Bile Salts

TDS Total Dissolved Solids

UNEP United Nations Environmental Programme

WHO World Health Organization

ABSTRACT

A study was carried out to determine the heavy metal concentrations in the wetlands of Lake Victoria basin and their effect on bacterial resistance to ten commonly used antibiotics. Water and sediment samples for heavy metal analysis and microbiological assays were randomly collected from sewage outfalls and points bordering heavy metal activity settlements. Heavy metal analysis was done using Flame Atomic Absorption Spectrometer (F-AAS). The microbiological assays targeted heterotrophic bacteria, coliforms and enteric pathogens. The isolates were subjected to heavy metals;- mercury, nickel, chromium and copper in the laboratory to assess their levels of tolerance. Antibiotic susceptibility testing was carried out to check for antibiotic resistance. Sediment samples recorded a higher level of heavy metals with a mean range of 31.97-109.9, lead; 3.48-183.66, zinc; 3.01-17.03, nickel; 1.93-214.61, copper; 17.01-65.91, cobalt; and 2.08-25.89 mg/g chromium compared to water samples whose mean range was as follows;- lead; 0.77-0.94, manganese; 0.10-3.10, zinc; 0.23-1.16, cadmium 0.02-0.04, and copper 0.51-0.57 mg/l. There was a significant relationship in the amounts of heavy metals in water samples and the bacterial counts, $r = 0.637$, $p < 0.05$. The study

showed a significant difference in percentage tolerance to the four heavy metals tested. ($F = 4.25$, $P = 0.011$, $P < 0.05$). Mercury recorded the highest toxicity followed by chromium and nickel. Copper was the least toxic to the microorganisms. 53.8% of the total isolates showed multidrug resistance. They were significantly resistant ($p < 0.05$) to cefuroxime (67.7%), cotrimoxazole (65%), tetracycline (62.4%) and ampicillin (53%). They were however highly sensitive to gentamycin (98.9%), kanamycin (83.9%) and norfloxacin (79.6%). The study showed no significant difference in multidrug resistance in the three bacteria groups ($F = 1.75$ $P = 0.191$ $P > 0.05$). However, multidrug resistance in the enteric pathogenic bacteria (mean % multi drug resistance 65.15%) was higher than in heterotrophs (mean 46.52%) and coliform bacteria (mean 36.36%). There was a significant relationship between chromium tolerance with resistance to cefuroxime ($p < 0.000$), Nickel tolerance with resistance to cefuroxime ($p < 0.05$) and mercury tolerance with resistance to ampicillin ($p < 0.05$). Plasmid DNA finger printing of the metal tolerant and antibiotic resistant isolates showed a positive relationship in number of isolates carrying plasmids and the number having multiple drug resistance ($r = 0.372$, $P = 0.261$) suggesting that heavy metal pollution in wetlands induces multidrug resistance. The high degree of resistance to common antibiotics like ampicillin, cotrimoxazole and cefuroxime can therefore be attributed to the inflow of heavy metal rich effluent into the wetlands of the Lake basin resulting to co-selection of both metal tolerant and antibiotic resistant microbial species. This requires intervention measures to curb the potential health hazard posed by heavy metal pollution in the aquatic ecosystems.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Lake Victoria which is the second largest fresh water lake in the world occupies an area of 68,800 km^2. The Lake is shared by the three East African countries, Kenya (6%), Uganda (51%) and Tanzania (43%). In Kenya the lake and its entire basin serve as the lifeline for about two million people who derive their livelihood directly or indirectly from resources in the lake. Inflow into the lake occurs through a number of rivers which originate from both lowlands and highlands. These include Rivers Nzoia, Yala, Nyando, Kagera, Kuja, Migori and other inlets draining into the Lake. The causes of the rising pollution levels in the lake are as many as they are diverse. The lake has for along time been a sink to excess untreated effluent of both industrial and municipal origin (Nzomo, 2005).

The major towns of Kisumu, Eldoret, Kakamega, Homabay, and Kericho have malfunctional sewage plants which discharge inadequately treated sewage in rivers draining into Lake Victoria. Kisumu sewage plant at Kisat with a design capacity of 9000 cubic meters now receives 15000 cubic meters of effluent, much of which flows into the lake without treatment. Agro based industries like sugar, paper, coffee, tea, dairy, and

fish tanneries discharge semi-treated effluents with high BOD to the rivers (Nzomo, 2005). Among the pollutants into the ecosystems are heavy metals such as mercury, cadmium, chromium, lead, copper, zinc, arsenic and other chemicals which affect aquatic life including microbial communities (UNEP, 2006). Introduction of heavy metals in various forms in the environment produces considerable modifications of microbial communities and their activities (Doelman *et al*., 1994). Some of the metals like copper, cobalt, zinc nickel and molybdenum are naturally present in low concentrations in rocks, soils, waters and the atmosphere and are used as nutrients by micro-organisms. In high concentrations however they produce toxic effects. Some heavy metals like vanadium and nickel, which are constituents of crude oil, are toxic even at low concentrations (Babich and Stotzky, 1985).

Micro-organisms have evolved mechanisms to detoxify heavy metals, and some even use them for respiration. To survive under metal stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation, complexation and reduction of the metal ions to a less toxic state (Nies, 1999). These microbial interactions with metals may have several implications for the environment. The microbes may play an important role in the biogeochemical cycling of toxic metals as well as remediating metal contaminated environments.

Toxic metals in the environment lead to selective pressure among the microbial communities resulting in the development of metal resistant populations (Mahler *et al.,* 1986). There is evidence of a correlation between tolerance to heavy metals and antibiotic resistance, a global problem currently threatening the treatment of infections in plants, animals and humans (DeRore *et al.,* 1994). Resistance genes to both antibiotics and heavy metals may be located closely together on the same plasmid in bacteria and are more likely to be transferred together in the environment. Metal tolerance and antibiotic resistance in bacteria have been shown to increase proportionally along industrial contamination gradients (Osborn *et al*., 1997). It has been shown that bacterial isolates resistant to vanadium show increased resistance to quoinolones (Ciprofloxacin and Norfloxacin), which are crucial in the management of salmonella infections. The genes can be transferred to indigenous populations of microorganisms occurring frequently in the environments thereby enhancing the spread of antibiotic resistance (CCAR, 2002).

Antibiotic resistance has become a major concern as a medical and an economic problem. The dissemination of resistance traits could reduce the efficacy of drugs to only a few antibiotics and this could increase health care costs. It is estimated that resistant bacterial infections increase health care costs by four billion dollars per year in the United States (AAM, 1999).

This study was carried out to investigate the extent of metal induced drug resistance in environmental bacteria within the Lake Victoria basin with a view to promoting human health and improving the sustainable management of the lake basin resources.

1.2 Statement of the problem

Heavy metals and other chemicals that enter the environment influence the microbial structure and diversity (Pennanen *et al.*, 1996). Aquatic ecosystems serve as a sink for metal pollution loads from point and diffuse sources. Therefore heavy metal loading in the wetlands of Lake Victoria basin represents a serious health threat to the local communities. The toxic metal flux into the ecosystem occurs through mining activities, discharge of raw and inadequately treated industrial and municipal effluent, vehicle service station/garage effluent and agricultural inputs like chemical fertilizers, herbicides and pesticides.

Under such environmental conditions of metal stress, metal and antibiotic resistant populations adapt fast by spread of R-factors rather than by mutation and natural selection (Bhattacherjee *et al.*, 1988). This has potential health implications since bacteria generally considered harmless may possess simultaneous metal and antibiotic resistance determinants that can confer multi-drug resistance to pathogenic bacteria resulting in infections that are expensive and difficult to manage (Ramteke, 1997). A previous study

within the Lake Victoria basin has shown increased incidence of multiple drug resistance among Gram negative bacteria to commonly used antibiotics such as streptomycin and nalidixic acid (Boga *et al*., 2007).

1.3 Research questions

i. Does the heavy metal concentration within the Lake Victoria basin affect the heterotrophic plate count bacteria?

ii. Is there relationship between heavy metal tolerance and antibiotic resistance in environmental bacteria from the lake basin?

iii. Does the antibiotic resistance pattern in heterotrophic bacteria, enteric pathogens and coliform bacteria from the lake basin vary?

iv. How does the plasmid DNA profile pattern of the multi-drug resistant environmental bacteria from the lake basin compare?

1.4 Hypotheses

i. Heavy metal loading in the wetlands of the Lake Victoria basin has no effect on heterotrophic plate count bacteria.

ii. There is no relationship between heavy metal tolerance and antibiotic resistance in environmental bacteria from the Lake Victoria basin.

iii. Antibiotic resistance pattern in heterotrophic bacteria, enteric pathogens and coliform bacteria from the lake basin does not vary.

iv. The plasmid DNA profile pattern of the multi-drug resistant environmental bacteria does not vary.

1.5 Objectives

1.5.1 General objective

To determine the association between heavy metal tolerance and antibiotic resistance in common environmental bacteria from the Lake Victoria basin.

1.5.2 Specific objectives

i. To determine the physico-chemical parameters of the targeted wetlands of the Lake Victoria basin

ii. To determine the status of heavy metal such as Lead, Cadmium, Chromium, Zinc and Copper in selected wetlands of the Lake Victoria basin and relate their levels to the standard plate count bacteria.

iii. To isolate heterotrophic bacteria, coliforms and enteropathogenic bacteria from the environment.

iv. To establish if there is a correlation between heavy metal tolerance and antibiotic resistance in the three groups of the environmental bacteria from the lake basin.

v. To assess antibiotic resistance in heterotrophic bacteria, enteric pathogens and coliform bacteria from the lake basin.

vi. To compare the plasmid DNA profile pattern of the multi-drug resistant bacterial isolates

1.6 Significance and anticipated output of the study

i. Assist environmental authorities to develop and enforce strategies for reducing the loading of heavy metals and other chemical pollutants into the ecosystem in order to improve the quality of the environmental resources.

ii. Sensitize the stake holders in the agro based industries and the municipal council authorities on the impact of raw effluent discharge into the environment.

iii. Create awareness among the communities on the significance of environmental management and conservation in view of promoting public health.

iv. Address the increasing global concern on antimicrobial resistance by establishing the drug resistance trends in environmental bacteria to provide the basis for making policy decisions.

1.7 Scope and limitations of the study

i. Due to the vast nature of the Lake Victoria basin, this study targeted to cover three main rivers discharging their water into Lake Victoria. These included River Nyando, River Yala and River Nzoia and a few of their tributaries with the target sites being mainly sewage outfalls and some of the points suspected to be hot spots of pollution due to industrial activities.

ii. The study was aimed at establishing the heavy metal status and the microbiological quality of surface water hence the analysis did not include water from under ground sources.

iii. Higher plants and animals were not included as part of the analysis of the presence of heavy metals.

iv. The study did not take into account seasonal variations to account for the differences in physico-chemical parameters and the variations in the concentrations of the heavy metals in the wetlands as well as the differences in the microbial numbers as determined by the plate counts.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Pollution in Lake Victoria

Millions of liters of untreated sewage sludge flow into Lake Victoria everyday from major urban centres along the lake shore. This contamination coupled with chemical and fertilizer run-off from lakeside industries and agriculture have has a devastating effect. Poor sanitary and drainage conditions have led to outbreak of water borne diseases such as Schistosomiasis, cholera, diarrhea and skin diseases (UNEP, 2006).

Some areas of the rivers feeding the lake and the shoreline are particularly polluted by municipal and industrial discharges. Some information is available from local and national authorities on the scale and location of polluting industries, and there are a number of basic industries that are common to most of the major urban areas. For example, breweries, tanning, fish processing, agro processing (sugar and coffee) and

abattoirs. Some of these have implemented pollution management measures but in general, the level of industrial pollution control is low.

Small scale gold mining is increasing and this is leading to some contamination of the local waterways by mercury which is used to amalgamate and recover the gold (UNEP, 2006). Some traces of other heavy metals such as chromium, cadmium and lead are also found in the lake although the problem has not yet reached major proportions.

Rivers draining in Lake Victoria are heavily laden with nutrients, organic and sediment pollution loads. Rivers Nzoia, Nyando and Kisat are more polluted than others (Nzomo, 2005). The major sources of the lake pollution can be categorized into three:-

Industrial pollution

This occurs mainly from agro based industries – Sugar, Paper, Coffee, Tea, Dairy, Fish tanneries which discharge semi- treated effluents with high BOD to rivers (Table 1). This is coupled with poorly maintained waste treatment plants releasing liquid wastes that don't meet stipulated standards.

Table 1: Data from industrial wastes

INDUSTRY	DATE SAMPLED	TEMP ^{o}C	pH	COND µs/cm	TDS mg/l	BOD mg/l
Mumias Sugar Company	12-2-01	25.6	7.8	1064	686	180
Chemelil Sugar Company	17-2-01	26.7	6.2	1142	549	670
East Africa Sugar Industries (Muhoroni)	17-2-01	28.3	6.8	955	459	350

Webuye Panpaper Millers	13-2-01	24.5	8.9	1440	786	240
Agro-Chem & Food Allied Company	17-2-01	27.6	7.6	8350	4650	3850

SOURCE OF DATA: LBDA Water Quality Surveillance Reports (2001).

Municipal pollution

The major towns of Kisumu, Eldoret, Kakamega, Homabay and Kericho have malfunctional sewage plants (Table 2). These release effluents into the rivers. River Kisat is heavily loaded with liquid wastes from Kisumu sewage plant and discharges the raw effluent directly into the lake.

Table 2: Data from municipal wastes

INDUSTRY	DATE SAMPLED	TEMP ^{o}C	pH	COND µs/cm	TDS mg/l	BOD mg/l
Kisumu Conventional Treatment Plant	11-2-01	27.2	7.5	855	410	200
Eldoret Sewage Lagoons	12-2-01	20.9	7.8	90	42	132
Kericho Conventional Treatment Plant	18-2-01	20.4	8.17	708	335	40
Kakamega Sewage Lagoons	14-2-01	25.4	9.3	237	112	80
Homa Bay Sewage Lagoons	19-2-01	25	8.5	881	420	100

SOURCE OF DATA: LBDA Water Quality Surveillance Reports (2001).

Land use pollution

Nutrients and sediment loads from agricultural fields are deposited in the rivers and washed to the lake. Nitrogen and phosphorus input is the major cause of progressive

eutrophication in the lake. Total phosphorus and total nitrogen input in the lake stand at between 3,860 and 77,200 tonnes respectively per year (Nzomo, 2005).

2.2 Microbial interaction with metal pollutants

Toxic metals are among the most problematic wastes loaded in the environment primarily through indiscreet disposal of chemical wastes from industrial, agricultural and mining activities. This results in surface contamination and subsequent transport to ground water (Cornish *et al.,* 1995). The non-biodegradability of heavy metals is responsible for their persistence in the environment and subsequent bioaccumulation in the food chain (Ishibashi *et al.,* 1990). Microbiological activity is of primary importance in the bioremediation of metal contaminated ecosystems as microbial communities represent substantial biomass and play major roles in virtually all biogeochemical pathways (Gadd, 1997).

The structure and diversity of microbial communities is known to change in the presence of elevated levels of heavy metals as the communities adapt to pollutant loads (Babich & Stotzky, 1985; Pennanen *et al.,* 1996). In naturally polluted environments, the response of microbial communities to heavy metals depends on the concentration and availability of the metals and is also dependent on the actions of complex processes controlled by multiple factors such as the type of metal, the nature of the medium and the microbial species (DeRore *et al.,* 1994; Hachemi *et al.,* 1994).

Several studies have found that heavy metals influence micro-organisms by adversely affecting their growth, morphology and biochemical activities, resulting in decreased biomass and diversity (Barkay *et al.,* 1985; Mahler *et al.,* 1986; Heggo and Angle, 1990). Heavy metals generally exert an inhibitory action on microorganisms by blocking essential functional groups. For example, mercury compounds affect metabolism by binding SH-groups of proteins (Brock and Madigan, 1991) and interfering with membrane transport (Huges and Poole, 1989). Other inhibitory mechanisms include displacement of essential metal ions and modification of active conformations of biological molecules (Doelman *et al.,* 1994; Gadd and Grifths, 1978).

It was demonstrated that copper and silver blocked an enzyme system involved in respiration (Liebe and Stuchr, 1972; Rahn *et al.,* 1973). At relatively low concentrations, some metals like copper, cobalt, zinc, nickel are essential for microorganisms since they provide vital cofactors for metallo-proteins and enzymes (Eiland, 1981).

2.3 Metal tolerance mechanisms

Resistance mechanisms to toxic metals are known to exist (Rouch *et al.,* 1995). Metal resistance in bacteria is controlled by resistance genes that are found on chromosomes, plasmids and transposons. Although plasmid mediated resistance is the most common, other resistance systems have been described (Bruins *et al.,* 2000). For instance mercury resistance in *Bacillus* spp, cadmium efflux by p-type ATPase also in *Bacillus* spp *and* arsenic efflux in *E.coli* are chromosomal metal resistance systems (Silver and Phung, 1996).

There is considerable evidence that micro-organisms can rapidly adapt to the toxic metals by altering their chemistry and mobility (Beverigde and Doyle, 1989; Lovley, 1994). The mechanisms of resistance include metal reduction or transformation to more volatile or less toxic forms. Some bacteria including *Pseudomonas*, *E.coli* and *Clostridium* enzymatically reduce Hg^{2+} to Hg^{0} which is highly volatile and diffuses away from the bacterial cell. Others have specific metal efflux systems, which are the most commonly found mechanism of plasmid mediated metal resistance (Silver, 1992). Other microbes tolerate metals through binding by extracellullar polysaccharides (precipitation and exclusion) mediated by production of low molecular weight binding proteins such as phytochelatins (Angle and Chaney, 1989). Chelation and complexion of metal species with the media components and organism induced pH changes can also contribute to metal tolerance. Chromate tolerance is achieved through methylation, reduction and precipitation at the cell surface (Wood and Wang, 1983), blocking cellular uptake by altering the uptake pathway and removal from cytoplasm by efflux pumps. In many cases these responses appear to be plasmid mediated (Silver *et al.,* 1989).

Cadmium binds to sulfhydryl groups on essential proteins thus interfering with important cellular functions (Nies, 1992). It can also cause single-stranded breakage of DNA in E.*coli* (Trevors *et al.,* 1986). Two cadmium plasmid mediated resistance efflux systems, that is, *cad* in *Staphylococcus aureus* and *czc* in *Acinatobacter eutrophus* are well characterized and documented (Smith and Novick, 1972; Nies, 1992). The *czc* system

also confers resistance to zinc and cobalt. Another system that confers resistance to zinc and cobalt that is chromosomally encoded is known to exist (Bruins *et al.,* 2000). Cadmium resistance genes located on transposons have been reported in *Listeria monocytogenes* (Lebrum *et al.,* 1994).

Mercury resistance is widely distributed among Gram positive and Gram negative bacteria. Some bacteria have the Hg (II) (*mer*) resistance operon. The *mer* operon detoxifies Hg (II) transports, and self regulates mercury resistance (Misra, 1992; Ji and Silver, 1995). Resistance to mercury is based on its redox potential and its low vapour pressure. Resistant bacteria are able to reduce Hg^{2+} to metallic mercury (Hg^{o}), which does not remain in the cell but leaves the cell by passive diffusion (Silver and Phung, 1996).

Bacteria tolerance to heavy metals has been reported in both Gram positive and Gram negative bacteria (Foster, 1983). It is generally believed that Gram positive bacteria are less tolerant to heavy metal stress than Gram-negative bacteria. Some bacterial species like *Bacillus* spp may be resistant owing to their ability to sporulate, *Corynebacterium* spp have unique membrane lipids that can protect the cells from environmental stress (Beverigde and Doyle, 1989).

Resistance of bacteria to heavy metals is conditioned by the presence or absence of the metal in the environment as the absence of metals from the environment reduces

noticeably its power of resistance (Timoney *et al.,* 1978). Baath (1989) found that although exposure to metals enhances resistance, the fact that resistant bacteria are found in environments never exposed to high concentrations of heavy metals indicates that heavy metal tolerant species already exist in non- pollutant habitats.

2.4 Microbial resistance to antibiotics

Antibiotic resistance is the ability of a microorganism to resist the effects of an antibiotic to which it is normally susceptible. It evolves by natural selection, plasmid transfer and mutation. The use of antibiotics gives a selective advantage to resistant bacteria and over time the population is composed of entirely resistant strains. Treatment of these resistant strains with antibiotics becomes ineffective (Laxminarayan and Brown, 2001)

Antibiotic resistance genes are normally carried on chromosomes, plasmids or conjugative transposons. Resistance genes are acquired through conjugation from antibiotic producers (Saylers and Whitt, 1994). Plasmids are autonomous genetic elements that replicate independently of the main chromosome. Plasmids carry genes that govern their replication, segregation and copy number as well as additional phenotypic functions such as antibiotic resistance, metal resistance, synthesis of bacteriocins and ability to mediate cell to cell conjugation among others (Silver, 1992).

Antibiotic resistant bacteria can transfer resistance to indigenous bacteria through plasmids or conjugative transposons. Transfer of plasmids occurs within or between species (Mach and Grimes, 1982). Conjugative transposons are located in the bacterial chromosome and can transfer themselves from the chromosome of the donor bacteria to the chromosome of the recipient bacteria. Conjugative plasmids also integrate within plasmids. Conjugative plasmids can transfer antibiotic resistance among species within Gram positive or Gram negative bacteria as well as between Gram positive and Gram negative bacteria. Unlike plasmids, conjugative plasmids are not easily detected. Plasmid transfer is believed to be responsible for the geographical spread of antibiotic resistance (Laxminarayan and Brown, 2001).

Sensitive microorganisms resist antibiotics by modification of the target reaction so that it no longer responds to the antibiotic or cause a change that prevents the antibiotic from reaching the target reaction. Some bacteria produce enzymes that destroy the antibiotics activity. Resistance to β-lactams, aminoglycosides and tetracyclines is achieved by enzymes whose activity inactivates the antibiotic (Saylers and Whitt, 1994; Mckane and Kandel, 1996). A modification in the plasma membrane wall may reduce its permeability to an antibiotic (Mckane and Kandel, 1996).

Some bacteria have developed the ability to actively pump the antibiotic out of the cytoplasm. This is termed as the efflux mechanism, which was first described in bacteria that were able to pump tetracycline out of their cells (Salyers and Whitt, 1994). Antibiotic

resistance is also acquired when a structure that is normally attacked by an antibiotic is modified so that it is no longer recognized by the drug. Bacteria resistant to streptomycin produce modified ribosomes to which the antibiotic cannot bind (Mckane and Kandel, 1996). Antibiotic resistance can also be achieved by by-passing the metabolic step inhibited by the drug.

A microorganism which develops cross-resistance becomes insensitive to all related antibiotics (Mckane and Kandel, 1996). The multiple antibiotic resistance (*mar*) locus of *E.coli* and *Salmonella* is one of the most researched and understood chromosomal multiple drug resistance systems (Randall and Woodward, 2002). The *mar*-locus is involved in resistance to antibiotics such as chloramphenicol, cephalosporins, nalidixic acid and flouroquinolones, penicillins, puromycin, rifampicin and tetracycline (Cohen *et al.,* 1989). Development of antibiotic resistance is associated with the use of antimicrobial agents in human medicine, veterinary medicine, animal husbandry, plant agriculture, aquaculture and with environmental contamination by industrial effluents (CCAR, 2002).

An increasing number of diseases are resisting treatment due to the spread of drug resistance within bacterial populations. This poses a serious threat to successful treatment of many microbial infections (Ramteke, 1997; Bhattacherjee *et al.,* 1988). For example gonorrhea caused by *Neisseria gonorrheae* was first treated successfully with sulfonamides in 1936 but by 1942 most strains had developed resistance and physicians

turned to penicillin. However, within 16 years a penicillin resistant strain had emerged in the Far East. (Harley and Prescott, 1996).

There is increasing concern over the use of bactericides, disinfectants and antiseptics in the community and health care facilities due to their potential to induce the expression of antimicrobial resistance genes which encode multi-drug efflux pumps and their regulators (CCAR, 2002). This mechanism almost always causes resistance to a wide variety of antibiotics especially in *E.coli*, *Salmonella*, *Pseudomonas* spp and other bacterial species (Moken *et al.*, 1997).

Rapid spread of genes resistant to antibiotics can occur in a bacterial population and from one ecosystem to another (Witte, 1997). Particular antibiotic resistance genes first described in human specific bacteria have been found in animal specific species of microorganisms and vice versa, suggesting that bacterial populations can share and exchange these genes (Sundin and Bender, 1996; Sternberg, 1999).

2.5 Correlation of metal tolerance and antibiotic resistance

Bacterial resistance to antibiotics and other antimicrobial agents is an increasing problem in today's society (AAM, 1999). Our current antibiotics are becoming less useful but used more heavily against resistant pathogenic bacteria, infectious diseases are becoming more difficult and more expensive to treat. This is due to creation of selective pressure in

the environment that leads to mutations in microorganisms that allows them better to survive and multiply (Baquero *et al.,* 1998).

According to Lawrence's (2000) discussion of the Selfish Operon Theory, clustering of genes on a plasmid, if all the genes clustered are useful to the organism interms of survival of its species, those genes are more likely to be transferred together in the event of conjugation. In an environment with multiple stresses, for example antibiotics and heavy metals, it would be more ecologically favorable, for a bacterium to acquire resistance to both stresses. If the resistance is plasmid mediated, those bacteria with clustered resistance genes are more likely to simultaneously pass on those genes to other bacteria, and those bacteria would then have a better chance of survival. In such a situation, one may suggest an association of antibiotic resistance with metal tolerance.

Association between resistance to antibiotics and heavy metals has been reported by several workers where genes encoding resistance to metals were located on transmissible plasmids (Novick and Roth, 1968; Scottel *et al.,* 1974; Dhalkephalker and Chopade, 1994). Genes that code for antibiotic resistance traits and genes that code for metal resistance are often carried on the same plasmids or mobile genetic elements (Wireman *et al.,* 1997). Transferable plasmids encoding resistance to various heavy metals and antibiotics in *Salmonella abortus equi* have been reported (Ghosh *et al.,* 2000).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 The study area

Lake Victoria with a surface area of 68,800 km^2 and an adjoining catchment of 184,000 km^2 is the world's second largest body of fresh water, and the largest in the developing world, second only to Lake Superior in size. Lake Victoria touches the equator in its northern reaches and is relatively shallow, reaching a maximum depth of about 80 m and an average depth of about 40 m. The lake shoreline is long (about 3,500 km) and convoluted, enclosing innumerable small shallow bays and inlets, many of which include swamps and wetlands which differ a great deal from one another and from the lake itself.

Some 85% of the water entering the lake does so from precipitation directly on the lake surface, the remainder coming from rivers which drain the surrounding catchment. The most significant of these rivers in the Kenyan section include River Nzoia and River Yala which drain into the lake north of the town of Kisumu and rivers Nyando, Migori, Sondu and Kuja draining into the lake south of Kisumu town. This study targeted a section of the lake basin covering three main rivers draining into the lake and their inlets (Figure 1). They included River Nyando, River Nzoia, and River Yala. The smaller rivers studied were River Mbogo, River Kisat, River Woroya and Hippo Point River. These Rivers are characterized by indiscreet damping of domestic waste and raw sewage from the towns and urban centres through which they traverse.

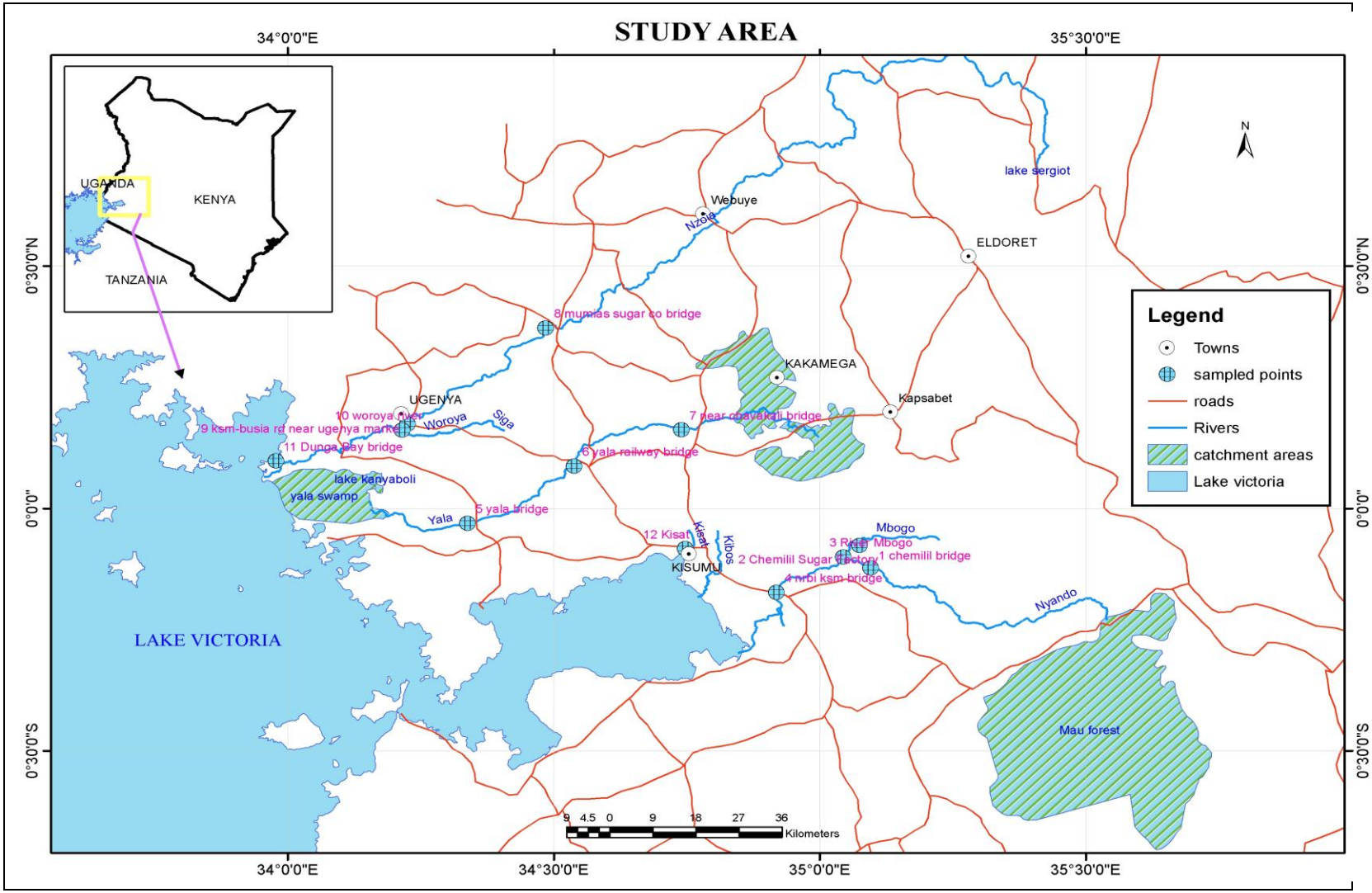

Figure 1: Map of the Study area; Source: Centre for Mapping and Regional Development-Nairobi (2007).

3.2 Sampling and field measurements

Certain points along these rivers were selected for the study based on the physical appearance of the water, land use patterns, industrial and economic activities and accessibility. A non probability sampling technique of convenience sampling was used. Two points along river Nyando were sampled together with the effluent from Chemelil sugar processing plant which drains into river Nyando through river Mbogo. The surrounding of the upper part of the river sampled at the Chemelil bridge is characterized by sugarcane farming while the lower section around the Nairobi – Kisumu highway is dominated by rice fields along side subsistence farming of maize, beans and vegetables.

River Yala was sampled at two points, the upper part near Chavakali bridge characterized by tea growing and the lower part near the railway bridge dominated by subsistence farming. River Nzoia was sampled at the Mumias sugar company bridge on its upper part whose surrounding is characterized by sugarcane and subsistence farming. The lower part was sampled at the bridge near Ugunja market on the Kisumu – Busia road. The other inlets into the lake which were sampled were River Kisat and Hippo point which drain much of the effluent from the town of kisumu into the lake.

Field measurements included water pH, water conductivity, water temperature, total dissolved solids and dissolved oxygen. The parameters were measured by their respective electrodes using a universal multiline P4 WTW (Weilheim Germany) meter. Samples collected for analysis included river water and sediments. Water

samples for microbiological assays and heavy metal analysis were collected just below the water surface into separate sterile Teflon bottles using aseptic techniques. Sediments were augured at a depth between 0 and 20 cm. Metals were preserved in solution by adding 2 drops of pure nitric acid to each sample. The water and sediment samples were transported in ice cooler box to Kenya Medical Research Institute (Kisumu) and analyzed for microbiological quality in the laboratory within six hours. The microbiological assays included heterotrophic plate counts, coliform status and isolation of enteric pathogens (Harley and Prescott, 1996).

3.2.1 Analysis of heavy metals

The Flame Atomic Absorption Spectrometer (F-AAS noVAA 350) facility was used to determine presence and concentration of heavy metals in the water and the sediment samples. The procedure applied analyzed heavy metals, lead, manganese, zinc, nickel, copper, cobalt and chromium (APHA, 1992).

3.2.2 Digestion of the total metals

Three ml of concentrated nitric acid was added to a 60 ml of acid-preserved sample in a conical flask, placed on a hot plate and evaporation done until 5 ml were obtained while making sure the sample did not come to boiling. The 5 ml sample was then cooled to room temperature and a further 5 ml concentrated redistilled nitric acid added then covered with a watch glass and refluxed with additional acid until the diluent was complete. Evaporation was done to less than 5 ml, cooled and

1:1 Hydrochloric acid (equal volume of water and HCL) was added. Heating was done for 13 min to dissolve any precipitate and any residue removed from the digested sample by filtration into a 100 ml volumetric flask. Matrix modifier was added then dilution done appropriately and the sample analyzed using the AAS. The concentrations of the heavy metals in milligrams per liter (water samples) and milligrams per gram (sediment samples) were reported as 'total' (APHA, 1992).

3.3 Heterotrophic plate counts by the standard spread plate technique

Nutrient agar medium containing peptic digest of animal tissue 5.0 g, NaCl 5.0 g beef extract 1.50 g, yeast extract 1.50 g and agar 15.00 g was prepared by dissolving 28 g in 1000 ml distilled water and sterilized in the autoclave at 121 ^{o}C for 15 min before dispensing in sterile petridishes. Serial dilutions of both water and sediment samples drawn from each site were prepared ranging from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Using a sterile pipette, 1 ml of the water sample was transferred into each of the tubes containing 9 ml of sterile physiological saline upto the highest dilution of 10^{-5}

One g of the sediment sample from each site was similarly dissolved in 9 ml sterile isotonic saline and serially diluted upto a highest dilution of 10^{-5}. 0.1ml sample from each of the last two dilutions (10^{-4} and 10^{-5}) were pipetted on the agar plates in duplicates and uniformly spread using a bend glass rod by the standard spread plate technique. The plates were incubated at 37 ^{o}C for 24 to 48 h. The colonies

appearing on each agar plate were counted after 48 h. Only plates containing between 30-300 cfu/ml and cfu/g were considered. The dilution factor was used to calculate the number of total viable counts in the water and sediment samples (APHA, 1998).

3.4 Isolation of coliforms and enteric bacteria on MacConkey agar medium

MacConkey agar medium containing peptone 20.0 g, lactose 10 g, bile salts 5.0 g, Sodium Chloride 5.00 g, neutral red 0.04 g and agar 12.0 g was prepared by dissolving 52 g in 1 litre of distilled water and sterilized in the autoclave at 121 ^{o}C for 15 min before dispensing into sterile Petri dishes. 1 ml of the water samples and 1 g of the sediment samples were serially diluted in 9 ml sterile physiological saline tubes from 10^{-1} to 10^{-4}

0.1 ml of both the water and sediment samples from the highest dilution (10^{-4}) were each pipetted on the MacConkey agar plates and aseptically spread using the standard spread plate technique. The plates were incubated at 37 ^{o}C for 24 h. This medium was used for the selective isolation, cultivation and differentiation of coliforms and enteric bacteria based on the ability to ferment lactose.

The lactose fermenting colonies were plated on nutrient agar and incubated at 44.5 ^{o}C for 24 h to detect the presence of thermotolerant *E. coli* (Cheesbrough, 1990). Organisms growing at this temperature were suspected to be *E. coli* and were

transferred to agar slants for further confirmation. The non-lactose fermenting colonies were also transferred to agar slants and confirmed using the classical biochemical tests.

3.4.1 Coliform analysis by multiple fermentation tube technique (MPN index)

Three tests, presumptive test, confirmed and completed test were used to determine the presence of coliform bacteria in the water samples (APHA, 1998).

3.4.2 Presumptive test

The presumptive test was used to determine the total coliforms present in the water samples. Double and single strength lactose broth was prepared and dispensed into tubes in 20 ml volumes. Durham tubes were inverted in the broth, which was sterilized at 121 ^{o}C for 15 minutes. The double strength lactose broth was inoculated with 10 ml of the water sample in triplicate. Single strength lactose broth was inoculated with 1 ml and 0.1 ml of the water sample in triplicate. All the tubes were incubated at 37 ^{o}C for 48 h. Presence of gas in the Durham tube as well as acid production evidenced by colour change to yellow was the presumptive evidence of the presence of coliform bacteria in the water sample.

3.4.3 Confirmed test

The confirmed test was used to confirm the presence of coliform bacteria in a water sample for which the presumptive test was positive. A sterile inoculating loop was

used to transfer a loopful of the inoculum from the positive lactose broth tubes into brilliant green lactose bile broth dispensed into tubes containing Durham tubes and sterilized at 121 ^{o}C for 15 min. The brilliant green broth tubes were incubated for 48 h at 37 ^{o}C. The formation of gas any time within 48 h constituted a positive confirmed test. The distribution of the positive tubes was used to estimate the total coliforms per 100 ml of water using the most probable number (MPN) index (APHA, 1995).

3.4.4 Completed test

A sterile inoculating loop was used to streak eosin methylene blue (EMB) agar plates from the positive brilliant green lactose broth tubes. The plates were incubated for 24 h at 37 ^{o}C. The presence of pink mucoid colonies and green metallic sheen colonies was recorded as a suspected indicative result for coliform bacteria. Well-isolated colonies were further inoculated into brilliant green lactose bile broth and a streak done in a nutrient agar slant. A Gram stain on the cultures stored in the agar slants was done. The formation of gas in the lactose broth and the demonstration of Gram- negative, nonsporing rods in the agar culture indicated a positive completed test.

The coliform bacteria, which were primarily, identified using their colony morphology, cultural characteristics and Gram stain were differentiated and confirmed using the IMViC (indole, methyl red, Voges-Proskauer, and citrate) test.

E. coli laboratory strain ATCC no 25922 was used as the control (Harley and Prescott, 1996).

3.4.5 Indole production

Trypticase soy broth medium containing tryptone 17.0 g, soytone 3.0 g, dextrose 2.5 g sodium chloride 5.0 g, and dipotassium phosphate 2.5 g in 1000 ml of distilled water was dispensed into tubes and autoclaved at 121 ^{o}C for 15 min. Using aseptic techniques, each tube was inoculated with a loopful of culture and the tubes were incubated at 37 ^{o}C for 24 h. 0.5 ml of kovacs reagent was added to each tube and the tube shaken gently.

3.4.6 Methyl-red test

MR-VP broth medium containing peptone 7.0 g, dextrose 5.0 g and potassium phosphate 5.0 g in 1000 ml of distilled water was dispensed into tubes and autoclaved at 121 ^{o}C for 15 min. Using aseptic techniques each tube was inoculated with a loopful of culture and the tubes incubated at 37 ^{o}C for 24 to 48 h for the slow fermenters. A third of each culture was transferred into a sterile empty tube for the Voges – proskauer test. To the two thirds of the culture remaining in each tube, 0.2 ml of methyl red indicator was added (Harley and Prescott, 1996).

3.4.7 Voges-Proskauer test

The one third aliquot from the methyl red test was used for this test. 0.8 ml of Barrits reagent containing 40 % KOH and a 5 % solution of alpha-naphthol in absolute ethanol was added to each culture and shaken vigorously to aerate.

3.4.8 Citrate utilization test

Simmons citrate agar containing ammonium dihydrogen phosphate 1.0 g, dipotassium phosphate 1.0 g, sodium chloride 5.0 g, sodium citrate 2.0 g magnesium sulphate 0.2 g, bromothymol blue 0.08 g and agar 15.0 g in 1000 ml of distilled water was prepared as slants by autoclaving at 121 ^{o}C for 15 minutes and allowing to solidify. Using aseptic technique each tube was inoculated with the bacteria culture by means of a stab-and-streak and the tubes were incubated at 37 ^{o}C for 24 to 48 h. The slant cultures were examined for the presence or absence of growth and for any change in colour from green to blue (Cheesbrough, 1990).

3.5 Isolation of enteric pathogens

3.5.1 *Salmonella* and *Shigella* Species

To screen for these pathogens, 10 ml of Selenite F broth medium (oxoid) containing tryptone 5.0 g, lactose 4.0 g sodium phosphate 10.0 g sodium acid selenite 4.0 g was prepared by dissolving 23 g in 1000ml of distilled water, dispensing into universal bottles and heating with agitation for 15 min.

1 ml of the water sample was inoculated into the selenite – F broth tubes for enrichment of the pathogens. 1 g of the sediment sample was dissolved in 9 ml sterile distilled water, vortexed and 1 ml of the sample inoculated into the selenite F broth. The tubes were incubated at 37 ^{o}C for 24 h with one control (uninoculated tube) per sample. The tubes showing turbidity after 24 h were considered to have positive growth. The positive tubes were selected for inoculum transfer into salmonella-shigella (SS) agar medium, which is selective for these organisms.

The SS agar containing beef extract 5.0 g, peptone 5.0 g, lactose 10.0 g, bile salts 8.5 g sodium citrate 8.5 g, sodium thiocitrate 8.5 g ferric chloride 1.0 g brilliant green 0.0033 g, neutral red 0.025 g and agar 13.5 g was prepared by dissolving 63 g in 1000ml of distilled water and heating close to boil before dispensing into sterile petridishes. Using a sterile inoculating loop, a loopful was transferred from the positive selenite F broth tubes and streaked on the SS agar plates. The plates were incubated at 37 ^{o}C for 24 h. Cream colonies with dark centers characterized by production of hydrogen sulfide on the plates were suspected to be *Salmonella* species while colourless colonies were suspected to be *Shigella* species (Cheesbrough, 1990). The suspected colonies were purified into fresh SS agar plate, Gram stained and transferred into nutrient agar slants for confirmation by motility and biochemical test.

3.5.2 Triple sugar iron (TSI) agar test

Triple sugar iron agar containing beef extract 3.0 g, yeast extract 3.0 g, peptone 15.0 g, peptose-peptone 5.0 g, lactose 10 g, saccharose 10.0 g, dextrose 1.0 g ferrous sulfate 0.2 g, sodium chloride 5.0 g, sodium thiosulfate 0.3 g, phenol red 0.024 g and agar 12.0 g was prepared as slants by dissolving 65 g in distilled water, dispensing into tubes and autoclaving at 121 ^{o}C for 15 minutes. Each TSI agar slant was labelled with the bacterial isolate to be inoculated and one tube (uninoculated) was used as a negative control.

An aseptic technique was used to streak the slant and stab the butt with the bacterial culture using a straight inoculating needle. *Salmonella* species ATCC no. N2202 and *Shigella* species (local isolate) were used as the positive controls. The tubes were incubated for 18 to 24 h at 37 ^{o}C to detect the presence of sugar fermentation, gas production and H_2S production. The tubes were incubated and checked daily for upto seven days in order to observe blackening. The cultures were examined for the colour of the slant and butt and for the presence or absence of blackening within the medium (Harley and Prescott, 1996).

3.5.3 Motility test

This test was done to differentiate between *Salmonella* and *Shigella* species. A motility test medium containing tryptose 10.0 g, sodium chloride 5.0 g and agar 5.0 g in 1000 ml of distilled water was dispensed into tubes, autoclaved at 121 ^{o}C for 15

minutes and allowed to cool as agar deeps. Each agar deep tube was inoculated with the bacterial culture aseptically by stabbing the medium to the bottom with a straight inoculating needle. The tubes were incubated at 37 ^{o}C for 24 to 48 h. The cultures were examined for the presence or absence of motility. Motility was present when the growth of the culture was not restricted to the stab line of inoculation. Growth of nonmotile bacteria was confined to the line of inoculation.

3.6 *Vibrio* species

Ten ml alkaline peptone water medium containing peptone 5.0 g, tryptone 5.0 g and NaCl 5.0 g was prepared by suspending 15 g in 1000 ml distilled water. It was dispensed into universal bottles and sterilized at 121 ^{o}C for 15 min. 1 ml of the water samples was inoculated into the alkaline peptone water for enrichment of *Vibrio cholerae*. 1 g of the sediment sample was dissolved in 9 ml sterile distilled water, vortexed and 1 ml of the sample inoculated into the alkaline peptone water tubes for enrichment. The tubes were incubated at 37 ^{o}C for 24 h with one control (uninoculated) tube per sample.

Tubes showing turbidity after 24 h were considered to have positive growth. The positive tubes were selected for inoculum transfer into Thiosulphate citrate bile sucrose salts medium (TCBS). The TCBS medium (oxoid) containing Yeast extract 5.0 g, Peptone 10.0 g, Sodium thiosulphate 10.0 g, Sodium citrate 10.0 g, ox bile 8.0 g, Sucrose 20.0 g, Sodium chloride 10.0 g, ferric citrate 1.0 g, Bromothymol

blue 0.04 g, Thymol blue 0.04 g and agar 14.0 g was prepared by dissolving 89 g in 1000ml of distilled water and heating to boil by agitation before dispensing into sterile Petri dishes. A loopful from the positive peptone water tubes was aseptically streaked onto the TCBS agar plates. The plates were incubated at 37 ^{o}C for 24 h. The colonies were purified, Gram stained and confirmed using the oxidase test.

3.6.1 Oxidase test

Nutrient agar medium was prepared by autoclaving at 121 ^{o}C for 15 minutes and dispensed into sterile petridishes. The agar plates were each divided into two sections and each labelled with the bacterial isolate to be inoculated. A single streak-line inoculation on the agar surface was made aseptically with the cultural isolate. The plates were incubated at 37 ^{o}C for 24 h. Using a wooden applicator stick, an oxidase disk (p-aminodimethylaniline) was placed on an isolated colony and the colony observed for colour change (APHA, 1998). *Pseudomonas aeruginosa* ATCC no 27853 was used as the control.

3.7 Assessment of bacterial heavy metal tolerance

One hundred and six bacterial cultures were investigated for heavy metal tolerance with seven isolates as certified reference materials. The isolates were tested for metal tolerance using five different concentrations of four different metal salts each. The salt concentrations used were mercury (II) chloride crystals, ACS Reagent Grade (25 mg/l, 50 mg/l, 100 mg/l, 200 mg/l, 400 mg/l), Pentahydrated cupric sulfate ($CuSO_4.5H_2O$), fine

crystals ACS Reagent grade (100 mg/l, 200 mg/l, 400 mg/l, 800 mg/l, 1600 mg/l), nickel (II) sulfate crystals, ACS Reagent Grade (100 mg/l, 200 mg/l, 400 mg/l, 800 mg/l, 1600 mg/l) and potassium dichromate ($K_2Cr_2O_7$) CAS 7778.50-9 Reagent Grade (300 mg/l, 600 mg/l, 1200 mg/l, 2400 mg/l, 4800 mg/l). The minimal inhibitory concentration (MIC) of the metal ions at which no growth was observed was determined by the broth dilution method (Calomiris *et al.,* 1984).

Bacterial cultures stored in agar slants were aseptically transferred into nutrient broth tubes. The tubes were incubated at 37 ^{o}C for 24 h. Nutrient broth containing peptic digest of animal tissue 5.0 g, sodium chloride 5.0 g, beef extract 1.5 g, yeast extract 1.5 g was prepared by dissolving 13 grams in 1000 ml distilled water and dispensed into tubes in volumes of 5mls.

A set of 5 tubes for each metal salt was prepared in duplicate. A stock solution of each metal salt was prepared. A two fold dilution was done by transferring 5 ml of the metal stock solution into the first tube of 5 ml nutrient broth and mixing the solution. This was followed by a 5 ml transfer from the broth tube with the highest concentration of the metal to the last tube receiving the least metal concentration. 5 ml from the last tube was discarded. The nutrient broth tubes amended with the different concentrations of the metal ions were autoclaved at 121 ^{o}C for 15 min.

The concentration of the bacterial cultures was prepared to match the McFarland standard. 0.1 ml of the culture was inoculated into the metal emended broth tubes and the tubes incubated at 37 ^{o}C for 36 - 48 h and checked for growth. The minimum concentration of each metal ion at which no turbidity was observed in the tube was considered as the MIC of the isolate. A positive control consisted of a metal deficient medium inoculated with a microorganism and a negative one consisted of a broth medium without the microorganism.

3.8 Antibiotic susceptibility testing

All the bacterial isolates, heterotrophs, coliforms, and the enteric pathogens were screened for antibiotic resistance. Ten antibiotics were used in this test. The selection of the antibiotics was based on the frequency of use in Kenya, antibiotic family and the spectrum of activity. The main classes of the antibiotics used included Beta-lactams (Penicillins and Cephalosporins), Aminoglycosides, Flouroquinolones, Sulfonamides, Tetracycline and Chloramphenicol. The antibiotic discs (Abtek Biologicals Ltd) used were Ampicillin 25 µg, Cotrimoxazole 25 µg, Augmentin 30 µg, Tetracycline 25 µg, Kanamycin 30 µg, Gentamicin 10 µg, Cefuroxime 30 µg, Chloramphenicol 30 µg, Nalidixic acid 30 µg and Norfloxacin 10 µg.

Ninety nine bacterial isolates were investigated for resistance to various antibiotics. Ninety three isolates from the Lake Victoria basin and six isolates from National

Public Health Laboratories as certified reference materials were screened for antibiotic resistance. The standardized disc-agar diffusion technique (Finegold and Martin, 1982) was used for the antibiotic susceptibility test. Bacterial cultures stored in agar slants were aseptically transferred into nutrient broth tubes. The tubes were incubated at 37 ^{o}C for 24 h. 0.1 ml of each bacterial culture was uniformly spread on a sterile Mueller-Hinton agar plate using the standard spread plate technique. The culture was allowed to dry on the plate for 5 to 10 minutes at room temperature with the top in place.

Using an alcohol-flamed forceps, the antibiotic impregnated discs where placed aseptically on the bacterial lawns on agar plates. The plates were incubated for 18 to 24 h at 37 ^{o}C. The diameter of the zones of inhibition realized was measured to the nearest millimeter for each of the antibiotics tested. The inhibition zone sizes were interpreted using standard recommendations of the National Committee for Clinical Laboratory Standards (NCCLS), (2000).

3.9 Plasmid DNA preparation and isolation

Plasmid DNA was prepared and isolated by the alkaline lysis method, a protocol which is a modification of the methods of Birnboim and Doly (1979). A single bacterial colony from a 24 h culture was inoculated into 2 ml of Luria-Bertani (LB) broth containing tryptone 10 g, NaCl 10 g, yeast extract 5 g and 1000 ml distilled water and incubated overnight at 37 ^{o}C with vigorous shaking. The bacterial cells were harvested by

centrifuging 1.5 ml of the culture in microfuge tubes for 30 seconds at 4 ^{o}C and 12000 (rpm) revolutions per min. The supernatant was discarded and a dry pellet retained. The bacterial cells were resuspended in 100 µl of ice-cold cell resuspension buffer consisting of Tris- EDTA by mixing with a pipette. The cells were lysed by adding 200 µl of freshly prepared solution II (NaOH and SDS) and the tubes were inverted 4 to 6 times to ensure proper mixing. The tubes were stored in ice for 3 to 5 min. 150 µl of ice-cold buffer (Potassium acetate- solution III) was added. The tubes were inverted for 10 seconds to disperse solution III through the viscous bacterial lysate and stored on ice for 3 to 5 min. The tubes were centrifuged at 12000 rpm for 5 minutes at 4 ^{o}C and the supernatant transferred to a fresh tube by pipetting.

The DNA was precipitated with 2 voumes of 95% ethanol at room temperature by inverting the tubes 4 to 6 times. After standing for 2 min at room temperature the tubes were centrifuged at 12000 rpm at 4 ^{o}C for 5 min. The supernatant was discarded gently and the tubes placed in an inverted position on a paper towel to allow all the fluid to drain away. The pellet of double stranded DNA was rinsed with 1 ml of 70% ethanol at 4 ^{o}C. The supernatant was discarded and the pellet of nucleic acid allowed to dry in the air for 10 min. The nucleic acid was redissolved in 50 µl of TE (pH 8.0) and the DNA stored at -20 ^{o}C.

3.9.1 Agarose gel electrophoresis of DNA

Agarose (sigma) 1.0 g was dissolved in 100 ml of tris boric acid buffer (TBA). The mixture was heated in a microwave for 2 min while shaking at intervals of 30 sec to release any gas. A comb with well spaced teeth was placed on an agarose electrophoresis plate to create wells. The agarose solution was allowed to cool slightly and stained with 5 µl of ethidium bromide before pouring on the plate. The stained agarose gel was allowed to polymerise for 1 h in the plate. The comb was carefully removed and the plate was placed in the electrophoresis chamber. The TBA buffer was added until the gel was completely submerged. About 20 µl of plasmid DNA mixed with 10 µl of loading buffer was pippeted into the wells. The chamber was connected to a power supply and the gels were run at 100 volts for three h (Umolu *et al.*, 2006).

3.9.2 Analysis of Plasmid DNA

The agarose gels stained with ethidium bromide were visualized by UV-transillumination. Plasmid DNA appeared as bright bands on the lanes. The gels were photographed and the plasmid sizes estimated by comparing with previously characterized plasmids of the control strain of *E. coli* V517 used as the genetic marker.

3.10 Data management and analysis

Field data was recorded in a table in a notebook at the sampling sites while laboratory data was collected and recorded stepwise in laboratory notebooks. Quantitative and qualitative analysis of the laboratory results was done using a computer package, minitab

13.0, and SPSS (statistical package for social science) version 11.5. The data was presented using tables, graphs and charts. Analysis of variance (ANOVA) was used to determine the variations in the number of bacteria tolerant to the heavy metals and resistant to antibiotics. Pearson correlation was used to establish the relationships investigated. A probability value, $p < 0.05$ was considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Physico-chemical parameters

The water temperatures ranged from 19.3 ^{o}C to 26.5 ^{o}C recorded at the upper part of River Yala and the upper part of River Nyando (Table 3). The water pH was neutral to slightly alkaline. The pH ranged from 7.32 observed at the Hippo Point River to 8.24 at the lower part of River Nzoia.

There was however a wide range of the dissolved oxygen (0.92 to 10.2 mg/l) observed. These were recorded at the lower and the upper parts of River Yala respectively.

The highest concentration of the total dissolved solids (244 mg/l) occurred at the upper part of River Nyando with the lowest concentration observed at the upper part of River Yala (0.08 mg/l). Conductivity varied greatly with the highest (>200 micro siemens) recorded at River Nyando (lower) and the lowest (0.08 µs) at the upper part of River Yala during the sampling period.

Table 3. Physico – chemical parameters of the study sites

	Site	Temp ^{o}C	pH	Conductivity µs	TDS mg/L	Dissolved O_2 mg/L
1.	R. Kisat	25.2	7.6	1.29	1.0	7.2
2.	R. Mbogo	22.8	8.17	0.21	150	6.7
3.	Chemelil effluent	26.3	7.67	1.0	1.12	0.7
4.	Upper Nyando	26.5	8.23	0.38	244	8.0
5.	Upper Yala	19.3	7.91	0.08	0.08	10.2
6.	Upper Nzoia	22.8	8.02	139	92	9.1
7.	Lower Nzoia	24.4	8.24	132.7	0.21	9.5
8.	R. Woroya	22.4	8.1	102.2	0.52	9.2
9.	Lower Yala	21.4	7.85	81.2	0.58	0.92
10.	Lower Nyando	25.9	8.08	>200	151	7.4
11.	Hippo Point River	24.4	7.32	115	77	8.5

4.2 Heavy metals in selected wetlands of the Lake Victoria basin

The eleven study sites recorded varying concentrations of the metals investigated (Table 4). The mean range of heavy metals in water samples was 0.77-0.94 mg/l (lead), 0.10-3.10 mg/l (manganese), 0.23-1.16 mg/l (zinc), 0.02-0.04 mg/l (cadmium) and 0.51-0.57 mg/l (copper). Out of the water samples studied, effluent from the Chemelil sugar factory recorded the highest levels of the heavy metals of 5.08 mg/l, River Kisat, 3.28 mg/l and the upper part of River Yala, 2.94 mg/l. The lower part of River Nyando and the Hippo

Point River recorded the least levels of the heavy metals of 1.7 mg/l and 1.62 mg/l respectively.

Table 4. Mean heavy metal concentrations (mg/l) in the water samples (N=3)

Site	Lead	Manganese	Zinc	Cadmium	Copper
R.Kisat	0.77	1.71	0.26	0.007	0.53
R.Mbogo	0.80	0.52	0.39	0.05	0.55
Chemelil effluent	0.85	3.10	0.55	0.006	0.57
Upper Nyando	0.77	0.16	0.23	0.002	0.52
Lower Nyando	0.77	0.19	0.23	0.00	0.51
Upper Yala	0.94	0.31	1.16	0.00	0.53
Lower Yala	0.81	0.44	0.23	0.006	0.52
Upper Nzoia	0.83	0.33	0.23	0.04	0.53
Lower Nzoia	0.79	0.29	0.23	0.02	0.53
R.Woroya	0.85	0.75	0.25	0.00	0.54
Hippo-Point River	0.77	0.10	0.23	0.00	0.52

Heavy metal levels were remarkably high in sediment samples compared to the water samples in all the sites (Table 5). The mean range of these metals in the sediments was 31.97-109.9 mg/g (lead), 3.38-183.66 mg/g (zinc), 3.01-17.03 mg/g (nickel), 1.93-214.61 mg/g (copper), 17.01-65.91 mg/g (cobalt) and 2.08-25.89 mg/g (chromium). Sediments from River Kisat, River Nyando (lower) and the Chemelil sugar factory effluent recorded

the highest levels of the heavy metals of 10401.2 mg/g, 1930.12 mg/g and 1352.45 mg/g respectively. River Nzoia recorded the lowest level of the metals of 474.64 mg/g (Lower) and 315.18 mg/g (Upper)

Table 5. Mean heavy metal concentrations (mg/g) in the sediment samples (N=3)

Site	Lead	Manganese	Zinc	Nickel	Copper	Cobalt	Chromium
R.Kisat	109.91	10225.3	26.05	4.03	1.93	33.98	0
R.Mbogo	37.09	839.2	36.65	9.16	22.72	20.01	2.08
Chemelil effluent	72.99	798.25	183.66	17.03	214.61	65.91	0
Upper Nyando	41.88	898.71	71.99	14.98	16.58	55.94	0
Lower Nyando	44.61	1720.95	53.79	12.24	40.54	49.95	8.04
Upper Yala	39.49	892.15	26.45	3.01	8.95	33.98	2.08
Lower Yala	37.78	956.83	19.91	17.71	3.93	22.01	19.94
Upper Nzoia	32.99	237.66	6.08	9.85	9.51	17.01	2.08
Lower Nzoia	31.97	376.94	3.48	14.98	4.35	17.02	25.89
R.Woroya	39.83	350.50	0.007	10.53	32.68	41.97	0

Generally the metals investigated in both water and sediment samples recorded amounts above the permissible limits stipulated by the World Health Organization (WHO, 1985) and the Kenya Bureau of Standards (KEBS, 1996). Manganese in the water sample remained above the permissible limit of 0.1 mg/l (WHO, 1985; KEBS, 1996). Small amounts of cadmium ranging from 0.02 to 0.05 mg/l in water samples were recorded

which is above the critical limit of 0.005 mg/l (WHO, 1985; KEBS, 1996). Lead recorded levels above the critical limit of 0.05 mg/l while Copper remained within the stipulated limit by WHO however the levels were above the 0.1 mg/l stipulated by the Kenya Bureau of standards (KEBS, 1996). Among the sediments sampled, River Kisat recorded the highest levels of lead (109.91 mg/g) and Manganese (10225.3 mg/g) while Chemelil effluent recorded the highest levels of Zinc (183.66 mg/g) and Copper (214.61 mg/g). Low levels of both Nickel and chromium were recorded in all sediment samples with chromium remaining below detectable levels in some of the sites.

4.3 Microbiological studies

A total of ninety nine pure cultures were obtained from the study sites (Figure 2). Fifty one of the cultures were obtained from the water samples while the other forty eight were drawn from the sediment samples. Based on their colony morphology, cultural characteristics and biochemical reactions, the cultures were grouped into heterotrophs, coliforms and enteric pathogens. The cultures of the heterotrophic bacteria were selected from nutrient agar medium based on their frequency of growth from each study site.

The coliform bacteria identified based on their IMViC reactions were mainly *E. coli* and *Enterobacter* species (Appendix 1a). The enteric pathogens identified upto the genus level were classified as *Salmonella* and *Shigella* species based on their TSI reaction and motility (Appendix 1b), while cytochrome oxidase positive organisms were identified as *Vibrio* species (Appendix 1c).

4.3.1 Total coliform bacteria and the standard plate count bacteria

Most of the sites sampled recorded high numbers of total coliforms (> 1100 per 100 ml of water) except Rivers Yala (lower), River Nyando (lower) and Hippo Point River which recorded 150, 28 and 150 total coliforms per 100 ml of water respectively (Table 6). The highest total viable counts from water samples were observed from River Kisat (4.8×10^6 cells/ml), effluent from Chemelil sugar factory (2.8×10^6 cells/ml) and River Mbogo (1.65×10^6 cells/ml). The lower part of River Nyando and River Nzoia (lower) recorded the lowest viable counts (1.0×10^8cells/ml and (2.0×10^9 cells/ml).

A higher number of the plate count bacteria was however observed from the sediment samples compared to the water samples (Table 6). The highest count was recorded from the effluent sediments near the Chemelil sugar factory followed by sediments from River Kisat. These were 5.25×10^6 and 1.6×10^6 cells/g respectively. Low viable counts in sediment samples were observed in the upper part of River Yala (2.4×10^8 cells/g) and River Woroya (2.0×10^8 cells/g).

Table 6. Coliform counts and Total plate counts in the study sites

Site	Coliforms MPN/100 ml	Total plate count Water- cfu/ml	Total plate count Sediment- cfu/g
R. Kisat	> 1100	4.8×10^6	1.6×10^6
R. Mbogo	> 1100	1.65×10^6	1.3×10^6
Chemelil effluent	> 1100	2.8×10^6	5.25×10^6
Upper Nyando	> 1100	2.1×10^7	1.02×10^6
Lower Nyando	28	1.0×10^8	6.1×10^7
Upper Yala	> 1100	2.0×10^8	2.4×10^8
Lower Yala	150	3.0×10^8	2.5×10^8
Lower Nzoia	> 1100	8.0×10^8	4.8×10^7
Lower Nzoia	> 1100	2.0×10^9	1.6×10^7
R. Woroya	> 1100	6.0×10^8	2.0×10^8
Hippo Point River	150	1.48×10^7	_

(-) Not done

4.3.2 Diversity of bacterial isolates from the study sites

The bacterial isolates obtained from both water and sediment samples for the study were distributed as follows; R. Kisat (9), R.Mbogo (9), Chemelil effluent (13), Upper Nyando (9), Lower Nyando (9), Upper Yala (6), Lower Yala (10) Upper Nzoia (10), Lower Nzoia (12), River Woroya (8) and Hippo Point River (4) (Figure 2).

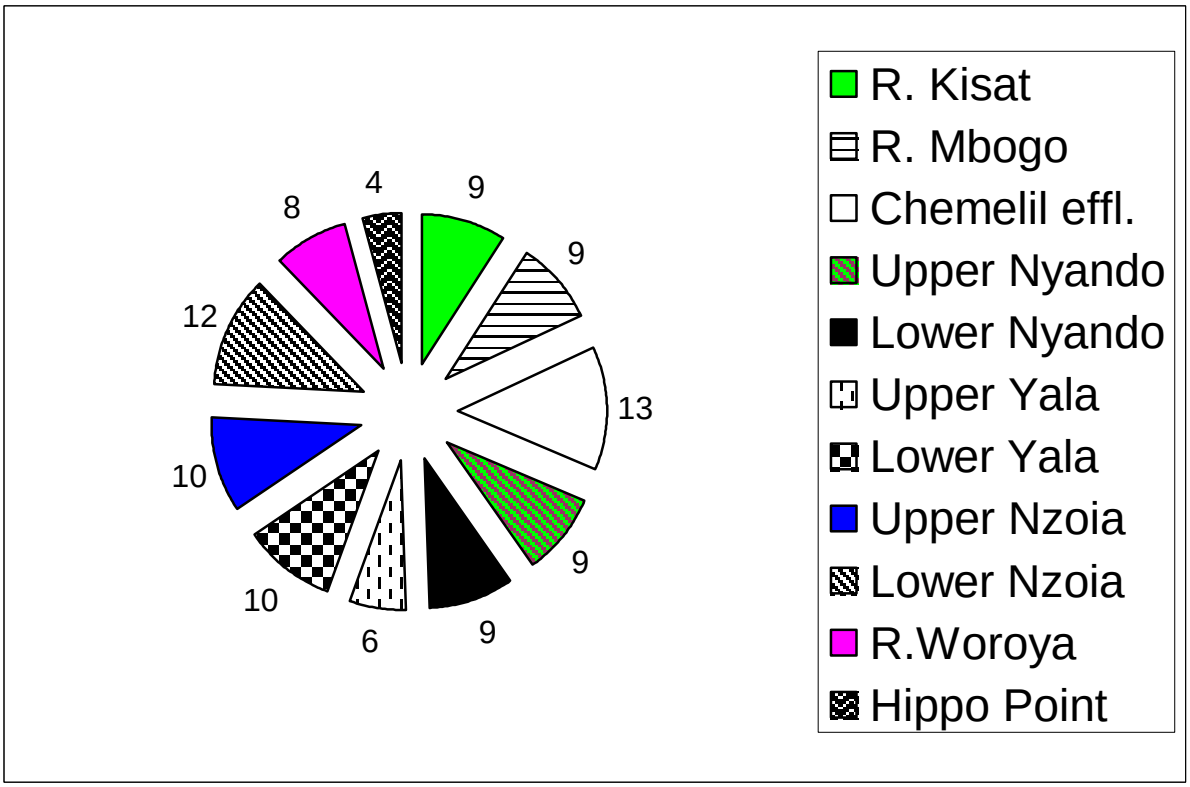

Figure 2: Diversity of the Isolates from the study sites

4.4 Heavy metal levels and the standard plate count bacteria

The water samples studied showed a significant relationship between the total amounts of heavy metals present in the rivers and the bacterial counts from these rivers, $r = 0.673$, $p < 0.05$ (Figure 3). The sites with high concentrations of heavy metals also had high levels of bacterial cell counts.

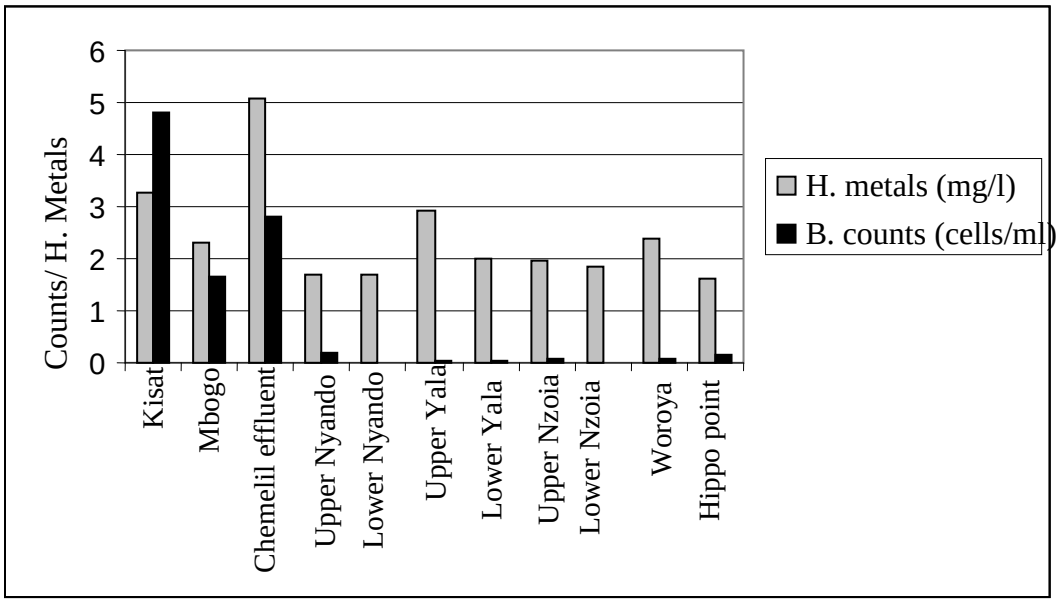

Figure 3: Heavy metal concentrations (mg/l) and bacterial plate counts (cells $\times 10^6$ /ml) in the water samples

The study on the sediment samples however showed no significant relationship between the total amounts of heavy metals presence in the sediments and the bacterial counts from the sediments, r = 0. 175, p > 0.05 (Figure 4). The sites with high heavy metal content did not necessarily have high levels of bacterial cell counts.

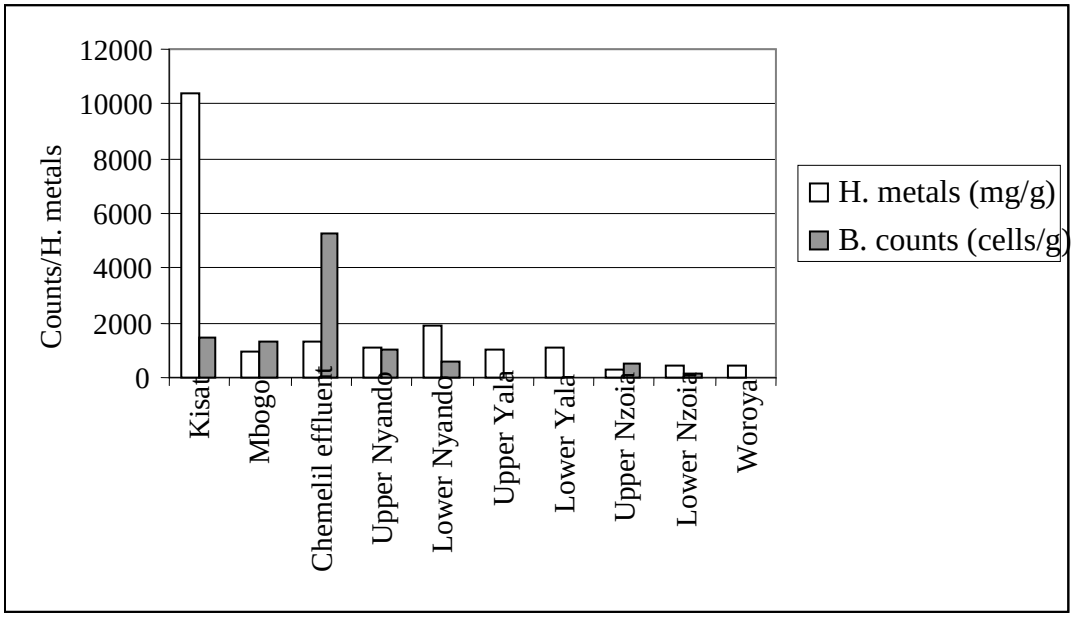

Figure 4: Heavy metal concentrations (mg/g) and bacterial plate counts (cells × 10^6 /g) in the sediment samples

4.5 Tolerance of the bacterial isolates to heavy metals

4.5.1 Nickel toxicity

There was a significant effect of nickel concentration on the percentage number of bacteria tolerant to $NiSO_4$ from all the sites, $F_{50} = 143.424$, $p < 0.05$ (Table 7). Concentration of 800 mg/l and 1600 mg/l had significantly lower number of tolerant bacteria than the lower concentrations. Although concentration of 1600 mg/l had fewer

tolerant bacteria, there was no significant difference on the tolerance level of the bacteria with that in 800 mg/l. The bacterial isolates from upper part of river Yala (mean % tolerance 36.8) were less tolerant to $NiSO_4$ than all the other isolates whereas bacterial isolates from Chemelil effluent, River Mbogo and Hippo Point river (mean % tolerance 60) were more tolerant to $NiSO_4$ compared to isolates from the other sites.

Table 7. Levels of tolerance to $NiSO_4$ by bacterial isolates from the study sites

	$NiSO_4$ Concentrations and bacterial % tolerance					
Site	**100mg/l**	**200mg/l**	**400mg/l**	**800mg/l**	**1600mg/l**	**Mean % Tolerance**
R. Kisat	9 (100%)	9(100%)	8(89%)	0(0%)	0(0%)	57.8
R. Mbogo	9 (100%)	9 (100%)	9 (100%)	0(0%)	0(0%)	60*
Chemelil effl.	13(100%)	13(100%)	10(77%)	2(15%)	1(8%)	60*
Upper Nyando	10(100%)	10(100%)	7(70%)	0(0%)	0(0%)	54
Lower Nyando	9(100%)	8(89%)	8(89%)	0(0%)	0(0%)	55.6
Upper Yala	6(100%)	4(67%)	1(17%)	0(0%)	0(0%)	36.8*
Lower Yala	10(100%)	9(90%)	7(70%)	0(0%)	0(0%)	52
Upper Nzoia	10(100%)	8(80%)	3(30%)	0(0%)	0(0%)	42
Lower Nzoia	12(100%)	12(100%)	8(67%)	2(17%)	1(8%)	58.4
R. Woroya	8(100%)	7(88%)	6(75%)	1(13%)	0(0%)	55.2
Hippo Point R.	4(100%)	4(100%)	4(100%)	0(0%)	0(0%)	60*

(*) Denotes highest and lowest bacterial mean percentage tolerance

4.5.2 Mercury toxicity

A small number of bacteria from all the sites were able to tolerate higher concentrations of $HgCl_2$ (200 mg/l and 400 mg/l) (Table 8). There was a significant effect of mercury concentration on the percentage number of tolerant bacteria from all the study sites, $F_{50} = 12.896$, $p < 0.05$. However, there was no significant difference in the number of bacteria tolerant to concentration of 200 mg/l and 400 mg/l even though concentration of 400 mg/l had a lower number of tolerant bacteria. The bacterial isolates from upper part of river Yala were the most sensitive to mercury (mean % tolerance 0) as they did not tolerate any of the concentrations of the $HgCl_2$ tested. Those from lower Yala were all sensitive to concentrations above 100 mg/l. 25% of the bacterial isolates from lower Nzoia, River Woroya and Hippo Point tolerated the highest concentration of $HgCl_2$ (400 mg/l) tested. 11% of the isolates from River Kisat and lower Nyando also tolerated this concentration. Isolates from lower Nzoia were the most tolerant to mercury (mean % tolerance 63.4).

Table 8. Levels of tolerance to $HgCl_2$ by bacterial isolates from the study sites

	$HgCl_2$ Concentrations and bacterial % tolerance					
Site	**25mg/l**	**50mg/l**	**100mg/l**	**200mg/l**	**400mg/l**	**Mean % Tolerance**
R. Kisat	5(56%)	4(44%)	4(44%)	3(33%)	1(11%)	37.6
R. Mbogo	9(100%)	7(78%)	4(44%)	3(33%)	0(0%)	51
Chemelil effl.	9(69%)	6(46%)	4(31%)	3(23%)	0(0%)	33.8
Upper Nyando	10(100%)	5(50%)	3(30%)	3(30%)	0(0%)	42
Lower Nyando	7(78%)	6(67%)	6(67%)	3(33%)	1(11%)	51.2
Upper Yala	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0*
Lower Yala	5(50%)	1(10%)	0(0%)	0(0%)	0(0%)	12
Upper Nzoia	8(80%)	7(70%)	5(50%)	4(40%)	1(10%)	50
Lower Nzoia	12(100%)	10(83%)	8(67%)	5(42%)	3(25%)	63.4*
R. Woroya	7(88%)	5(88%)	4(50%)	2(25%)	2(25%)	50.2
Hippo Point River	3(75%)	2(50%)	1(25%)	1(25%)	1(25%)	40

(*)Denotes highest and lowest bacterial mean percentage tolerance

4.5.3 Chromium toxicity

There was a significant effect of concentration on the percentage number of bacteria tolerant to chromium $F_{50} = 51.711$, $p < 0.05$ (Table 9). Few bacteria were able to tolerate higher concentrations of $K_2Cr_2O_7$. However, there was no significant difference in the number of bacteria tolerant to concentrations of 2400 mg/l and 4800 mg/l. It was noted

that concentration of 4800 mg/l had much smaller number of tolerant bacteria. Only 11% and 8% of the isolates from River Mbogo and Chemelil effluent tolerated this concentration. The bacterial isolates from upper part of River Yala were less tolerant (mean % tolerance 10) to $K_2Cr_2O_7$ than all the isolates from other rivers. Bacterial isolates from River Mbogo and Hippo Point were the most tolerant (mean % tolerance 60) to chromium.

Table 9. Levels of tolerance to $K_2Cr_2O_7$ by bacterial isolates from the study sites

	$K_2Cr_2O_7$ Concentrations and bacterial % tolerance					
Site	**300mg/l**	**600mg/l**	**1200mg/l**	**2400mg/l**	**4800mg/l**	**Mean % Tolerance**
R. Kisat	9(100%)	8(89%)	5(56%)	0(0%)	0(0%)	49
R. Mbogo	9(100%)	8(89%)	7(78%)	2(22%)	1(11%)	60*
Chemelil effl.	13(100%)	10(77%)	6(46%)	3(23%)	1(8%)	50.8
Upper Nyando	9(90%)	8(80%)	5(50%)	1(10%)	0(0%)	46
Lower Nyando	9(100%)	8(89%)	8(89%)	1(11%)	0(0%)	57.8
Upper Yala	3(50%)	0(0%)	0(0%)	0(0%)	0(0%)	10*
Lower Yala	10(100%)	9(90%)	6(60%)	0(0%)	0(0%)	50
Upper Nzoia	9(90%)	7(70%)	3(30%)	1(10%)	0(0%)	40
Lower Nzoia	12(100%)	11(92%)	9(75%0	0(0%)	0(0%)	53.4
R. Woroya	7(88%)	7(88%)	3(38%)	1913%)	0(0%)	45.4
Hippo Point River	4(100%)	4(100%)	3(75%)	19(25%)	0(0%)	60*

(*) Denotes the highest and the lowest bacterial mean percentage tolerance

4.5.4 Copper toxicity

A small number of bacterial isolates tolerated higher concentrations of Copper Sulphate (Table 10). 20% of the isolates from R.Nyando (upper) tolerated the highest concentration (1600 mg/l) with only 10% and 8% of the isolates from Upper Nzoia and Chemelil effluent tolerating this concentration. Bacterial isolates from Upper Yala were less tolerant (mean % tolerance 43.4) to copper sulphate than all the isolates from other rivers. Isolates from Upper Nyando were the most tolerant to copper sulphate, followed by rivers Kisat and Lower Nzoia (mean % tolerance 74, 68.8 and 68.4 respectively). There was a significant effect of concentration on the percentage number of bacteria tolerant to $CuSO_4$ from the study sites, $F_{50} = 73.592$, $p < 0.05$.

Table 10. Levels of tolerance to $CuSO_4$ by bacterial isolates from the study sites

	$CuSO_4$ Concentrations and bacterial % tolerance					
Site	**100mg/l**	**200mg/l**	**400mg/l**	**800mg/l**	**1600mg/l**	**Mean % Tolerance**
R. Kisat	9(100%)	9(100%)	9(100%)	4(44%)	0(0%)	68.8*
R. Mbogo	9(100%)	9(100%)	9(100%)	3(33%)	0(0%)	66.6
Chemelil effl.	13(100%)	11(85%)	8(62%)	5(38%)	1(8%)	58.6
Upper Nyando	10(100%)	10(100%0	9(90%)	6(60%)	2(20%)	74*
Lower Nyando	9(100%)	8(89%)	5(56%)	1(11%)	0(0%)	51.2
Upper Yala	6(100%)	4(67%)	2(33%)	1(17%)	0(0%)	43.4*
Lower Yala	9(90%)	8(89%)	7(70%)	0(0%)	0(0%)	48
Upper Nzoia	9(90%)	8(80%)	8(80%)	3(30%)	1(10%)	54
Lower Nzoia	12(100%)	11(92%)	11(92%)	6(50%)	1(8%)	68.4
R. Woroya	8(100%)	8(100%)	7(88%)	2(25%)	0(0%)	62.6
Hippo Point River	4(100%)	4(100%)	1(25%)	0(0%)	0(0%)	45

(*) Denotes the highest and the lowest bacterial mean percentage tolerance

The study established that, there was a significant difference in percentage tolerance of the bacterial isolates to the four heavy metals tested ($F = 4.25$, $P = 0.011$, $P < 0.05$) (Table 11). The bacterial tolerance to copper (mean percentage tolerance 58.24) was higher than tolerance to Nickel (mean 53.80%), chromium (mean 47.49%), and Mercury (mean 39.20%).

Table 11. Mean percentage tolerance per heavy metal for the four metals by the bacterial isolates from all the sites (N=99)

Site	$NiSO_4$	$HgCL_2$	$K_2Cr_2O_7$	$CuSO_4$
R.Kisat	57.8	37.6	49	68.8
R.Mbogo	60	53	60	66.6
Chemelil effl.	60	33.8	50.8	58.6
Upper Nyando	54	42	46	74
Lower Nyando	55.6	51.2	57.8	51.2
Upper Yala	36.8	0	10	43.4
Lower Yala	52	12	50	48
Upper Nzoia	42	50	40	54
Lower Nzoia	58.4	63.4	53.4	68.4
R.Woroya	55.2	50.2	45.4	62.6
Hippo Point River	60	40	60	45
Mean % tolerance	**53.80**	**39.20**	**47.49**	**58.24**

At the various sites, there was a significant difference in heavy metal tolerance ($F = 3.04$, $P = 0.008$, $p < 0.05$). Bacterial tolerance to the heavy metals in Upper Yala (mean 22.55%) (Table 12) was lower than in all the other sites. Isolates from lower Nzoia (mean 60.90%) were the most tolerant to the heavy metals tested.

Table 12. Mean percentage bacterial heavy metal tolerance from the sites

Site	**Percentage tolerance**	**s.e.d**
R. Kisat	53.30a	13.23
R. Mbogo	59.40a	6.41
Chemelil Effl.	50.80a	12.03
Upper Nyando	54.00a	14.24
Lower Nyando	53.95a	3.30
Upper Yala	22.55b	20.85
Lower Yala	40.50ab	19.07
Upper Nzoia	46.50ab	6.61
Lower Nzoia	60.90a	6.45
R. Woroya	53.35a	7.35
Hippo Point River	51.25a	10.31

NB Mean denoted by similar letters is not significantly different at $P \leq 0.05$

4.6 Heavy metal tolerance in the bacterial isolates and the standard isolates

The level of tolerance to heavy metals in the environmental isolates from the lake basin compared to that of isolates (Certified Reference Materials) obtained from the National public health laboratories showed a relationship value, $r = 0.965$, $P < 0.05$. This showed a significant relationship in the levels of heavy metal tolerance in both categories as they recorded similar trends. Although all the metals at various concentrations exhibited

varied levels of toxicity to the microorganisms, Mercury was the most toxic to the microorganisms followed by Chromium and Nickel. Copper was the least toxic to the microorganisms obtained from the Lake Victoria basin. Copper however recorded a higher toxicity compared to nickel in the standard isolates (Figure 5).

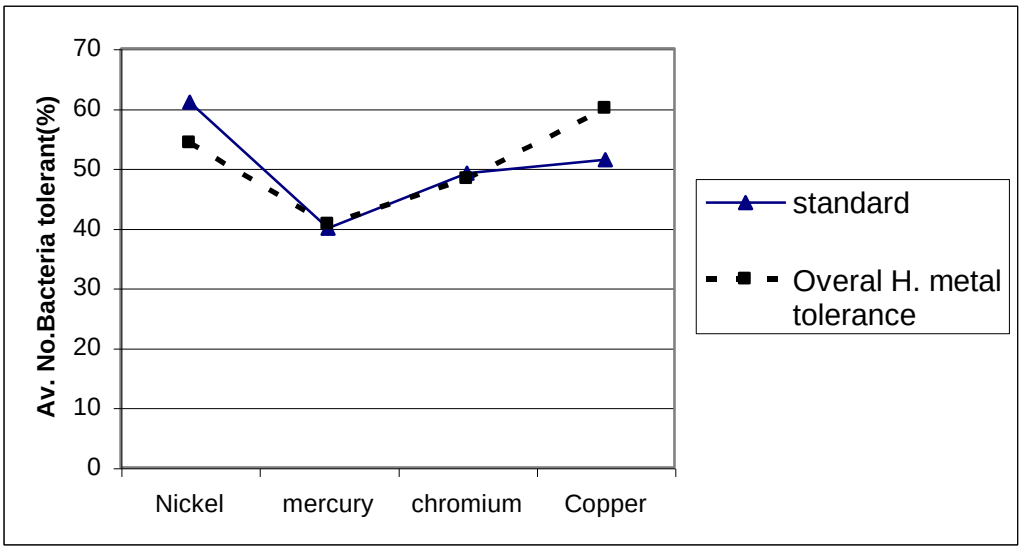

Figure 5: Lake Basin bacterial isolates tolerance to heavy metals and standard isolates from National Public Health Laboratories

4.7 Antibiotic resistance in the bacterial isolates from the Lake Basin

A total of ninety three bacterial isolates from the various study sites were tested with ten antibiotics to establish their levels of resistance to antibiotics (Table 13). A statistical analysis using ANOVA to find the variation in the number of bacteria resistant to antibiotics indicated a significant difference in resistance to various antibiotics, $F, = 35.743$, $p < 0.05$.

Table 13. Antibiotic susceptibility of the bacterial isolates from the lake basin

Antibiotics	**Resistant isolates**	**Sensitive isolates**	**Intermediate isolates**
Ampicillin	49 (53%)	36 (38.7%)	8 (8.6%)
Tetracycline	58 (62%)	21 (22.6%)	14 (15.1%)
Cotrimoxazole	60 (65%)*	27 (29.0%)	6 (6.5%)
Augmentin	41 (44%)	26 (27.9%)	26 (27.9%)
Kanamycin	2 (2.1%)	78 (83.9%)	13 (13.9%)
Gentamicin	1 (1.1%)	92 (98.9%)	0 (0%)
Cefuroxime	63 (67.7%)*	25 (26.9%)	5 (5.4%)
Chloramphenicol	28 (30.1%)	55 (59.1%)	10 (10.8%)
Nalidixic acid	32 (34.4%)	11 (23.7%)	39 (41.9%)
Norfloxacin	1 (1.1%)	74 (79.6%)	18 (19.4%)

(*) Denotes the most resisted antibiotics

The environmental isolates from all the sites were highly sensitive to gentamicin (98.9%), kanamycin (83.9%) and norfloxacin (79.6%) (Table 12). Cefuroxime had significantly higher bacterial resistance recorded (67.7%) followed by cotrimoxazole (65%), tetracycline (62%) and ampicillin (53%). A small number of the isolates were intermediate to nalidixic acid (41.9%) and augmentin (27.9%)

The highest incidence of antibiotic resistance was recorded in isolates from River Nzoia, both its lower and upper parts (44.0% and 40.1%) (Table 14). Isolates from Upper

Nyando and Chemelil effluent similarly recorded a marked antibiotic resistance (40.0% and 35.7%). River Yala recorded the least incidence of resistance to antibiotics (upper 24%, lower 29.8%).

Table 14. The average percentage bacterial resistance to antibiotics in all the sites (N=93)

Site	Mean % ± SE	Rank (Site with the Highest % resistant bacteria)
R. Kisat	33.3 ± 7.63	7
R. Mbogo	33.4 ± 9.13	6
Chemelil effluent	35.7 ± 9.27	4
Upper Nyando	40.0 ± 9.43	3
Lower Nyando	34.0 ± 9.58	5
Upper Yala	24.0 ± 6.53	11
Upper Yala	29.8 ± 6.85	10
Upper Nzoia	40.1 ± 10.15	2
Lower Nzoia	44.0 ± 11.27	1
R. Woroya	30.3 ± 8.0	8
Hippo Point River	30.0 ± 10.41	9

The certified reference materials (isolates) from the National Public Health Laboratories (NPHL) tested with the same antibiotics showed similar trends of resistance as the lake basin environmental isolates. Cefuroxime, cotrimoxazole and ampicillin recorded the

highest incidence of resistance at 71% each. 86% of these isolates were sensitive to gentamycin and 71% sensitive to chloramphenicol (Figure 6).

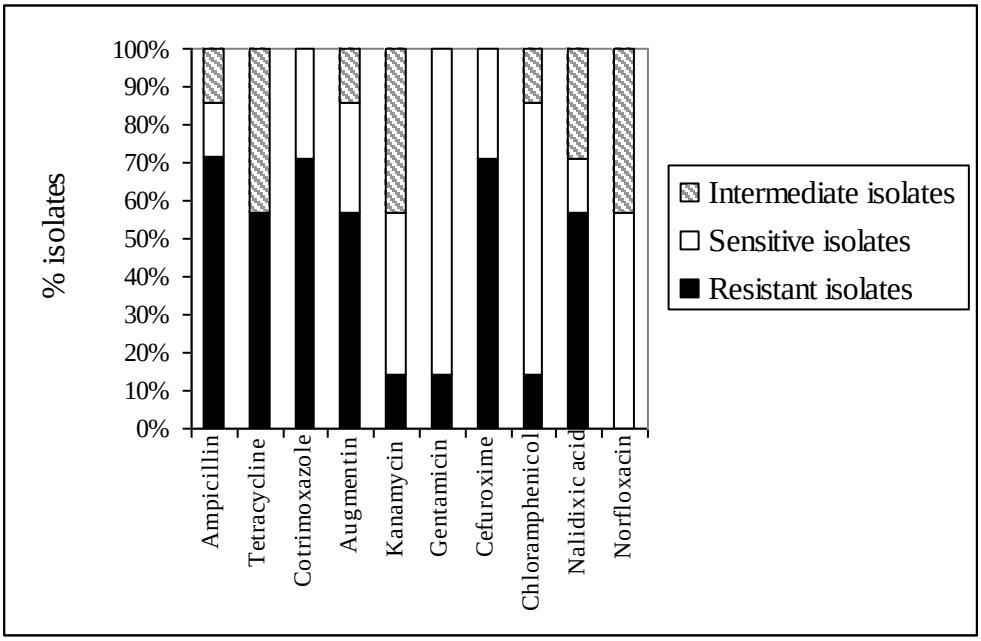

Figure 6: CRM percentage numbers of resistant, sensitive and intermediate levels to the antibiotics

4.8 Multiple drug resistance in the bacterial isolates

Fifty three (53.8%) of bacterial isolates from all the sites showed resistance to four or more of the antibiotics tested (MDR) (Table 15). The highest incidence of multiple drug resistance (80%) was observed in isolates from River Nzoia lower. Similarly River Nyando (upper), River Nzoia (upper) and the Chemelil effluent recorded marked incidence of multi-drug resistance of 70%, 66.6% and 58.3% respectively. Isolates from River Yala (lower) and River Nyando (lower) showed the least incidence of multi-drug

resistance of 33.3% and 37.5%. The isolates were mainly resistant to cefuroxime, cotrimoxazole, tetracycline, ampicillin and augmentin. 71% of the CRM isolates recorded almost similar trends of multi-drug resistance with *Pseudomonas aeruginosa* showing resistance to nine of the ten antibiotics tested.

Table 15. Number of bacterial isolates showing resistance to 4 or more antibiotics (multiple drug resistance) from all the sites

Site	**Total No. of isolates**	**% multi-drug resistance**
R. Kisat	9	4 (44.4%)
R. Mbogo	9	4 (44.4%)
Chemelil effluent	12	7 (58.3%)
Upper Nyando	10	7 (70%)
Lower Nyando	8	3 (37.5%)
Upper Yala	5	2 (40%)
Upper Yala	9	3 (33.3%)
Upper Nzoia	9	6 (66.6%)
Lower Nzoia	10	8 (80%)
R. Woroya	8	4 (50%)
Hippo Point River	4	2 (50%)

4.9 Association of metal tolerance and antibiotic resistance in the bacterial isolates

Association between metal tolerance and antibiotics resistance was observed in three metals. Tolerance to chromium and resistance to cefuroxime was highly significant (r =

0.878, P = 0.000). Nickel tolerance was also significantly related to percentage resistance to cefuroxime (r = 0.672, P = 0.0230). Mercury tolerance was significantly related to resistance to ampicillin (r = 0.683, P = 0.020). Resistance to other antibiotics was not significantly related to tolerance to these metals. Tolerance to copper was not significantly related to resistance to any of the antibiotics tested.

4.10 Antibiotic resistance in heterotrophic bacteria, enteric pathogens and coliforms

A total of fifty three heterotrophic isolates, thirteen isolates of coliform bacteria and twenty five isolates of enteric pathogens were investigated (Figures 8, 9 and 10). An analysis of variation (ANOVA) showed that there was no significant difference ($P > 0.05$) in antibiotics resistance in the three bacterial groups. However, there was higher number of resistance by heterotrophic bacterial isolates than in both coliform and enteric pathogens. Similarly more coliform bacterial isolates were resistant than Enteric pathogens. This study showed that the number of resistant bacterial isolates significantly differed with the number showing sensitive and intermediate responses, $F_{87} = 11.782$, $P < 0.05$.

The heterotrophs were 100% sensitive to kanamycin and gentamicin but recorded 84.9% resistance to cefuroxime (Figure 7). The most resistant of these isolates were from both water and sediment samples drawn from River Nzoia, Chemelil effluent, River Mbogo and upper Nyando.

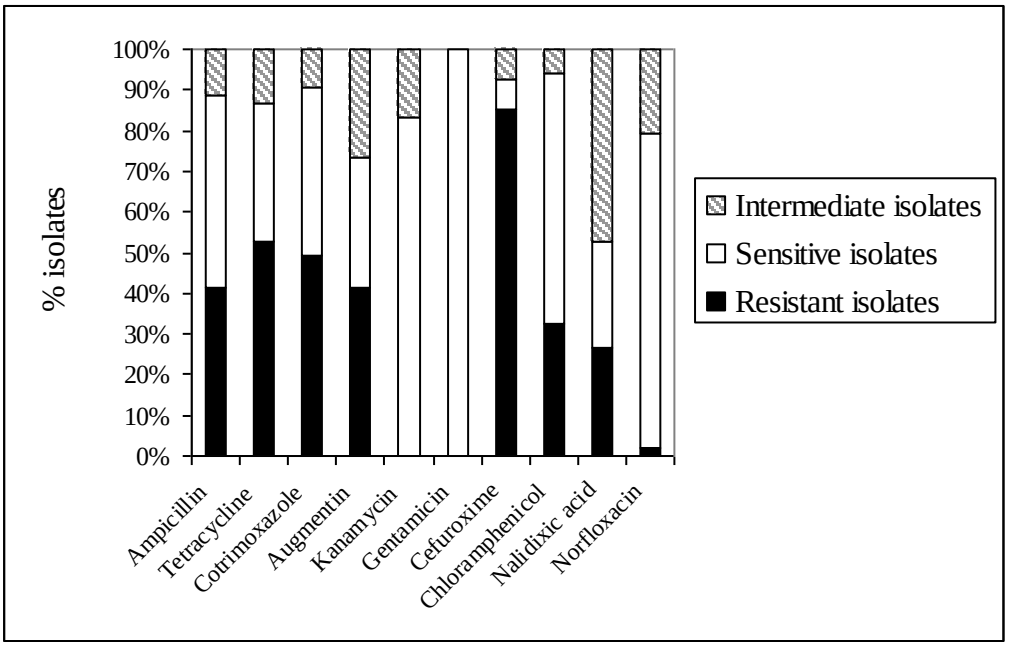

Figure 7: Antibiotic resistance in heterotrophic bacteria

The coliform bacteria recorded a remarkable resistance to cotrimoxazole (92.3%) but showed similar sensitivity to kanamycin and gentamicin as the heterotrophs (Figure 8). Coliforms isolated from River Nzoia (water) and Chemelil effluents (water) were the most resistant.

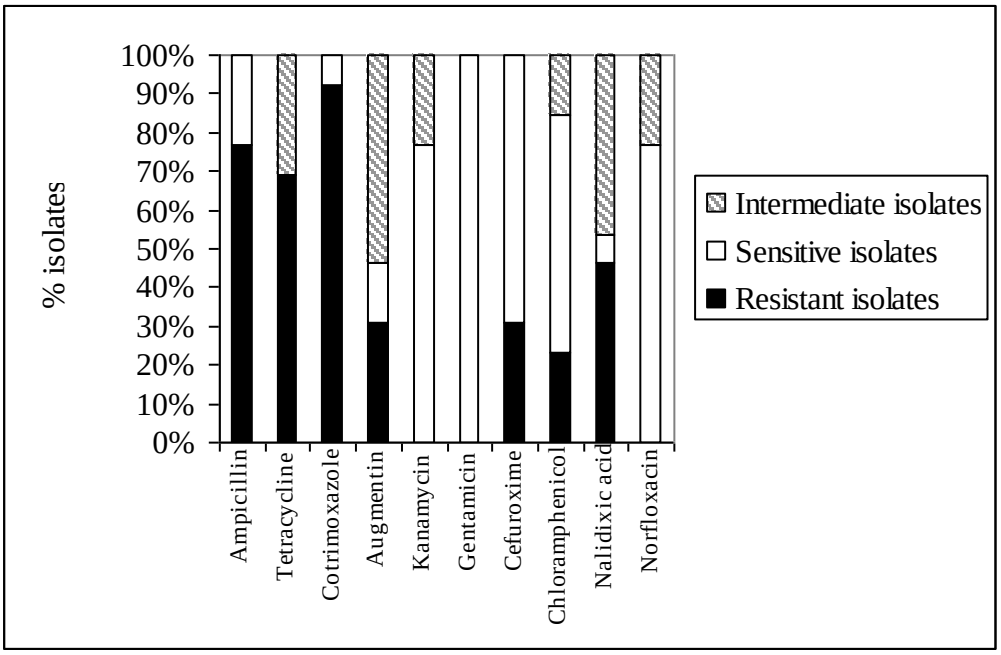

Figure 8: Antibiotic resistance in Coliform bacteria

Multiple drug resistance was similarly recorded among the enteric pathogens (Figure 9). These microorganisms had marked resistance to tetracycline (76%), ampicillin (68%), cotrimoxazole (68%) and augmentin (68%). They were however highly sensitive to norfloxacin (100%), gentamicin and kanamycin. The most resistant enteric pathogens were isolated from water and sediment samples drawn from River Kisat, Chemelil effluent, River Nzoia and River Yala.

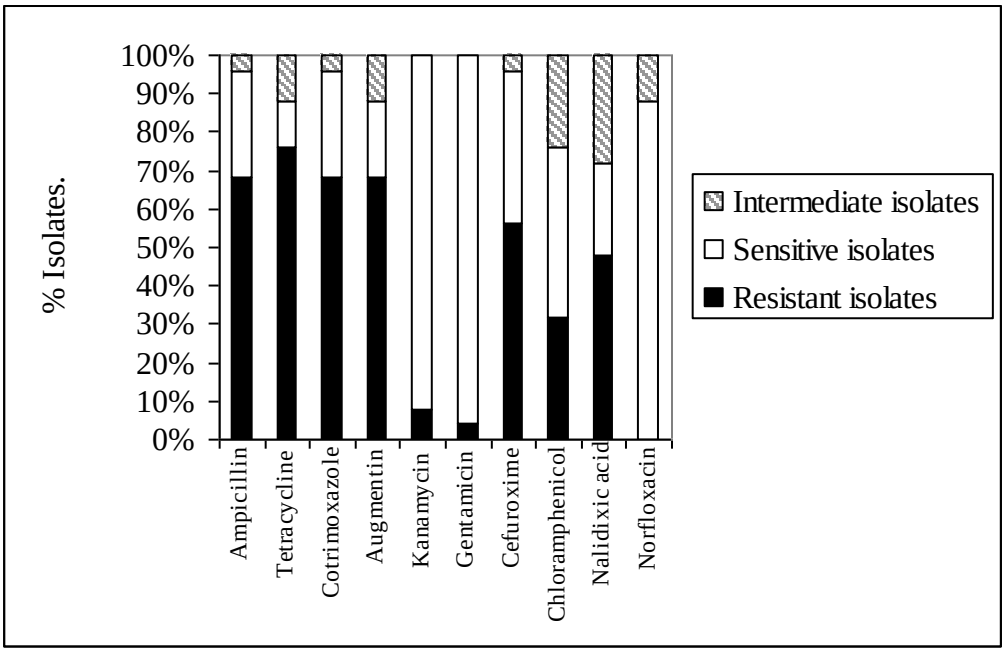

Figure 9: Antibiotic resistance in enteric pathogenic bacteria

The study showed no significant difference in multidrug resistance in the three groups of bacteria (F =1.75 P = 0.191 P > 0.05）. However, multidrug resistance in the entero-pathogenic bacteria (mean % multi drug resistance 65.15%) was higher than in heterotrophs (mean 46.52%) and coliform bacteria (mean 36.36%) (Figure 10).

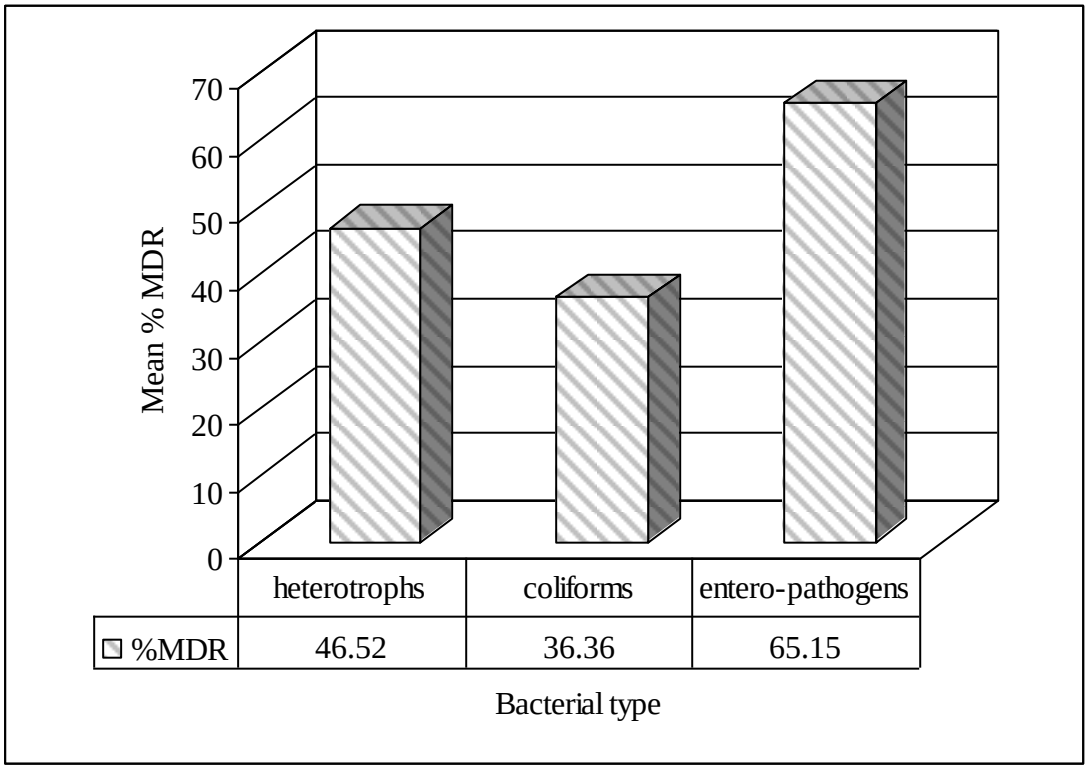

Figure 10: Mean percentage MDR in the three bacteria groups

4.11 Plasmid DNA isolation

The multiple drug resistant isolates were screened for the presence of plasmid DNA (Plate 1). A number of isolates yielded plasmids whose molecular sizes ranged from about 28.5 to 0.8 mDa. A *salmonella* species (isolate no.090) from R. Kisat carried two plasmids at the level of about 3.4 mDa on the *E.coli* v517 standard marker and 0.8 mDa. Another unidentified isolate (no 118) from R. Mbogo carried two plasmids of about 3.6 and 2.7 mDa. Isolate number 108 (unidentified) from the sediments of R.Nyando (lower) yielded two plasmids estimated to be about 28.5 and 3.6 mDa. A number of some other bacteria from some of the other sites carried a plasmid each while other strains did not

show presence of plasmid DNA (Table 16). The study established that there was a positive relationship in number of isolates carrying plasmids and the number having multiple drug resistance (r = 0.372, P = 0.261), However the relationship was not found to be significant at 95% CI. Sites having higher number of isolates showing multidrug resistance also had higher number of isolates carrying plasmids.

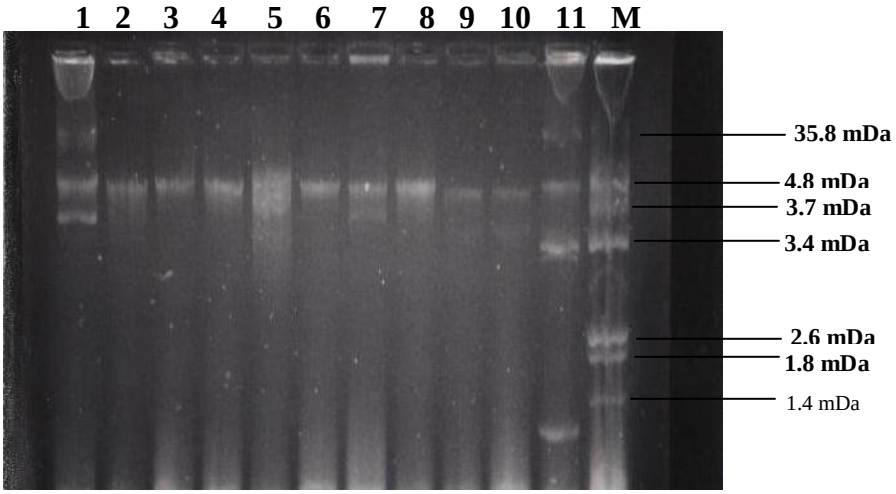

Plate 1: A photograph of the plasmid DNA of the multiple drug resistant bacteria.
Lane M is the control *E. coli* strain V517

Table16. Plasmid DNA of the multiple drug resistant bacteria from the study sites

Serial number	Number of plasmids	Plasmid size (mDa)	Source/River
007	0		Chemelil effluent
004	0		R.Kisat
009	1	3.5 mDa	R.Mbogo
036	0		Lower Yala
014	0		Upper Nyando
023	0		Upper Nzoia
071	1	3.7 mDa	R.Woroya
090	2	3.4 mDa 0.8 mDa	R.Kisat
046	1	2.3 mDa	Lower Nzoia
118	2	3.6 mDa , 2.7 mDa	R.Mbogo
043	1	1.8 mDa	Upper Nyando
069	1	2.6 mDa	Upper Nzoia
108	2	28.5 mDa, 3.6 mDa	Lower Nyando
040	0		Lower Nyando
037	0		Lower Nyando
073	0		Upper Yala
010	0		R.Mbogo
053	0		R.Mbogo

CHAPTER FIVE

5.0 DISCUSSION

Physico-chemical characteristics influence the growth and diversity of microbial populations. The high water temperature at the Upper River Nyando was probably due to the high concentration of total dissolved solids as the same river also recorded the highest concentration of the total dissolved solids. Turbid waters absorb heat hence are likely to be warmer (Kato, 1941; Khan and Siddiqui, 1970). The pH of the water in the rivers was generally neutral or slightly alkaline. Neutral pH is suitable for growth of bacteria such as *Caulobacter* spp, *Gallionella* spp, and *Pseudomonas* spp, which predominate in streams with low nutrient composition. However with increased pH levels there is a tendency of bacteria to die (Mwachiro, 1993).

The low dissolved oxygen at lower Yala was possibly due to the higher water temperature. The solubility of oxygen decreases with increasing temperature (Ellis, 1989). The low dissolved oxygen can as well be attributed to the sluggish flow of the water as it enters Yala Swamp. The high value of total dissolved solids (TDS) at the Upper River Nyando was possibly due to dumping of industrial wastes, as this part of the river is fed by streams carrying effluent from the Chemelil sugar factory. Although electrical conductivity is a function of total dissolved solids (Rainwater and Thatcher, 1960; Cole, 1979) this study observed that River Nyando (Upper) which had the highest total dissolved solids recorded a low electrical conductivity. However its lower part

which recorded high total dissolved solids had the highest electrical conductivity. It is probable that the total dissolved solids recorded at lower Nyando are more of inorganic origin as this area is a rice farming zone where chemical herbicides, fertilizers and pesticides are used on the farms. Some of the metal concentrations detected in the wetlands of the Lake Victoria basin were above the permissible limit by the World Health Organization (WHO, 1985) and the Kenya Bureau of Standards (KEBS, 1996). The levels of Manganese, cadmium and lead were above the permissible limit by WHO (1985) and KEBS (1996). Therefore metal pollution in the wetlands can be attributed to these elements. The levels of zinc and copper were below the critical limit of WHO and KEBS, hence there is no metal pollution in the wetlands studied which is due to these metals.

Chemelil sugar factory effluent and River Kisat recorded the highest levels of heavy metals indicating these are hot spots of pollution from industrial discharge and municipal wastes. The occurrence of cadmium and lead in the wetlands is of grave concern due to their toxic effects. The results obtained in this study are to a certain extent consistent with data reviewed by Biney *et al*. (1994) who reported that metal concentrations in African rivers and lakes were lower than those reported in other parts of the world. They also found out that there was no difference between coastal and inland waters. This study showed a relationship between the amounts of heavy metals in the rivers and the bacterial counts, where the sites with high concentrations of heavy metals in the water also had high levels of bacterial cell counts. It is probable that since the heavy metal content was

relatively low in water compared to the sediments, some of these levels were appropriate for use by the water micro organisms in their metabolic processes. Eiland (1981) observed that at certain concentrations some metals like copper, zinc, cobalt and nickel are essential for microorganisms since they provide vital cofactors for metallo- proteins and enzymes. However the trend was not observed in sediments possibly due to elevated heavy metal levels in sediments which may have inhibited microbial growth due to toxicity.

Most of the isolates were able to tolerate high concentrations of nickel sulphate, though very few tolerated the highest concentration tested. The microorganisms were able to grow in concentrations much higher than those detected at the sites. Isolates from upper Yala were less tolerant to nickel ions possibly due to the low level of nickel detected at the site compared to Chemelil where high levels of nickel were detected and this was reflected in the isolates being the most tolerant to nickel which may have been due to selection pressure.

Although Mercury was not analyzed in the field samples, a significant number of the isolates were found to tolerate high concentrations of mercuric chloride, suggesting that mercury tolerance may have been linked with tolerance to other metallic ions. A similar observation was also made by Basu *et al*, (1997). A study done by Boga *et al*. (2007) in the wetlands of the Lake Victoria basin found that 47.04% of the isolates were tolerant to mercury and 89.9% were tolerant to lead. Multiple heavy metal resistance determinants,

namely cd-co-zn genes (*czc*) the co-ni-cr genes (*cnr*, *chr*) and the Hg (*mer*) have been isolated from plasmids (Nies, 1992). The combined resistance to Ni, Cr and Zn was reported by Margesin and Schinner (1996). Low levels of chromium were detected at some of the sites, however of the total isolates tested for chromium tolerance, a significant proportion tolerated high $K_2Cr_20_7$ concentration. Tuhina *et al.* (2000) analyzed tannery effluents for chromium and nickel. They obtained organisms tolerant to a higher level of chromium than the chromium present in the effluent. Chemelil effluent was the most polluted site and it is probable that due to combined resistance to metal ions, isolates from this site were tolerant to chromium. Tolerance to these ions was highest in River Mbogo where the effluent from Chemelil sugar processing plant empties before discharging into River Nyando.

Most bacterial strains studied tolerated copper sulphate concentrations which were far much higher than the concentration detected at the study sites. Isolates from upper Nyando were more tolerant to copper ions though low levels of copper were detected at the site. This tolerance may be attributed to combined resistance to other metal ions which may have resulted in selection pressure among the microbial populations over time.

Its worth noting that the following increasing order of toxicity Hg>Cr>Ni>Cu realized in this study does not differ from that reported in other studies. Duxbury (1981) made a similar observation. The isolates investigated in the present study were tolerant to three or

the four metals tested and all the sites studied had multiple metal tolerant isolates. Dressler *et al.* (1991) found that copper resistant *Alcaligenes* strains isolated from waste water also contained resistance determinants for zinc, calcium, chromium and mercury although the corresponding metal ions were not used for selection. Timoney *et al*. (1978) also demonstrated linkage between Hg^{2+}, Zn^{2+} and Cd^{2+} tolerance. Multiple metal tolerance occurs because toxic metals do not occur singly in the environment. Usually a major cation is accompanied by other ions. For instance zinc is often accompanied by cadmium while nickel is accompanied by cobalt and chromium. In response to this challenge multiple metal ion tolerant bacteria have evolved which contain a variety of plasmid encoded metal tolerance determinants (Dressler *et al*., 1991).

The rivers recorded high coliform counts possibly due to inadequate and malfunctional sewage treatment systems in towns next to the river points from where samples were drawn. Some of the places are high density residential areas and due to inadequate or no sewage systems, most of the human and animal wastes ends up in streams and eventually into the rivers especially during the rain season as it was witnessed during this study .

In this study the lowest coliform counts were recorded at lower Nyando and lower Yala, these are agricultural areas with minimal sewage pollution. At these points the rivers have gone through a stage of self-purification and most of the coliforms have died. Similarly at this lower part of the rivers there is increased water volume and hence its dilution. Dilution effect could have contributed to the low coliform count. The coliforms recorded

in all the river points sampled exceed the world health organization (WHO, 1985) and Kenya Bureau of Standards (KEBS, 1996) recommendations that total coliforms of drinking water should not exceed 10 per 100ml of water. Boga *et al*. (2007) studied wetlands south of the Lake Victoria basin and made a similar observation. The water in all the rivers studied is therefore polluted and unfit for human consumption.

The high viable counts recorded from River Kisat, effluent from Chemelil sugar factory and River Mbogo suggest that there is more pollution in these rivers. Raw sewage from the malfunctional Kisumu sewage treatment plant and effluent from the town flows into Lake Victoria through River Kisat. This increases the biochemical oxygen demand indicating the presence of large numbers of microorganisms. The higher the BOD the higher the usage by aerobic microbes (Vesilind *et al.,* 1990). Effluent from Chemelil sugar processing plant empties into River Mbogo carrying pollutants of both organic and inorganic origin which contribute to increased BOD and hence high microbial numbers.

The lower parts of River Nyando and River Nzoia recorded the lowest viable counts possibly due to increased dilution downstream which lowers the biochemical oxygen demand hence lowering the heterotrophic bacterial populations. The high counts recorded in sediment samples from the same sites, Kisat and Chemelil effluent are possibly due to high levels of pollutants embedded more in sediments than in water as the same sites also recorded the highest level of heavy metal concentrations, some of which may have been essential for microbial growth.

It has been observed that antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment (Hassan, 1985). The varying concentrations of the different antibiotics are based on the degree of toxicity exerted by a particular antibiotic on a microbial cell depending on the target site, where different antibiotics are required in relatively low concentrations to exert either bactericidal or bacteriastatic effect.

In this study resistance to aminoglycosides such as kanamycin and gentamicin which are commonly used was quite low. This is inline with the observation made by Boga *et al*. (2007). Flouroquinolones such as norfloxacin which is also quite commonly in use recorded an equally low resistance. Resistance to ampicillin, augmentin and chloramphenicol was modest (53%, 44% and 30.1%). A similar pattern of resistance to ampicillin among Gram negative bacteria from the lake basin was observed by Boga *et al.* (2007). Cefuroxime, Cotrimoxazole and tetracycline are extensively used and the bacterial isolates tested in this study showed a marked resistance to these antibiotics (67.7%, 65% and 62%).

The widespread occurrence of drug resistant microorganisms especially pathogens in our environment has necessitated the need for regular monitoring of antibiotics susceptibility trends to provide the basis for developing rational prescription programs, making policy decisions and assessing the effectiveness of both (Omigie *et al.,* 2006).

Semi-treated and untreated effluent of industrial and municipal origin from the towns of Webuye, Mumias, Ugunja market and other urban centers flows to River Nzoia through its tributaries. Chemical fertilizers and herbicides used in sugar cane farming and other subsistence crops such as maize and vegetables also find their way to River Nzoia. This possibly results in microbial selection for metal tolerance and hence high incidence of multiple antibiotic resistance recorded in this river.

There is evidence of correlation between tolerance to heavy metals and antibiotic resistance (DeRore *et al.,* 1994). Association between nickel and chromium tolerance with resistance to cefuroxime, mercury tolerance and ampicillin resistance was recorded in this study. Microorganisms tolerant to metals and resistant to antibiotics have been isolated by other workers from clinical and metal contaminated environment such as estuaries, soils and sewage (Henriette *et al*., 1991). Sabry *et al.* (1997) reported multiple antibiotic resistance of a natural bacterial population isolated from a polluted marine environment.

They showed that in the population there was association of resistance between zinc and ampicillin, copper and penicillin, cobalt and ampicillin, cadmium and erythromycin, Nickel and ampicillin as well as mercury and bacitracin. Calomiris, *et al*. (1984) observed a positive correlation between tolerance to Cu^{2+}, Pb^{2+} and Zn^{2+} and multiple antibiotic resistance. They also reported that water isolates resistant to Kanamycin were also tolerant to Cu^{2+}, Pb^{2+} and Zn^{2+}.

Microorganisms undergo selection pressures in the presence of toxic compounds and develop resistance (Hideomi *et al*., 1977). The most common resistance is to metal and antibiotics, which can be a result of bio-essentiality or of abuse of the metal and/or antibiotics. Metal tolerance is known to be associated with antibiotic resistance (Belliveau *et al*., 1991). Both resistances are carried on the same plasmid and are transferable among organisms through conjugation or transduction (Nakahara *et al*., 1977). Metal resistance was reported before the use of antibiotics and is therefore postulated to have evolved earlier (Ji and silver, 1995).

Effluent from Chemelil Sugar factory and wastes from Chemelil urban centre find their way into River Nyando. Chemical fertilizers and herbicides used in sugar cane and rice farming in these areas eventually reach the river. The high levels of metals detected in these sites may have contributed to high incidences of antibiotic resistance. Metal pollution leads to the emergence of metal resistant bacteria and the associated multiple antibiotic resistance (Bhattacherjee *et al.*, 1988).

The results obtained in this study are consisted with the data collected by other workers. Bhattacherjee *et al.* (1988) reported that multiple antibiotic resistant bacteria always occurred in polluted downstream water. This is inline with the high incidence of multidrug resistance recorded in lower Nzoia River which is far downstream as the river nears L. Victoria. This however is contrary to the findings from River Yala (lower) and

R. Nyando (lower) where the least incidences of multiple drug resistance occurred despite substantial levels of metal pollution, Implying metal pollution did not impact on antibiotic resistance. Enteric pathogenic bacteria recorded the highest incidence of multiple drug resistance in the present study followed by heterotrophic bacteria. Coliforms recorded the lowest incidence. The results obtained by other workers in similar studies vary.

Boon and Cattanach (1999) compared the antibiotic resistant native and feacal bacteria isolated from rivers, reservoirs and sewage treatment facilities. They found that the incidence of resistance to ampicillin, chloramphenicol, kanamycin, nalidixic acid, and streptomycin was greater in native heterotrophic bacteria than *E.coli* isolated from Yarra River in Australia. In contrast Jones *et al*. (1986) reported that *E. coli* Isolated from English lake waters had a greater incidence of antibiotic resistance than native aquatic bacteria.

The high incidence of resistance in bacteria isolated from sites away from human influence is possible due to the production of antibiotics by native bacteria which gives the bacteria a competitive advantage (Lancini *et al*., 1995). In this study the highest incidence of multidrug resistance in enteropathogens and coliforms occurred in R. Nzoia, R. Kisat and Chemelil effluent. These sites serve as the sink to excess untreated effluent of both industrial and municipal origin from the towns of Webuye, Kisumu, and from Chemelil sugar factory. Such effluent has elevated metal pollution which co-selects for

antibiotic resistance. Native heterotrophs registering high incidence of multidrug resistance occurred in R. Mbogo and Upper Nyando. These sites receive raw and semi-treated effluent directly from Chemelil sugar factor whose pollution of the sites may be contributing to antibiotic resistance. In the present study some of the antibiotic resistant isolates tolerated high concentrations of the metals tested, however a few isolates were found to carry plasmids. Some of the isolates found to be metal tolerant and multiple drug resistant did not carry plasmids. There is the possibility that metal tolerance genes are carried on chromosomes or transposons which were not investigated during this study.

Usually essential metal tolerance genes are chromosome borne while toxic metal tolerance systems are plasmid mediated (Bruins *et al*., 2000). Three bacterial isolates, two from R. Mbogo and one from R. Nyando (lower) were found to carry two plasmids each. These isolates tolerated high concentrations of the metals tested and were also multiple drug resistant suggesting that these characteristics may have been plasmid mediated. Multiple metal tolerance and multiple antibiotic resistance in Gram negative bacteria from the Lake Victoria basin was observed by Boga *et al.* (2007) although the relationship could not be established.

Previous studies have demonstrated the role of plasmids in conferring resistance to both antibiotics and metals. Mc Hugh *et al*. (1975) have shown plasmids conferring antibiotic and metal resistance to be present in *Salmonella typhimurium* isolates from human burn wounds treated with silver nitrate solution. Others have demonstrated genetic linkages

(presumably by plasmids) between antibiotic resistance in *Enterobacter aerogenes* and tolerance to Cd^{2+} and Zn^{2+} (Pickett *et al.*, 1976). Timoney *et al*. (1978) demonstrated linkage between Hg^{2+}, Cd^{2+}, Zn^{2+} and ampicillin resistance. Novick and Roth (1968) have demonstrated penicillinase producing plasmids of *S. aureus* to be responsible for resistance to erythromycin and various inorganic ions, including Cd^{2+}, Pb^{2+}, Hg^{2+} and Zn^{2+}. However, these researchers have emphasized that complicated sets of relationships exist between the host cell and the plasmid with respect to resistance to metals. For example, some *S. aureus* strains possessing plasmids conferring Cd^{2+} resistance were shown to mutate and become Cd^{2+} sensitive with the mutation not being plasmid linked. Also, *S. aureus* isolates not containing plasmids were shown to mutate and become Cd^{2+} resistant.

5.1 CONCLUSIONS

i. There are elevated levels of various metals like lead, manganese, zinc and copper in different environmental compartments within the Lake Victoria basin especially in Chemelil, R. Kisat, and R. Nyando

ii. The levels of these heavy metals are higher in sediments than in water.

iii. The presence of heavy metals in the water has enriched growth of the native heterotrophic bacteria but the elevated levels in sediments have adversely affected the heterotrophic bacteria.

iv. Exposure to varying concentrations of these heavy metals to the native environmental bacteria has led to evolution of metal tolerant strains.

v. The selection of metal tolerant bacteria is reflected in the high degree of resistance to common antibiotics like ampicillin, tetracycline, cotrimoxazole, and cefuroxime.

vi. Multiple drug resistance strains are abundant in the wetlands and this poses a potential health hazard and requires intervention measures.

vii. The pattern of antibiotic resistance in different bacterial groups from the wetlands is more or less the same which implies that different bacterial strains carry similar antibiotic resistance determinants.

5.2 RECOMMENDATIONS

i. Conjugation experiments which were not done in this study should be conducted to account for the frequent incidence of metal tolerance and antibiotic resistance.

ii. There is need for rehabilitation and maintenance of waste treatment facilities in Kisumu, Webuye and Chemelil in order to reduce pollution in the ecosystems.

iii. Environmental protection agencies should develop a long term comprehensive and coordinated river and lake water quality monitoring programme as a tool for water quality management/adopt and enforce water quality standards.

iv. Bring into enforcement all environmental related laws to protect and conserve the wetlands and natural resources within the Lake basin.

v. Communities/stakeholders should be sensitized on the significance of environmental management and conservation through various fora.

5.3 RECOMMENDATIONS FOR FURTHER RESEARCH

i. The study should be extended to cover areas where gold mining is ongoing to establish the impact of the activity on the environment due to use of mercury because use of a mercuric compound on microorganisms during the in-vitro experiments recorded the highest toxicity despite the study samples having not been analyzed for mercury.

ii. More flora and fauna including food crops and fish which are known to accumulate plenty of heavy metals should be sampled for heavy metal analysis to shed more light on the extent of metal pollution within the lake basin.

iii. Ground water sources such as wells and boreholes which were not included in this study should be analyzed for heavy metals and microbiological quality to assess the extent of the pollution levels.

iv. Molecular techniques should be used to characterize bacteria from the environment for accurate identification in order to take care of the limitations involved during use of biochemical tests.

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APPENDICES

Appendix 1a: Results of the identification of coliform bacteria using IMViC tests

Isolate code	Indole test	Methyl-red test	Voges-proskauer test	Citrate utilization test
043	+	+	+	+
044	-	+	-	-
045	-	+	-	-
046	-	+	-	-
047	+	-	+	+
048	-	-	+	+
049	-	+	-	-
050	+	-	-	-
051	-	+	+	+
052	-	+	-	-
053	+	+	+	+
054	+	+	+	+
055	-	+	+	+
056	+	-	-	-
057	-	+	+	+
058	+	+	+	+
059	-	+	-	-
060	-	+	+	+
061	-	+	+	+
062	+	+	-	-
063	+	-	+	+
064	-	-	+	+
065	-	-	-	-

Indole test	Methyl-red test	Voges-proskauer	Citrate test
+ Red colour change	+ Red colour	+ red colour	+ deep blue colour
- Yellow colour	- Yellow colour	- no colour change	- green colour

Appendix 1b: Motility test for *Salmonella* and *Shigella* species

Isolate code	Test result
066	Non motile
067	Non motile
068	Non motile
072	No growth
077	Non motile
079	Motile
080	Non motile
081	No growth
082	Motile
084	Non motile
085	Motile
086	Motile
088	Motile
089	Non motile
090	Motile
091	Non motile
092	Non motile

Non motile -*Shigella*
Motile - *Salmonella*

Appendix 1c: Confirmation of *Vibrio* species using oxidase test

Isolate code	Test result
093	+
094	+
095	-
096	+
097	+
098	+ after 30 seconds
099	+
100	+
101	+ after 30 seconds
102	+
103	-
104	-
105	+ after 30 seconds

+ Deep purple colour change
_ no colour change/white

Appendix 2: Heavy metal minimal inhibitory concentrations of the bacterial isolates–g/l

Metal salt	**Heavy metal concentrations used**				
Nickel sulphate	0.1	0.2	0.4	0.8	1.6
Mercuric chloride	0.025	0.05	0.1	0.2	0.4
Potassium dichromate	0.3	0.6	1.2	2.4	4.8
Copper sulphate	0.1	0.2	0.4	0.8	1.6

Site	**Isolate code**	**$NiSO_4$**	**$HgCl_2$**	**$K_2Cr_2O_7$**	**$CuSO_4$**
River Kisat	098	0.8	< 0.025	2.4	0.8
	105	0.8	0.05	2.4	1.6
	090	0.8	0.4	2.4	1.6
	050	0.8	0.2	0.6	1.6
	113	0.8	> 0.4	1.2	0.8
	001	0.8	0.4	2.4	1.6
	002	0.8	< 0.025	2.4	0.8
	003	0.2	< 0.025	1.2	0.8
	004	0.8	< 0.025	1.2	0.8
River Mbogo	010	0.8	1.4	> 4.8	1.6
	107	0.8	0.1	2.4	0.8
	118	0.8	0.4	2.4	0.8
	011	0.8	0.4	2.4	0.8
	114	0.8	0.05	2.4	0.8
	012	0.8	0.1	4.8	1.6
	009	0.8	0.2	0.6	1.6
	094	0.8	0.05	0.6	0.8
	103	0.8	0.05	1.2	0.8
	059	0.8	0.4	2.4	0.8

Minimal inhibitory concentrations continued...

	Isolate no.	$NiSO_4$	$HgCL_2$	$K_2Cr_2O_7$	$CuSO_4$
Chemelil effluent	100	0.8	0.05	1.2	1.6
	064	0.8	< 0.025	0.6	1.6
	049	0.8	0.4	1.2	0.2
	109	0.8	0.1	1.2	0.4
	110	0.4	0.2	> 1.6	> 4.8
	115	>1.6	0.05	4.8	1.6
	005	1.6	< 0.025	1.2	0.8
	006	0.4	0.4	0.6	0.2
	007	0.8	< 0.025	2.4	0.4
	008	0.8	0.1	2.4	0.8
	083	0.8	0.4	4.8	1.6
	086	0.8	0.05	0.6	0.4
	099	0.4	< 0.025	2.4	0.8

Upper Nyando	Isolate no.	$NiSO_4$	$HgCl_2$	$K_2Cr_2O_7$	$CuSO_4$
	043	0.8	0.05	> 4.8	1.6
	013	0.8	0.1	2.4	0.4
	014	0.8	0.4	2.4	0.8
	015	0.8	0.4	0.6	1.6
	016	0.8	0.1	1.2	1.6
	093	0.8	0.05	< 0.3	0.8
	102	0.4	0.4	2.4	0.8
	063	0.4	0.05	1.2	1.6
	052	0.8	0.05	2.4	1.6
	111	0.4	0.05	1.2	1.6
Lower Nyando	081	0.8	0.4	2.4	0.4
	108	0.8	< 0.025	2.4	0.8
	120	0.8	< 0.025	2.4	0.8
	092	0.8	0.4	2.4	0.4
	061	0.2	0.4	0.6	1.6
	037	0.8	0.2	4.8	0.4
	038	0.8	0.2	2.4	0.2
	039	0.8	0.05	2.4	0.8

	040	0.8	> 0.4	2.4	0.8
Upper Yala	017	0.4	< 0.025	< 0.3	0.2
	018	0.4	< 0.025	< 0.3	0.2
	019	0.4	<0.025	0.6	0.8
	044	0.2	< 0.025	0.6	0.4
	073	0.2	< 0.025	< 0.3	1.6
	116	0.8	< 0.025	0.6	0.4
Lower Yala	045	0.8	0.05	0.6	0.4
	070	0.4	< 0.025	2.4	0.8
	079	0.8	0.05	1.2	0.8
	106	0.8	0.05	2.4	0.2
	122	0.8	< 0.025	1.2	0.8
	033	0.8	< 0.025	2.4	0.8
	034	0.2	< 0.025	1.2	<0.1
	035	0.8	0.05	2.4	0.8
	036	0.4	0.1	2.4	0.8
	028	0.8	< 0.025	2.4	0.8

Upper Nzoia	Isolate	$NiSO_4$	$HgCl_2$	$K_2Cr_2O_7$	$CuSO_4$
	097	0.8	0.1	< 0.3	0.8
	069	0.4	0.4	0.6	0.8
	088	0.8	0.05	0.6	0.8
	060	0.8	0.1	1.2	1.6
	112	0.2	0.4	2.4	1.6
	119	0.4	< 0.025	2.4	1.6
	020	0.4	0.2	1.2	0.8
	021	0.4	< 0.025	1.2	< 0.1
	022	0.2	0.4	1.2	0.8
	023	0.4	> 0.4	4.8	0.2
Lower Nzoia	046	0.8	0.2	2.4	0.8
	095	0.8	0.2	2.4	0.8
	101	0.4	0.4	0.6	1.6
	090	0.4	0.4	2.4	> 1.6
	057	0.4	0.2	1.2	1.6
	051	0.8	> 0.4	2.4	1.6
	123	>1.6	0.05	1.2	1.6
	117	0.4	0.05	2.4	0.2
	024	0.8	> 0.4	2.4	0.8
	024	1.6	> 0.4	2.4	1.6

	026	0.8	0.1	2.4	0.8
	027	0.8	0.1	2.4	0.8
River Woroya	096	0.8	0.1	2.4	0.8
	0.8	0.4	0.2	1.2	0.8
	121	1.6	0.2	1.2	0.8
	029	0.2	< 0.025	< 0.3	0.4
	030	0.8	0.05	2.4	0.8
	031	0.8	> 0.4	1.2	1.6
	032	0.8	> 0.4	4.8	1.6
	071	0.8	0.05	1.2	0.8
Hippo Point	115	0.8	> 0.4	4.8	0.4
	055	0.8	0.1	1.2	1.6
	041	0.8	< 0.025	2.4	0.4
	042	0.8	0.05	2.4	0.4
NPHL(CRM)	125	0.8	0.05	1.2	0.4
	126	0.8	< 0.025	1.2	0.8
	127	1.6	0.4	2.4	0.8
	128	0.4	0.05	1.2	0.8
	129	0.8	0.1	2.4	0.8
	130	0.8	0.2	2.4	0.4
	131	0.8	0.2	1.2	0.4

Certified reference materials from the National public Health Laboratories (CRM-NPHL)

Code	**Isolate**	**Source**
125	*E.coli*	ATCC no 25922
126	*Pseudomonas aeruginosa*	ATCC no. 27853
127	*Shigella flexneri*	local isolate
128	*Klebsiella pneumoniae*	WHO
129	*Salmonella typhi*	N2202
130	*Bacillus subtilis*	local isolate
131	*Staphylococus aureas*	ATCC no. 20591

Appendix 3: Standards and guidelines for Metals in Drinking Water - mg/l

	WHO	KEBS	CEC	USA
Manganese	0.1	0.1	0.02	-
Zinc	5	5	0.1	-
Copper	1	0.1	0.1	
Cadmium	0.005	0.005	-	0.005
Lead	0.05	0.05	-	0.05
Iron	0.3	0.05	0.05	-
Chromium	0.05	0.05	-	0.02
Aluminum	0.2	0.1	0.05	0.2
Calcium	-	250	100	-
Magnesium	-	100	0	-
Sodium	200	200	20	-
Potassium	-	-	10	-
Mercury	0.001	0.001	-	0.001

WHO (1985), Guidelines for drinking water quality Vol. 1 Recommendations
KEBS -Kenya Bureau of Standards (1996) Kenya Standard. Specification for drinking water
CEC- council of the European Communities, Council directive relating the quality of intended for human
consumption (80/778/ECC)
USA -US Environmental protection agency national primary and secondary drinking water regulations.

Appendix 4: Molecular Biology buffers

Buffer	Components	Specification
Cells resuspension buffer (Solution I)	Tris	50 MM
	EDTA	10 MM
	pH	8 (adjust with HCL)
Cells lysis buffer (Solution II)	NaOH	200 MM
	SDS	1%
		Store at room temperature
Neutralization buffer (Solution III)	Potassium acetate	3.1M
	pH	5.5 (adjust with acetic acid) store at room temperature