JIMMA UNIVERSITY INSTITUTE OF HEALTH SCIENCE

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DEPARTMENT OF ENVIRONMENTAL HEALTH SCIENCE AND TECHNOLOGY

Human Health Risk Assessment Associated with Consumption of Vegetables Grown on Land Irrigated with Polluted Urban River Water in Jimma Town, South West Ethiopia

A Thesis Submitted to Jimma University, Institute of Health, Faculty of Public Health, Department of Environmental Health Science and Technology; in Partial Fulfillment for Requirements of Masters of Science in Environmental Science and Technology

BY Gutama Haile

November, 2018

Jimma, Ethiopia

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SCHOOL OF GRADUATE STUDIES

As thesis research advisors, we hereby certify that we have read and evaluated this thesis prepared under our guidance by Gutama Haile: Human Health Risk Assessment Associated with Consumption of Vegetables Grown on Land Irrigated with Polluted Urban River Water. We recommended that it be submitted as fulfilling the thesis requirement.

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ABSTRACT

Urban polluted river water used for irrigation can be contaminated by heavy metals and pathogenic microorganisms which contaminate soil and vegetables grown by it which brings health risk to consumers those consumed contaminated vegetables. The objective of this study was to assess human health risk associated with consumption of vegetables grown on land irrigated with polluted urban river water in Jimma town, south west of Ethiopia. The study was conducted in Jimma town from March to May 2018. The samples were collected during dry season for both heavy metal and microbial analysis from three sites (upper, middle and lower sites) and one control sample for each source from outside Awetu river catchment. The concentration of heavy metals was analyzed by atomic absorption spectrophotometer and microbial contaminants was analyzed following standard procedures. Health risks associated with these heavy metals were assessed based on total hazard quotients: that can be derived from concentrations of heavy metals in vegetables consumed in the area. The concentration of heavy metals in edible parts of vegetables increases in vegetables grown in downstream when compared with the upper stream metal concentration for both cadmium and lead. The mean metal concentrations for Pb were above the safe limits of world health organization's standards, while the mean Cd concentration was below safe limits. Health risk for Cd and Pb possess no potential risk to the local inhabitants through consumption of contaminated vegetables grown in the area as the value for total hazard quotient was less than 1, but the long term accumulation of these metal gradually increase the concentration in the environment and along the food chain accumulates in the body and thus can cause serious health problems. The study on microbial contaminants of vegetable indicates that all the vegetable samples were contaminated and none of them met the world health organization maximum permissible level for raw eaten vegetable consumption. The contaminated river water used for irrigation contaminated the vegetable. Thus, detail risk assessment should be conducted from production to consumption in order to provide complete intervention in reducing microbial diseases from vegetables.

Key words: Health risk, Heavy metals, Microbial contaminants, Soil, Vegetable, Water

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LIST OF ABBREVIATIONS AND ACRNOMYS

AAS	Atomic Absorption Spectrophotometer
ANOVA	Analysis of Variance
APHA	American Public Health Association
Cd	Cadmium
CFU	Colony Forming Unit
DMI	Daily Metal Intake
EC	Electrical Conductivity
EPA	Environmental Protection Agency
FAAS	Flame Atomic Absorption Spectrophotometer
FAO	Food and Agricultural Organization
HQ	Hazard Quotient
HRI	Health Risk Index
IDL	Instrumental Detection Limit
KIA	Kligler Iron Agar
LOQ	Limit of Quantification
MDL	Method Detection Limit
MSA	Mannitol Salt Agar
Pb	Lead
PCA	Plate Count Agar
PPM	Parts Per Million
RfD	Reference Dose
SD	Standard Deviation
SIM	Sulfur Indole Motility
SRM	Standard Reference Material
TF	Transfer Factor
THQ	Total Hazard Quotient
USEPA	United State Environmental Protection Agency
USPHS	United States Public Health Service
WHO	World Health Organization

CHAPTER ONE: INTRODUCTION

1.1 Background of the study

It is estimated that up to one-tenth of the world's population eats food cultivated by using wastewater. As population continue to grow and more freshwater is diverted to cities for domestic use, of which about 70% later returns as wastewater thus increasing the use of wastewater both in terms of the areas irrigated and in the volume applied.(Khan et al., 2011)

Food safety is a major public health concern worldwide. During the last decades, the increasing demand on food safety has stimulated research regarding the risks associated with consumption of food stuffs contaminated with pathogenic microorganism. (Benti et al., 2014)

Some heavy metals are essential for proper plant growth but the others are not essential so after accumulating in the soil they could be transferred to food chain and cause harmful effects. (Khan et al., 2013)

Vegetables are important ingredients in the human diet and contain essential nutrients and trace elements that have potential health benefits. (Deribachew et al., 2015). Heavy metal in vegetables is of growing concerns since some soils and irrigation waters are demonstrated to be polluted. Vegetables easily take up heavy metals and accumulate them in their edible parts. Once vegetables containing high levels of heavy metals are consumed by human, such metals can cause several clinical and physiological problems. (Pan & Jiang, 2016)

Long-term irrigation with wastewater leads to a build-up of heavy metals in soils and foods. Exposure of vegetables or plant products to various metal containing components has varying health implications. (Deribachew et al., 2015)

Presence of pollutants like heavy metals in urban and industrial wastewaters results in contamination of water and soil. Household effluents, drainage water, business effluents, atmospheric deposition and traffic related emissions transported with storm water into the sewage system carry a number of pollutants and enrich the urban wastewater with heavy metals (Getahun & Selassie, 2013)

The polluted river water being used for growing vegetables in the nearby areas of the cities without knowing their adverse impacts on the life of consumers (Daud et al., 2017). The access to clean water for irrigating vegetables is a major challenge. Consequently, urban and pre urban

vegetable farmers have no other choice than to use water from these contaminated and highly polluted sources. This raises public health concerns due to possible crop contamination with pathogens where vegetables are eaten uncooked (Amoah et al., 2006). In developing countries, continued use of untreated wastewater and manure as fertilizers for the production of vegetables is a major contributing factor to contamination that causes numerous foodborne disease outbreaks. (Benti et al., 2014).

Fresh vegetables and herbs, including those of the leafy variety, have been implicated as vehicles for the transmission of microbial foodborne disease worldwide. (WHO, 2008)

Large amount of waste substances, effluents, fertilizers, waste from pigments and industries, chemicals and energy are introduced into the environment through several sources. Some of these substances contain heavy metals such as cadmium, lead, and mercury, which are known to be toxic to human and wildlife. Generally, most heavy metals are not biodegradable; they have long biological half-lives and have the potential for accumulation in the different body organs leading to unwanted side effects. (Ogunkunle et al., 2014)

The use of indicator bacteria such as Fecal coliforms (FC) and Fecal streptococci (FS) for assessment of fecal pollution and possible water quality deterioration in fresh water sources is widely used (APHA, 1995). Currently, coliforms and E. coli are of great importance among bacterial indicators used in water quality definition and health risk. (Badawy & Osman, 2013)

A study conducted on vegetables cultivated by contaminated water (Lettuce and Carrots) was positive for Salmonella, Vibrio spp and *E. coli* following irrigation with water that also tested positive for the same pathogens. (Hedberg et al., 1999). Application of irrigation water directly influences whether the organisms can be found associated with the edible portion of the plant at harvest (Green et al., 2008)

Study conducted on bacteriological quality of reclaimed wastewater showed the presence of Salmonella, Shigellae, *E. coli*, Enterobacteriaceae and other unidentified bacterial species. (Amimi et al., 2014). Bacteriological contaminant causes public health concerns due to possible crop contamination with pathogens where vegetables are eaten uncooked (Amoah et al., 2006).

Previous studies conducted in Ethiopia indicated that soils and vegetables grown in the Akaki river catchments contained elevated concentration of heavy metals and hence it is unsafe to use vegetables and forages grown under such environment for both human and animal consumption.

Study conducted on fresh vegetable irrigated with Awetu river shows high contamination of vegetable with microbial organisms which can potentially constitutes a health risk to consumers. (Woldegzina and Mulleta, 2016)

The use of sewage contaminated municipal wastewater for irrigation of vegetables is common practice in Ethiopia, and Jimma is one of the town found in Ethiopia which was known with the absence of appropriate sewage disposal system and cultivating vegetable with wastewater for irrigation purpose without any treatment, which may cause soil pollution and contaminate vegetable grown on it.

This study was undertaken to assess the extent of microbial contamination and selected heavy metal contaminants in soil and selected vegetables grown under irrigation with Awetu river in Jimma town. The study was very important as large number of people consume the product and no study have been conducted to show the extent of contamination of soils, water and vegetables on irrigation site and health risk assessment of the heavy metal intake from vegetable in Jimma town, Oromia region, Ethiopia.

1.2 Statement of the problem

Shortage of surface and ground water for irrigation is an ever increasing problem around the world, leading to use of wastewater for agriculture has become a common reality in three-fourth of the cities of Asia, Africa and Latin America. (Getahun & Selassie, 2013) Presence of pollutants like heavy metals in urban and industrial wastewaters results in contamination of water and soil.

The application of sewage and sometimes industrial effluent on to agricultural lands are common practices throughout the world. The long term irrigation with effluents are known to have significant contribution to trace elements such as Cd, Cu, Zn, Cr, Pb, and Mn in surface soil in the agricultural fields as well as it improves the Physico-chemical properties in soil.(Roy & Gupta, 2016)

Wastewater carries appreciable amounts of trace toxic metals which often leads to degradation of soil health and contamination of food chain mainly through the vegetable grown on such soils. The toxic elements accumulated in organic matter in soils are taken up by growing plants and lastly exposing humans to this contamination. (Pradesh & Shukla, 2013)

Heavy metals contamination is a major problem of our environment and they are also one of the major contaminating agents of our food supply. This problem is receiving more and more attention all over the world, in general and in developing countries in particular. The wastewater contaminated soils have resulted in the growth of contaminated vegetables. (Chauhan & Chauhan, 2014)

Study conducted on the concentrations of some heavy metals in Spinach which were irrigated with treated and untreated wastewater of Bhiwadi industrial area by using Atomic Absorption Spectrometer (AAS) shows that all the metals studied were higher than those recommended by Food and Agricultural Organization (FAO) and the World Health Organization (WHO). (Sharma et al., 2014)

Study conducted on the concentrations of cadmium, chromium, iron and lead in water were recorded above the permissible limits set by WHO in water samples and the concentrations of heavy metals in soil were also above the permissible limits by WHO. (Nazir et al., 2015)

Study conducted by Al-jaboobi, Zouahri, et al (2014) on wastewater use have shown that more than 85% of the applied heavy metals are likely to accumulate in the soil, most at the surface. Samples of water, soil and crops were collected and analyzed for lead (Pb), copper (Cu), chromium (Cr), cobalt (Co) and nickel (Ni) using the Atomic Absorption Spectrometer (AAS) and the results indicated that the levels of heavy metals in wastewater, soil and food crops were above acceptable limits. (Al-jaboobi et al., 2014)

Study conducted by (Getahun & Selassie, 2013) on heavy metals such as cadmium, copper, lead, nickel and zinc shows that soils and vegetables grown in contaminated water contained elevated concentration of heavy metals and hence it is unsafe to use vegetables and forages grown under such environment for both human and animal consumption.

(Yeshiwas, 2017)conducted study on concentration of heavy metals in soils, as well as on the vegetable grown in the vicinity of industrial areas and contaminated irrigation water indicated that Vegetables grown in such lands, contaminated with heavy metals and unsafe for consumption. Serious health problems may develop as a result of excessive accumulation of heavy metals and even essential trace elements such as Cu and Zn in human body. (Woldetsadik et al., 2017)

Study conducted on bacteriological quality of reclaimed wastewater used for irrigation tested by membrane filtration technique showed the presence of Salmonella, Shigellae, *E. coli*, Enterobacteriaceae and other unidentified bacterial species. (Amimi et al., 2014).The maximum overall means of aerobic mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, staphylococci, and total and fecal coliform counts were 8.06, 7.10, 6.54, and 2.97 log CFU/g and 1036 and 716 MPN100/ ml. (Weldezgina and muleta, 2016).

The studies conducted to assess the level of contamination of fruits and vegetables in Jimma Town collected from four different local markets in the town. The results of the study showed that samples collected from “Hirmata Merkato” (29.8%) had high contamination rate followed by samples collected from “Bishishe” (28.4%), “Agip” (22.1%), and “Kochi” (19.7%) markets. (Fanos & Belew, 2015).

As the above studies shown like (Getahun & Selassie, 2013) , (Yeshiwas, 2017) , (Woldetsadik et al., 2016), (Woldetsadik et al.,2017) there was bacteriological contaminants and deposition of

heavy metals in soil, crops and vegetables grown in the vicinity of industrial areas and urban wastewater in different parts of the Ethiopia. The publications from different parts of Ethiopia related to heavy metals contamination of soil; irrigation water and their transfer to vegetable crops grown in the vicinity of industrial areas and urban wastewater indicate the presence heavy metal contamination.

Jimma town is one of the town in Ethiopia which have no wastewater treatment plant and directly disposing the urban waste in the nearby river and the local community was using this contaminated water for irrigation purpose. Vegetable farm irrigated by contaminated water in Jimma town was one of the area in Ethiopia in which research work related to heavy metals contamination has not been performed so far and study conducted on microbial contaminant by Woldegzina has not performed on the corresponding soil contaminants and health risk assessment for heavy metal. The main objective of the present study focusses on the status of heavy metals accumulation and microbial load in some vegetables those grown by using Awetu river and assessing human health risk related to vegetable consumption of the community.

1.3 Significance of the study

Jimma town was one of the town in Ethiopia which have large number of dwellers and different institutions with different social and economic activities which can contribute for waste production with high rate. Even though the town has such huge organization there was no urban water treatment and the waste handling trend was also poor. The community surrounding the town was using the urban contaminated wastewater for irrigation purpose and they consume the waste water irrigated vegetables without any home based treatment options like washing and cooking practice.

Study conducted in different parts of the world and different towns of Ethiopia shows that the urban wastewater contains different heavy metals and pathogenic microorganisms and the heavy metal can accumulate in soil and intern it will transfer to the vegetables and finally human beings can accumulate this heavy metals during consumption and finally it will pose health effects to humans.

There was no related study conducted in Jimma town on the status of heavy metal contamination of contaminated water entering to Awetu river, land on which vegetable grow and vegetables. The proposed study of heavy metal and microbial analysis was expected to deliver a base line data on the levels of heavy metals in water used for irrigation, soil and vegetables in Jimma town and microbial contaminants in vegetables and their respective health risk quantification. Therefore, determination of level of heavy metals and microbial contaminants in the irrigation water is very important to ensure individuals health status.

This study will help for:

- To reduce the health risk associated with consumption of contaminated vegetable by communicating the result with concerned bodies.
- To reduce contaminant from the water, soil and vegetable by knowing the status of the contaminants and disseminating the result with municipalities.
- Local government officials can use for monitoring and control of the river contamination.
- To put base line data for the next researcher on similar title.

CHAPTER TWO: LITERATURE REVIEW

2.1 Heavy metals

Heavy metals are metals that have density more than 5mg/cm^3 and the name heavy metal was used to show their effect.(Marie et al., 2009). Since in very small concentration they are toxic in nature the name heavy metal was given to them. (Gebresilassie & Gebremedhin, 2015)

Some part of heavy metals are very important for proper plant growth but the others are not essential so after accumulating in the soil they could transfer to food chain and cause harmful effects. (Khan et al., 2013)

Heavy metal incorporates an important class of toxic substance which are encountered in numerous occupational and environmental circumstances. The impact of these toxic agents on human health is currently an area of intense interest due to the ubiquity of exposure. (Hamid et al., 2017)

Heavy metals are harmful as a result of their non-biodegradable nature, long biological half-lives and their potential to accumulate in different body parts. Low concentrations of heavy metals have damaging effects to man and animals because there is no established mechanism for their elimination from the body. (Khan et al., 2013)

Heavy metals referred as common pollutants, which are widely distributed in the environment with sources from weathering of mineral soil, fertilizers run off from agricultural activities, industrial effluents and urban wastewater. (Shirkhanloo et al., 2015)

Long-term application of treated and untreated wastewater resulted in significant buildup of heavy metals in soil and vegetables and cereals and their subsequent transfer to food chain causing potential health risk to consumers. (Perveen et al., 2012)

2.1.1 Cadmium:

Cadmium is a naturally occurring metal and it can enter the environment from natural and anthropogenic activities and stays intact for long periods of time. Food is the major source of cadmium exposure in the general population. Chronic exposure to cadmium may cause several adverse health effects, including renal and bone damage. (Nazir et al., 2015)

Study conducted by (Bernard ,2008) shows cadmium as one of the most toxic elements to which man can be exposed at work or in the environment. Once absorbed, Cd is efficiently retained in the human body, in which it accumulates throughout life and it is primarily toxic to the kidney, especially to the proximal tubular cells, the main site of accumulation. The chronic effects of Cd consist of lung cancer, pulmonary adenocarcinomas, prostatic proliferative lesions, kidney dysfunction, bone fractures, and hypertension.(Mahmoud & Ghoneim, 2016)

Cadmium (Cd) is a hexagonal crystal, silver white malleable and a d-block metal. This is a transition metal belonging to period 5 and group 12. It has atomic number 48, atomic mass 112.2, density 8.65 g/cm3, melting point 594 K and boiling point of 1038 K. Together with Hg and Pb, Cd is one of the big three heavy metal poisons and is not known for any essential biological function. (Wuana & Okieimen, 2011)

Cadmium occurs naturally at low levels in the environment. Food, rather than air or water, represents the major source of cadmium exposure, although tobacco smoking adds significantly to the body's burden. Additional cadmium has been added to the environment through industrial processes such as cadmium metal production. Further cadmium has been added to agricultural soils through the use of phosphate fertilizers.(WHO, 1989). Other sources include farmyard manure, sewage sludge, metal working industries, waste incinerators, urban traffic and atmospheric deposition; cement factories, electroplating, pigments, plastic stabilizers, nickel-cadmium batteries, etc. (Wir et al., 2005)

Cadmium is naturally present in the environment: in air, soils, sediments and even in unpolluted seawater. Cadmium is emitted to air by mines, metal smelters and industries using cadmium compounds for alloys, batteries, pigments and in plastics and Cadmium accumulates in the human body affecting negatively several organs: liver, kidney, lung, bones, placenta, brain and the central nervous system. (Simone et al., 2010)

Inhalation of cadmium fumes or particles can be life threatening, and although acute pulmonary effects and deaths are uncommon and cadmium exposure may cause kidney damage. Animal experiments have suggested that cadmium may be a risk factor for cardiovascular disease, but studies of humans have not been able to confirm this. However, a Japanese study showed an excess risk of cardiovascular mortality in cadmium-exposed persons with signs of tubular kidney damage compared to individuals without kidney damage. (Järup, 2018)

2.1.3 Lead

Lead is a common metal that has been in many consumer products but it is now known to be harmful to human health if ingested or inhaled. It can be found in lead-based paint, air, soil, household dust, food, some types of pottery, and drinking water. Children could show slight deficits in attention span and learning abilities. (WHO, 1985)

Lead is a well-known non-biodegradable toxic metal in the environment and now, it has become a global health issue Lead poisoning occurs when people are exposed to lead and chemicals that contain lead, breathing air, taking drinks such as water and milk, eating foods such as fruits, vegetables, meats, grains and seafood, swallowing or touching dust or dirt that contains lead. (Tiwari et al., 2013). Excessive intake of the Pb to human body can damage the nervous, skeletal, endocrine, enzymatic, circulatory, and immune system.(Mahmoud & Ghoneim, 2016)

Lead (Pb) is cubic crystal, silver blue-white, soft and a p-block metal. It is located in period 6 and group 14. Lead has atomic number 82, atomic mass 207.2, density 11.4 g/cm3, melting point 601 K and boiling point 2013 K. (Wuana & Okieimen, 2011)

Lead (Pb) can exist in several valences and are of critical environmental importance. In urban areas, the principal source of Pb in wetlands comes from gasoline additives, metal plating, e-waste and battery cells, electrical equipment, textile mills, dye and pigments, paper mills, chemical and fertilizer industries, and ghee manufacturing industries. Lead toxicity leads to anemia both by impairment of hemoglobin biosynthesis and acceleration of red blood cell destruction in human beings. (Jumbe & Nandini, 2009).

Lead has no essential function in man. Food is one of the major sources of lead exposure; the others are air (mainly lead dust originating from petrol) and drinking water. Plant food may be contaminated with lead through its uptake from ambient air and soil; animals may then ingest the lead-contaminated vegetation. In humans, lead ingestion may arise from eating lead-contaminated vegetation or animal foods.(Simone et al., 2010)

2.2.1 Physico- chemical constituents of Water sample

Electric conductivity and pH influences the heavy metal concentration in water and soil. Irrigation water quality depend upon Physico-chemical properties of the water like pH and electrical conductivity (EC) those which used to identify and quantify toxicants and to provide

data that used for regulatory purposes and could be compared to allowable concentrations for particular recipient water. (Badawy et al., 2013)

2.2.2 Heavy metal in water.

Study conducted by (Mahmoud & Ghoneim (2016) on some plants, soil, water, and sediment samples to evaluate the contamination by heavy metals showed that the heavy metals, in the water of Zefta drain exceeded permissible limits for irrigation. In rice and maize shoots grown in soils irrigated by contaminated water from Zefta, the bioaccumulation factors for Cd, Pb, Zn, Cu, and Mn were higher than the permissible level for heavy metals.

Wastewater disposed contaminated with trace elements like lead (Pb), copper (Cu), chromium (Cr), and manganese (Mn) etc. Many of which are non-essential and over time toxic to plants, animals and human beings. They affect human life, lives of related other useful living things like animals and plants. (Khan et al., 2011)

Release of untreated effluents in water resources have resulted in the buildup of trace metals in the environment and their transport in soil and food produce .(Hamid et al., 2016). Transfer of heavy metals from water to soil and subsequently uptake and accumulation in edible parts of vegetative tissue from soil represent a direct pathway for incorporation of heavy metals into the human food chain. (Bashir et al., 2015)

Study conducted by (Deribachew et al.,2015) in Harar on crops (cabbage, radish, chandaliya) irrigated with untreated sewage water revealed the presence of toxic metals like Pb, Cr, Cd, Ni, Fe, Co, Zn, Co, thereby reducing soil fertility and agricultural outputs and negatively affecting human health. Similar findings have been documented from a study conducted in Harare, Zimbabwe, where farmers use wastewater for irrigation increased the contamination of Cd, Pb, and Ni in the edible portion of vegetables, potentially causing health risk in the long term. (Abaidoo et al., 2010)

The main sources of pollution that enter surface water bodies are industries, municipal solid waste and oily wastes from garages and fuel stations. Most of the water resources are gradually becoming contaminated due to the addition of foreign materials from the surroundings. These include organic matter of plant and animal origin, land surface washing and industrial and

sewage effluents. Rapid urbanization and industrialization with improper environmental planning often lead to discharge of industrial and sewage effluents into rivers. (Arora et al., 2008)

Worldwide, it is estimated that 20 million hectares of arable land are irrigated with contaminated water and wastewater. In several Asian and African cities, studies suggest that agriculture based on wastewater irrigation accounts for 50 percent of the vegetable supply to urban areas. Waste water has deleterious effects on soil and it cannot be properly used for agricultural practices due to salinity and solidity problems which impose harmful effects on seedlings of plants. Most of the leafy vegetables which were grown in contaminated soil accumulate higher amount of heavy metals in their leaves. (Malik et al., 2011)

Wastewater irrigation may lead to transport of heavy metals to soils and may cause crop contamination affecting soil flora and fauna. Some of these heavy metals may bio-accumulate in the soil while others, e.g., Cd may be redistributed by soil fauna such as earthworms (Pattnaik & Reddy, 2011)

2.3 Heavy metal in soil

Heavy metal pollution in soils refers to cases where the quantities of the elements in soils are higher than maximum allowable concentrations and this is potentially harmful to biological life at such locations (Anbu et al., 2016). Heavy metals occur at typical background in all ecosystems, however, anthropogenic releases can result in higher concentrations of these metals relative to their normal background values hence the pollution, (Anbu et al., 2016). Heavy metals released from vehicular emission can accumulate in surface soils and their deposition over time can lead to abnormal enrichment, thus causing metal contamination of the surface soils. (Khoder & Ghamdi, 2012)

The use of commercial fertilizers, pesticides, soil conditioners and hormones to improve the quality and quantity of agricultural production can cause pollution of the soils at different levels. In addition, discharge of wastewater from various industrial activities and urban area into the streams or water bodies without treating and then using this contaminated water for irrigation accelerates the process of soil pollution. (Kocaman et al., 2015)

Certain trace elements are essential in plant nutrition, but plants growing in a polluted environment can accumulate trace elements at high contaminations causing a serious risk to human health when they are consumed.(Pradesh et al., 2013)

2.4 Heavy metal in vegetables

Vegetables contains an essential part of the human diet since they contain proteins, vitamins, as well as carbohydrates, minerals, and trace elements. Green leafy vegetables are predominantly known for their high nutritional content and are mostly consumed for health and nutritional benefits. (Nagrar, 2014)

There is an inherent tendency of plants to take up toxic substances including heavy metals that are subsequently transferred along the food chain (Singh et al., 2012). Contamination of foods by heavy metals has become a challenge for producers and consumers. The main sources of heavy metals to vegetable crops are their growth media (soil, air and nutrient solutions) from which these heavy metals are taken up by the roots or foliage. Vegetables can take up and accumulate heavy metals in quantities high enough to cause clinical problems to humans (Alam, 2014). Leafy vegetables grown on heavy metal contaminated soils accumulate higher amounts of metals than those grown in uncontaminated soils because of the fact that they absorb these metals through their roots. (Alam, 2014)

Emission of heavy metals from the industries and vehicles may be deposited on the vegetable surfaces during their production, transport and marketing. Similarly, atmospheric deposition can significantly elevate the levels of heavy metals contamination in vegetables commonly sold in the markets. (Alghobar & Suresha, 2015)

The uptake and bioaccumulation of heavy metals in vegetables is influenced by many factors such as climate, atmospheric depositions, the concentrations of heavy metals in soils, the nature of soil and the degree of maturity of the plants at the season of the harvest (Scott & Faruqui, 2004)

Consumption of high quantities metals in vegetables causes clinical problems both to animals and human beings consuming these metal-rich plants because there is no good mechanism for their elimination from the human body.(Roy & Gupta, 2016). In plants heavy metal toxicity is

the result of complex interaction of major toxic ions with other essential or non-essential ions. (Khan et al., 2013)

Vegetables are an important constituent of diet. Comparison of vegetables, fruits and other grain crops shows that heavy metals are largely accumulated in the edible parts of vegetables. Vegetables absorb and store high quantity of these harmful metals and become source of health problems when ingested by humans and animals. (Hamid et al., 2017)

The use of wastewater for irrigation increased the contamination of heavy metal in the edible portion of vegetables, potentially causing health risk in the long term. (Abaidoo et al., 2010)

The determination of heavy metals in cultural vegetables has shown that metals such as chromium (Cr), cadmium (Cd) and lead (Pb) were above the standard level which was reported by Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO). (Shirkhanloo et al., 2015)

Heavy metal contamination of urban waste in Ethiopia is the major problem as it was discussed in the above literature since the waste management and waste treatment was very little and the

publication and study conducted on such area was minimum. It needs great attention to conduct study on this area.

2.5 Health risk of heavy metals

Heavy metals can directly influence behavior by impairing mental and neurological function, influencing neurotransmitter production and utilization, and altering numerous metabolic body processes. Many cases of heavy metal burden are associated with industrial exposure and waste water, but our food, drinking water and environment do not appear to be getting any purer. (WHO/FAO, 2014)

Certain trace elements are essential in plant nutrition, but plants growing in a polluted environment can accumulate trace elements at high contaminations rate causing a serious risk to human health when they are consumed. (Pradesh et al., 2013)

Consumption of high quantities metals in vegetables pose clinical problems both to animals and human beings consuming these metal rich plants because there is no good mechanism for their elimination from the human body.(Roy & Gupta, 2016)

Serious systemic health problems can develop as a result of excessive accumulation of dietary heavy metals such as Cd, Cr, and Pb in the human body. Consumption of heavy metals contaminated food can seriously deplete some essential nutrients in the body causing a decrease in immunological defenses, intra uterine growth retardation, impaired psychosocial behavior, disabilities associated with malnutrition and a high prevalence of upper gastrointestinal cancer. (Nagrar, 2014)

Elevated concentrations of Cd, Cu, Co and Pb in food stuff are basis of diseases such as bone cancer, high prevalence of upper intestinal cancer, reproductive effects, hypertension and renal failure.(Hamid et al., 2017)

Acute heavy metal toxicities may damage central nervous function, the cardiovascular and gastrointestinal systems, lungs, kidneys, liver, (Abakpa et al.,2013) endocrine glands, and bones. Heavy metals with adverse health effects in human metabolism present obvious concerns due to their persistence in the environment and human health consequences. (Shirkhanloo et al., 2015)

2.6 Bacteriological contaminants

Food safety is a major public health concern worldwide. During the last decades, the increasing demand on food safety has stimulated research regarding the risks associated with consumption of food stuffs contaminated with pathogenic microorganism. Several studies have revealed that contamination of vegetables with pathogens poses a threat for consumers. (Benti et al., 2014)

The polluted river water in many parts of the world used for growing vegetables in the nearby areas of the cities without knowing their adverse impacts on the life of consumers. (Daud et al., 2017). Furthermore, farmers, consumers, and some government agencies in many countries are not fully aware of the potential impacts of irrigation with wastewater. (Amoah et al., 2007)

The access to clean water for irrigating vegetables is a major challenge. Consequently, urban and pre urban vegetable farmers have no other choice than to use water from these highly polluted sources. This raises public health concerns due to possible crop contamination with pathogens where vegetables are eaten uncooked. (Amoah et al., 2007). In developing countries, continued use of untreated wastewater and manure as fertilizers for the production of vegetables is a major contributing factor to contamination that causes numerous foodborne disease outbreaks. (Baggs et al., 2001).

Study conducted on microbial quality of irrigation water and irrigated vegetable Shows the counts of fecal coliform in the water and irrigated vegetables exceeded the 1,000 CFU/100 ml guideline for water used in fresh produce, and the presence of fecal indicator bacteria in the irrigation water and vegetable samples suggests fecal pollution raising the possibility of the presence of pathogenic microorganisms in these vegetables and a threat to public health. (Umoh et al., 2013). Common urban parasitic pathogens that may be associated with urban farming include round, hook, whip, and tape worms; dysentery; salmonella bacteria; cholera bacteria; and schistosomiasis. (Nasr, 2001)

The harmful human microbial pathogens most frequently detected in wastewater are of enteric origin which transmitted by direct contact to farmers and also to the general public through consumption of irrigated produce, especially crops eaten raw (Ackerson & Awuah, 2016)

The study conducted on microbial quality of lettuce cultivated by waste water from Addis Ababa shows that irrespective of the farming sites, almost all irrigation water samples had a poor microbiological quality. In the studied sites, there are a number of factors that might potentially cause contamination of irrigation water with relatively high levels of fecal coliform, in particular the inflow from untreated wastewater in to the river. (Woldetsadik et al., 2017)

2.7 Atomic Absorption Spectroscopy

The technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It requires a standard with known analyte content to establish the relation between the measured and the analyte concentrations and relies on Beer Lambert's law (Skoog et al., 2005). (Melville & Mortensen, 2014)

The sample is converted into atomic vapors by a process known as atomization. The precision and accuracy of this method depends on the atomization step and therefore a good choice of the atomization method is required. The two types of atomizers are continuous and discreet atomizers. In continuous atomizers the sample is fed into the atomizer continuously at a constant rate giving a spectral signal which is constant with time. Atomization methods that are of continuous type are flame, inductively coupled argon plasma and direct current argon plasma. With the discrete atomizers, a measured quantity of a sample is introduced as a plug of liquid or solid. The spectral signal in this case rises to a maximum and then decreases to zero. An electro thermal atomizer is one of the discrete types. (Colorado State, 2017)

CONCEPTUAL FRAME WORK

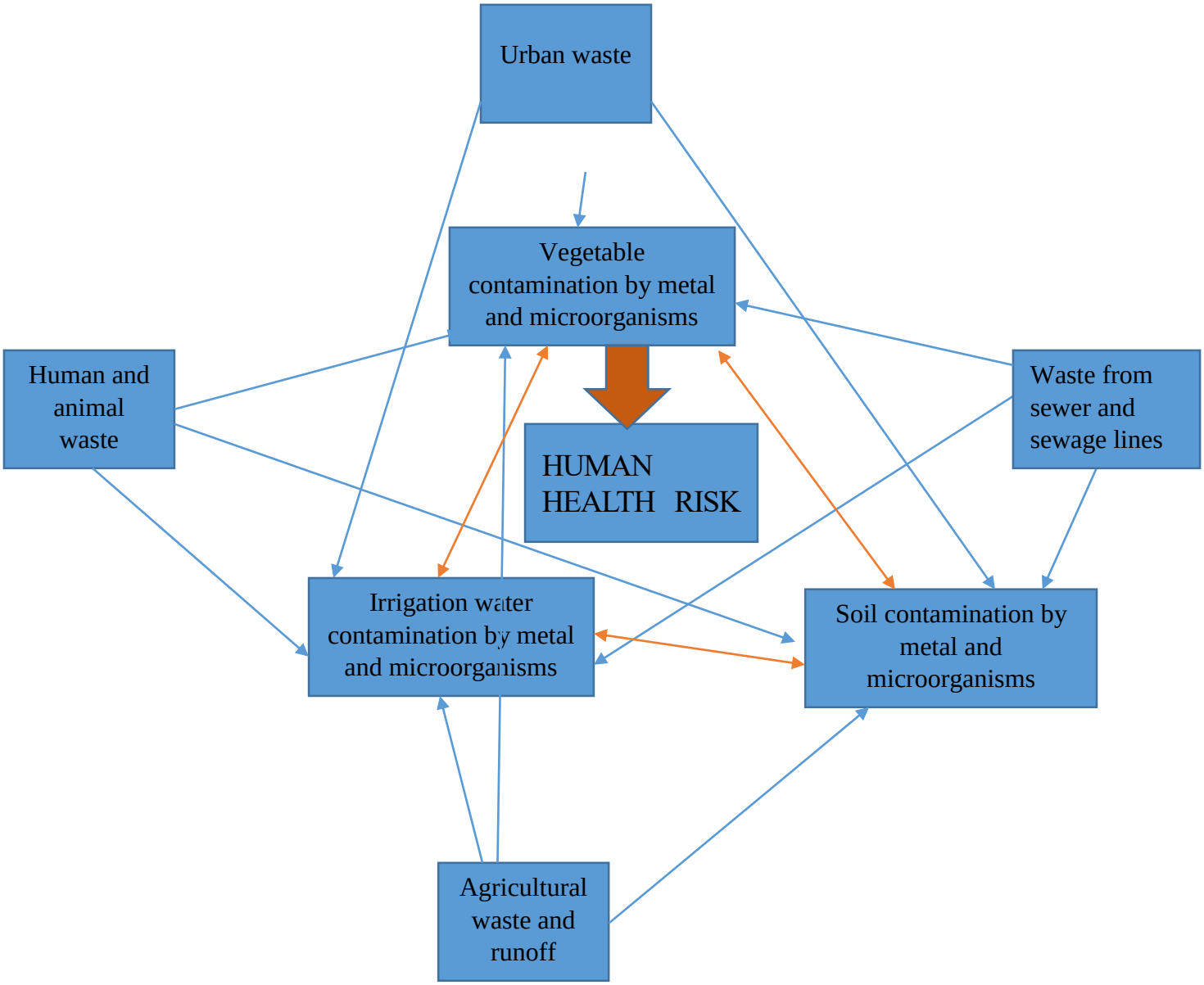

Figure 1: conceptual frame work

CHAPTER THREE: OBJECTIVE

3.1 General objective

The objective of this study was to assess human health risk associated with consumption of vegetables grown on land irrigated with polluted urban river water in Jimma town, south west of Ethiopia.

3.2 Specific objective

- To quantify the concentration of heavy metals (Cd and Pb) in water, soil and vegetable samples.
- To detect microbial contaminants of irrigation water, soil and vegetables samples.
- To assess the interaction between soil and vegetable metal contamination.
- To investigate the health risk of toxic metals through the consumption of vegetables.

CHAPTER FOUR: MATERIALS AND METHODS

4.1 Study Area

The study was conducted in Jimma town found at south western parts of Ethiopia located at 356 KM away from Addis Ababa the capital city of Ethiopia at $7^{o}40$" N latitude and $36^{0}60$`E longitude. Elevation within the town boundary ranges from the lowest 1720m from sea level of the airfield (kitto) to the highest 2010m from sea level at Jiren. It is bordered by Kersa Woreda in the east; with Manna Woreda in north, and Manna & Seka Chekorsa in west, Dedo woreda in south direction. According to Jimma town Finance and economic department 2014 annual report, the total population of the town was 192,256 from this male 94,205and female 98,051. The town have no modern sewerage system and the waste management system were very poor and there is no wastewater treatment plant in the town constructed by the municipality. The major socio economic activity of the town is trade, social service and urban agriculture. Concerning agricultural activities there is urban farming such as dairy, animal husbandry and poultry activities including town surrounding small scale irrigation which used wastewater and surface water as a source. There are 3 irrigation site around the town and they use wastewater as water source for irrigation, almost all of the cultivated vegetables around the town was used for human consumption in the raw form without cooking.

Map of study area

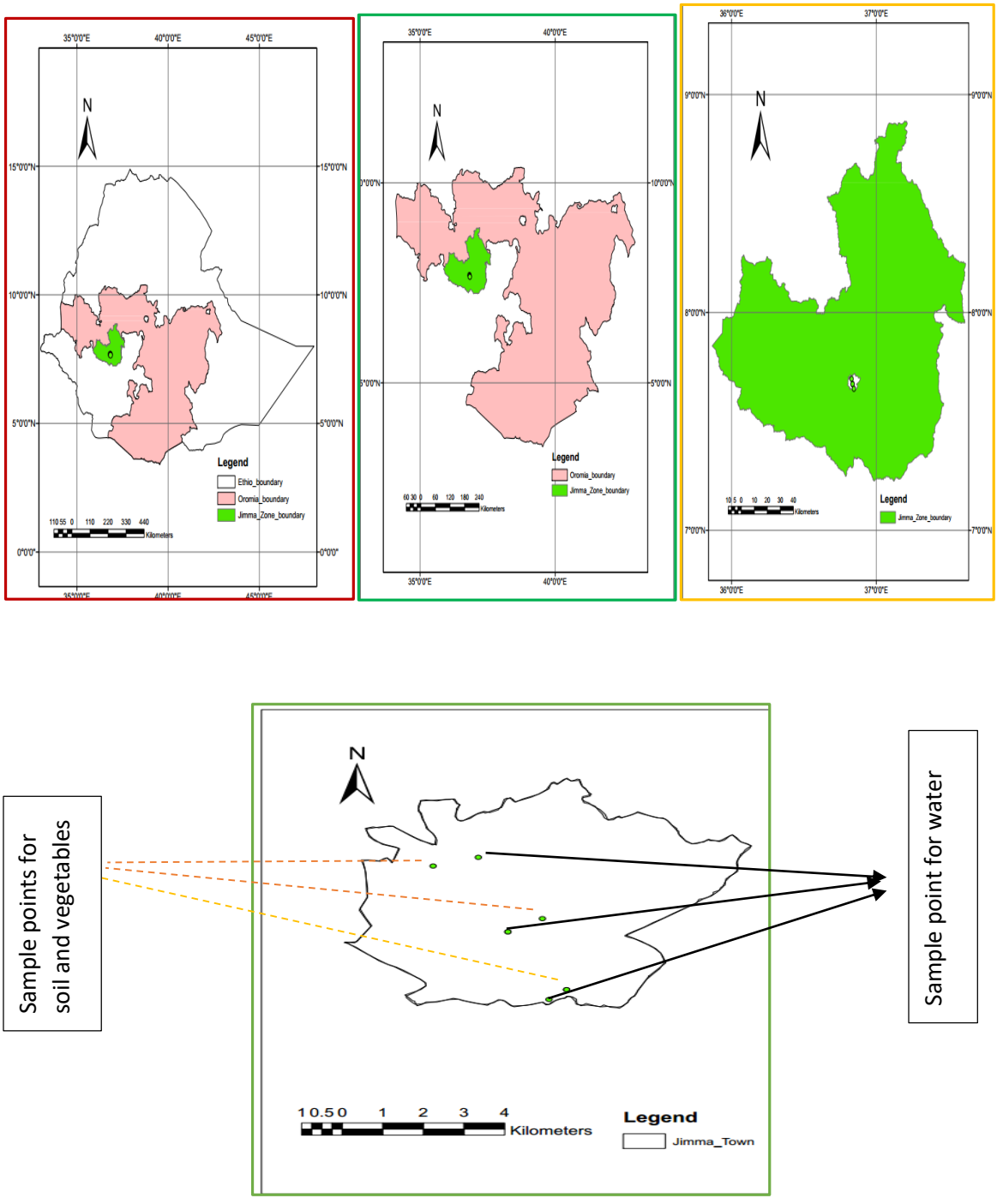

Figure 2: Map of the study area

4.2 Study Design and period

Laboratory based experimental study by atomic absorption spectrophotometer method for heavy metal and American Public Health Association, 2012 standard lab procedure method for microbial analysis used and the study was conducted from March to May 2018.

4.3 Experimental Methodology

Vegetables, soil and water samples was collected randomly from three different sites (upper, middle and lower) and control sample from non-urban water irrigated site was collected to analyze heavy metals i.e. Pb and Cd concentrations. Heavy metal analysis was carried out using atomic absorption spectrophotometer (APHA, 2012) and sample from similar site and source was taken for examination of bacteriological contaminants according guideline sated by American Public Health association (APHA, 2012).

4.4 Chemicals, Reagents and Instruments

4.4.1 Chemicals and Reagents

All chemicals and reagents used for the study was that of analytical grade. The chemical used for heavy metal analysis was HNO_3, 70% $HClO_4$, H_2SO_4, HCl, Stock standard solutions 1000 mg/L and 2% HNO_3, selected heavy metals were used for preparation of the working solutions (which were immediately prepared before analysis) for calibration and in spiking experiments. The glassware and polyethylene containers which used during analysis was washed with tap water, then soaked in 4 M HNO_3 solution and rinsed several times with deionized water. Different reagents such as, laurine sulfate, MacConkey agar, ethylene methylene blue, (EMB), nutrient agar, mannitol salt agar, MacConkey sorbitol agar, Kligner iron agar(KIA), Simon indole motility(SIM), Simon citrate, plate count agar, violet red bile glucose agar, peptone water, H_2O_2, crystal violet, Iodine, alcohol, safranin. Deionized water was used throughout the experiment for preparation and dilution of the sample solutions.

4.4.2 Apparatus and Equipment

The instruments used for this study was FAAS, technology with model no. 400 NOV for heavy metal determination of irrigation water, vegetable and soil samples and a Microprocessors based multi parameter probe (Hach-Model-HQ30d multi parameter digital meter) was used for the determination of pH and electrical conductivity.

The common laboratory apparatus which were used during the study include; different sized beakers, Erlenmeyer flasks, funnels, volumetric flasks, block digester, fume hood, centrifuge,

hydrometer, shaker, droppers, glass pipettes, spatula, measuring cylinders, plastic knife, vinyl gloves, stainless steel auger, stirrer, polyethylene bags, digital analytical balance, conical flasks and drying oven, vaccine carrier and ice box for sample transportation from field to laboratory and freezer for water samples. Freezer, incubator, microscope and safety cabinet was used in laboratory for microbial analysis.

4.5 Sampling and Sample Size

A composite sample was taken randomly from water used for irrigation, soil samples from irrigation site and selected vegetables. The total sample size for heavy metal and microbial analysis was shown in table1 and table 2 and the sampling target was contaminated water used for irrigation, soil contaminated by irrigation water and vegetables grown on contaminated soil and the selected vegetables was carrot, cabbage and tomato and one control sample each for irrigation water, soil and the selected vegetables,

Table 1 : Sample size and sampling source for heavy metal and microbial analysis

Sr. no	Media		Number of taste sample		Number of control sample		Total sample size	
			Sample for metal	Sample for microbial	Sample for metal	Sample for microbial analysis	Sample for metal	Sample for microbial analysis
1	Soil sample		3	9	1	3	4	12
2	Water sample		3	9	1	3	4	12
3	Vegetable sample	Carrot	3	9	1	3	4	12
		Tomato	3	9	1	3	4	12
		Cabbage	3	9	1	3	4	12
Total			15	45	5	15	20	60

4.6. Sample Collection and Preparation

4.6.1. Cleaning of Glassware and sampling materials

All sample containers and glassware used in the present study were washed in detergent and soaked in 30% nitric acid for 2 h to leach out adsorbed metal ion and then rinsed in tap water followed by deionized water before drying in dust free area (APHA, 2012).; The sampling material for microbial analysis was sterilized in the autoclave and all materials used was that of autoclavable.

4.7 Heavy metal analysis

4.7.1 Sampling and sample preparation for heavy metal analysis

4.7.1.1 Water sampling for heavy metal

The irrigation water samples were collected from upper, middle and lower points of Awetu river. A total of three taste samples in composite and one control sample were collected and measurement points for the sampling were designated as upper, middle and bottom. Water samples were collected at the upper point, middle point and lower or downstream (Singh et al., 2012). The water samples were filled in to plastic bottles (500 mL), which was rinsed with the sample water several times. Samples were collected in clean and dry polyethylene bottles. The bottles immediately acidified with 1 mL nitric acid, for later analysis of metal concentrations. The purpose of the acidification is to keep the metals in solution, to kill microorganisms and to avoid adsorption to the container walls (APHA, 2012; USEPA, 2002).

4.7.1.2 Soil sample collection and preparation for heavy metal analysis

About (100 g) Composite soil samples were collected from 10 cm, 15 cm and 20 cm depth (10-20) cm depth from the site where the vegetables were grown (for each site) with an auger from each point. (Roger, 1994) and the control soil sample was collected from area outside the Awetu river irrigated farm. Then the samples were placed in clean polyethylene bags and transported to the laboratory for analysis. Larger particles and other debris were removed from the soil and then soil samples were air dried in a dry and dust free place at room temperature (25 ^{0}C) for 5 days, followed by oven drying until getting constant weights. The samples were then grounded with a mortar and pestle to pass through a 2 mm sieve and homogenized. The dried, sieved, and homogenized soil samples were placed in polyethylene bags until the time of digestion.

4.7.1.3 Vegetable sample collection and preparation for heavy metal analysis

About 1000 g edible part of cabbage, carrot and tomato was collected from three different farming site along Awetu river based irrigation site and three composite subsamples were taken for collecting representative edible parts of the vegetables. The representative reputable samples were thoroughly mixed to give a composite sample as representative fraction of the vegetables. (Deribachew et al., 2015) The bruised or rotten portions were removed and the remaining samples were packed in polyethylene bags for transporting to the laboratory. In the laboratory, the collected vegetable samples were washed with tap water and then with distilled water to eliminate adsorbed dust and particulate matters. The vegetable samples were cut and chopped

into small pieces using plastic knife in order to facilitate drying. Accordingly, the samples were air-dried for six days and further dried in hot air oven at 105°C for 24 h, to remove moisture and maintain constant mass. The dried samples were ground into powder using acid washed commercial mortar and pestle and then sieved to 2 mm mesh size. The sieved samples were finally stored in polyethylene bags and kept in desiccators until the time of digestion.

4.8 Digestion of Soil, Water and Vegetable Samples for heavy metal analysis

4.8.1. Digestion of water sample for heavy metal analysis

The water samples from each sampling bottle were mixed thoroughly by shaking. A 50 mL filtered aliquot of water sample was transferred by pipet into a digestion flask. The metal percentage found in the water was determined by digesting in 3 mL concentrated HNO_3 and 3 mL H_2O_2 below 80 ^{0}C for 1 hour until a clear solution was observed. The clear solution was diluted to 100 mL volumetric flask with distilled water and blank digestion was also carried out in the same way. (Aga & Brhane, 2014). The blank solution contained all reagents except sample water. All samples were digested in triplicates and the digests were analyzed for the toxic heavy metals by using FAAS in Amhara Design and Supervision works Enterprise. The concentration of each metal was calculated using the formula below.(Aga & Brhane, 2014)

$$Final\ concentration\ (mg/L) = \frac{CM*DF*NV}{SV} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ Equation\ 1$$

Where: CM = Concentration of metal, DF = Dilution factor, NV = Nominal volume, SV= Sample volume (mL).

4.8.2 Digestion of soil samples for heavy metal analysis

The digestion of soil sample was performed by taking 0.5 g dried and homogenized soil samples transferred in to 100 mL digestion flask in triplicate. In each of these flasks, 5 mL of deionized water and 30 mL of a mixture HNO_3 (69%) and 37% HCl with volume ratio of 5:1 were added. The sample dissolved in the acid mixture was digested in digestion hood (at 200 ^{0}C) for 1 hour and kept to cool. After adding 2 mL of H_2O_2 to the cold digestion mixture, the final mixture was filtered out through What Man No. 42 filter paper to a 100 mL volumetric flask and finally diluted to the mark with distilled water (APHA, 2012; Loon,1985; Hizkeal,2012; Kedir, 2015).The varying filtrates obtained above were analyzed for the total content of each heavy metal by FAAS in Amhara Design and Supervision works Enterprise. The blank reagent was also digested following the same procedure as the taste soil sample.

4.8.3 Digestion of vegetable samples for heavy metal analysis

A 0.5 g of homogenized powdered vegetables sample was placed in borosilicate digestion flask to which 10 mL of acid mixture containing HNO_3- HCl-H_2O_2 (8:1:1, v/v/v) ratio were added. The mixture was heated at 120 ^{0}C over 3 hours on block digester. After digestion was completed, the clear and colorless solution was filtered out into 100 mL volumetric flask. Each digestion tube was rinsed with distilled water to collect any possible residue, and added to the volumetric flask and finally made up to volume with distilled water. All the dilute samples were stored in 100 mL plastic bottles (high density polyethylene) until analysis. Each vegetable sample was digested and analyzed in triplicate to confirm precision of the result. The blank solution was prepared by taking a mixture of 8 mL HNO_3, 1 mL HCl and 1 mL H_2O_2 and treating similarly as that of the sample (Street, 2008). Digestion of a reagent blank was performed along with the vegetable samples keeping all digestion parameters the same as that of taste sample. All the digested and diluted samples was stored in a refrigerator at 4ºC until analysis (Deribachew et.al., 2015). The heavy metal concentrations were analyzed by FAAS in Amhara Design and Supervision works Enterprise.

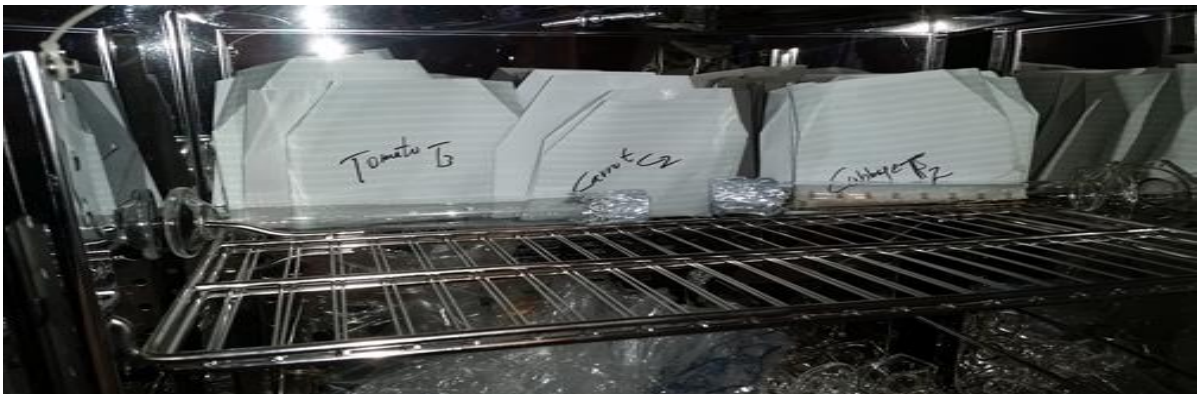

Figure 3: sample drying in oven for metal analysis

4.9 Calibration procedure

Calibration curves was prepared to determine the concentration of the heavy metals in the sample solutions. Intermediate standard solutions (10 mg/L) of each heavy metals were prepared from stock standard solutions containing 1,000 mg L^{-1} of selected heavy metals. Appropriate working standards were prepared for each of the metal solution by serial dilution of the intermediate solutions using deionized water. Each and every activity was adjusted according to the instrument operating manual to attain its better sensitivity and working standards was then

aspirated one after the other into the flame atomic absorption spectrometer and their absorbance were recorded. Calibration curves was plotted for each of the trace heavy metals standard using absorbance against concentrations (mg/L) (figure 18 and 19).

Table 2: Concentrations, absorbance and Coefficient determination for calibration curves

Metals	Concentration(ppm)	Absorbance	Coefficient of determination(R^2)
Cd	0.0,2.0,4.0,6.0,8.0	0.00,0.0537,0.1020, 0.1437,0.1802	0.9941
Pb	0.0,2.0,4.0,6.0,8.0	0.00,0.25594,0.53855, 0.90852,1.1720	0.9962

4.10 Physico chemical and trace metal analysis

3.10.1 Heavy metal analysis

The prepared transparent solutions of water, soil and vegetables samples was filtered through What man number 42 filter paper and diluted to 100 mL with distilled water. The concentrations of Cd, Pb in the filtrate was determined by using atomic absorption spectrophotometer in Amhara Design and Supervision works Enterprise. (APHA, 2012).

4.10.2 Physico chemical analysis of water and soil sample

Electrical conductivity is a measure of the ability of aqueous solution to carry an electric current that depends on the presence and total concentrations of ions, their mobility, valance and on the temperature. (Badawy et al., 2013). Electrical conductivity and pH were determined in soil with ratio of 1:1 and 1:2.5 soil to water ratio respectively, which was stirred for 30 minutes, allowed to stand for another 30 minutes, and stirred again for 30 second, the solution EC and pH were then measured.

4.11 Quality assurance

Appropriate quality assurance procedures and precautions was carried out to ensure reliability of the results. All chemicals used during analysis was that of analytical grade (AG) reagents. All solutions were prepared with double distilled water. Calibration standards for each metal was prepared by making appropriate dilution of stock solution of 1,000 ppm of E. Merck standards.(Roy & Gupta, 2016)

4.12 Quality control

Quality control measures was taken to assess contamination and reliability of data. For this Blank samples (zero metal concentration) was analyzed after fourth samples. Concentrations was calculated on a dry weight basis. All analysis was performed in triplicate. The accuracy and precision of metal analysis was checked against standards (Standard Reference Material) for every heavy metal.(Chauhan & Chauhan, 2014)

4.12.1 Accuracy

The accuracy of the method was determined by calculating percentage of recoveries. It was carried out by adding known quantity of analyte solution in to the sample by the proposed method. Spike recovery analysis of each metal was made to determine the recovery due to matrix effects.

$$R = \frac{Cs-C}{S} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 2}$$

Where; S= concentration equivalent of analyte added to the sample; Cs= metal content of the spiked sample; C = metal content of non-spiked sample; R = percent recover.

4.13 Method Detection Limit, Limit of quantification, instrumental detection limit

Blank samples were digested following the same procedures utilized for digesting the vegetable, soil, and water samples. Each blank was assayed for its metal contents (Cd, and Pb) by FAAS. The standard deviations (SD) of the replicate blanks was calculated to determine the method detection limit (MDL) and limit of quantification (LOQ). Method detection limit (MDL) was calculated as three times the standard deviations (MDL = 3SD) and LOQ was calculated as ten times the standard deviation (LOQ = 10SD). The MDL values obtained were compared with the instrument detection limit (IDL) and found to have greater values in all cases (Table 3). (APHA, 2012; Deribachew et al., 2015)

4.14 Analytical method validation

Analytical method validation Efficiency of the optimized procedure used for digesting the vegetable samples, soil, and water samples was checked by spiking the pre-treated vegetable, soil and water samples with standard solutions of each metal having a known concentration. The

spiked vegetables, soil, and irrigation water samples were digested following the same procedure employed in the digestion of the respective samples. Accordingly, a 0.5 g of cabbage, tomato and carrot sample was spiked with 5 mg kg^{-1} Pb and 1 mg kg^{-1} Cd. For the soil sample, 1 g was spiked with 10 mg kg^{-1} Pb and 1 mg kg^{-1} Cd. The irrigation water sample (50 mL) was spiked with 0.5 mg kg^{-1} Cd and 1 mg kg^{-1} Pb. For the vegetables, irrigation water, and soil samples, the percent recovery was performed in triplicates. (Deribachew et al., 2015)

4.15 Daily intake of heavy metal (DIM)

Daily intake of metals was calculated using the following equation. (Khan et al., 2013): (Ávila et al., 2016)

$$DMI = \frac{C\ metal * C\ factor * D\ food\ intake}{B\ average\ body\ weight} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad Equation\ 3$$

Where, C_{metal}, C_{factor}, $D_{\text{food intake}}$ and $B_{\text{average weight}}$ represent the heavy metals concentrations in plants (mg/kg), conversion factor, daily intake of vegetables and average body weight, respectively. The conversion factor 0.085 was used to convert fresh green vegetable weight to dry weight as described by (Rattan et al., 2005; Wang et al., 2013). The average daily vegetable intakes for adults and children were considered to be 0.0512 and 0.03443 kg/person/day (WHO, 2015); (Pem & Jeewon, 2015), respectively, while the average adult and child body weights were considered to be 53.057 and 31.04 kg. (Ratul,et.al, (2018) and (WHO, 2015).

4.16 Health risk index (HRI)

Health risk assessment of consumers from the intake of heavy metal contaminated vegetables were characterized by using HRI. The HRI > 1 for any metal in food crops means that the consumer population faces a health risk. The following formula was used for the calculation of HRI. (Khan et al., 2013):

$$HRI = \frac{DMI}{RfD} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad Equation\ 4$$

Where, DIM is the daily intake of metals and RfD is the reference dose. The RfD values for Cd and Pb are 0.001, 0.004 mg/kg bw/day, respectively. (Badawy et al., 2013), (Yang et al., 2017), (Gebreyohannes and Gebrekidan, 2018)

4.17 Transfer factor (TF)

Metal transfer factor (TF) denoting transfer of metals from soil to plant and it was computed as the ratio of the concentration of metals in plants to the concentration of metals in soil.

$$TF = \frac{Conc.in\ edible\ vegetable}{conc.in\ soil} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 5}$$

Where TF= Transfer factor, c_v = concentration of metal in edible part and C_s = concentration of metal in soil. Where, $C_{vegetable}$ and C_{soil} represent the concentration of heavy metals in extracts of vegetables and soils on dry weight (DW) basis, respectively.(Ratul et al., 2018).

4.18 Microbial sample collection and analysis

4.18.1 Water sampling and preparation for bacteriological analysis

Irrigation water samples were collected from the upper, middle and lower irrigation site of Awetu river and a total of nine taste and three control sample point in clean pre-sterilized poly ethylene bottle. 250 ml irrigation water was taken from each sampling point. The sample was taken to laboratory by using cold box and ice box to keep the temperature between 2-8 ^{0}c to prevent the microorganisms not to die.

4.18.2 Soil sample collection and preparation for bacteriological analysis

The soil sample was collected from three different sites and from each site three sample have taken comprising nine taste sample i.e. the upper river irrigated soil (upper), the middle river irrigated soil (middle) and the bottom Awetu river irrigated soil (lower) and one control sample was taken from areas non-Awetu river irrigated point at upper parts. About 25g of soil sample was taken from each point by using pre sterilized polyethylene plastic bag.

4.18.3 Vegetable sample collection and preparation for bacteriological analysis

Three vegetable type root , leaf and fruit edible vegetable i.e. cabbage, carrot and tomato sample was collected from three different irrigation sites i.e. upper site farm land (upper), middle site farm land (middle) and lower site farm land (lower/bottom) of the Awetu river and three points from each selected site were taken with a total of twenty-seven vegetable sample and one control sample site with three sample from each vegetable under study by using pre sterilized polyethylene bag and transported to Jimma university microbiology laboratory by maintaining its temperature in cold box (2 ^{0}C-8^{0}C) and analyzed within eight hours of sample collection.

Mixed vegetable samples (unprocessed and large sized) were aseptically chopped in to smaller pieces using sterile stainless steel knife prior to weighing. A 25g of subsample of each vegetable

sample have weighed and vigorously shaken in 225mL of sterile 0.1%(w/v) buffered peptone water for 3min separately to homogenize the samples and a tenfold serial dilution were made for each vegetable sample under study.

4.19.1 Preparation of water sample for bacteriological analysis

The collected water sample was prepared for bacteriological test according to WHO guidelines and the water sample was transferred to different size pre sterilized cylinders and test tubes and then proper serial dilution were made accordingly.

4.19.2 Preparation of soil sample for bacteriological analysis

A 25g of soil sample was weighed from each soil sample and placed in to a labeled plastic cup and the sample containing plastic cup was covered with plastic wrap to reduce moisture loss, and secured with a rubber band. Then wrap have been punctured several times with a probe to allow aeration without substantial moisture loss. The sample was weighed with the plastic wrap and the weighted results have been recorded to be used to determine the final soil moisture content. The sample were incubated at room temperature for one week.

4.19.3 Preparation of vegetable sample for bacteriological analysis

Mixed vegetable samples (unprocessed and large sized) was aseptically chopped in to smaller pieces using sterile stainless steel knife prior to weighing. A 25g of subsample of each vegetable sample have weighed and vigorously shaken in 225mL of sterile 0.1%(w/v) buffered peptone water for 3min separately to homogenize the samples and a tenfold serial dilution have been made for each vegetable sample under study. (Woldegzina and Mulleta ,2016).

Figure 4 : Cabbage, tomato and carrot sample preparation for bacteriological analysis

4.20 Bacteriological sample analysis

4.20.1 Analysis of the water sample

The multiple tube fermentation method was applied to analyze the water sample and a measured sub-sample (10 ml) were diluted with 10-fold serial dilution method. The remaining 10 ml is then diluted again and the process repeated. At the end of 5 dilutions this produces 50 tubes covering the dilution range of 1:10 through to 1:100000.

The tubes were then incubated at a pre-set temperature for a specified time and at the end of the process the number of tubes with growth in is counted for each dilution. To check the presence of different bacterial species the aliquots have dispensed on different medias and Statistical tables were then used to derive the concentration of organisms in the original sample. This method was applied by using indicator medium which changes color when acid forming species are present and by including a tiny inverted tube called a Durham tube in each sample tube. The Durham inverted tube catches any gas produced. The production of gas at 37 degrees Celsius is a strong indication of the presence of *E. coli*.

4.20.2 Analysis of soil sample

A 25g of soil sample was weighed from each soil sample and placed in to a labeled plastic cup and the sample containing plastic cup was covered with plastic wrap to reduce moisture loss and secured with a rubber band. Then wrap have been punctured several times with a probe to allow aeration without substantial moisture loss. The sample was weighed with the plastic wrap and the weighted results were recorded to be used to determine the final soil moisture content. The sample was incubated at room temperature for one week. Each of the soil sample have been re-weighted including the plastic wrap covering to allow for soil moisture calculation at the time of plating and 10-fold serial dilution of the soil sample was prepared. For each soil, 10g to a 95ml water blank was suspended and 1ml of the suspension was removed with a sterile pipette and added in to a 9ml water blank and serial dilution was made up to 10^5. Then 0.1 ml of suspension was transferred to pre prepared agar plate.

Figure 5 : serial dilution and sample spreading on media

4.20.3 Analysis of vegetable samples

A 25 g of subsample of each vegetable was aseptically weighed and vigorously shaken in 225mL of sterile 0.1%(w/v) buffered peptone water for 3min separately to homogenize the samples of each of the vegetable sample. The blender was carefully disinfected to prevent any cross contamination. Aliquots (0.5 mL) of each homogenate were serially diluted in sterile saline solution and the diluent of buffered peptone water have inoculated on the respective media. A 0.1ml volume of aliquot of appropriate dilution have been spread plated in duplicate on pre solidified plates of: Plate Count Agar for aerobic mesophilic bacteria, Violet Red Bile Glucose Agar for Enterobacteriaceae, Mannitol Salt Agar for staphylococci. Then was incubated at optimum temperature and time for counts.

Homogenized samples have heated at 80 ^{0}C for 10 minutes in a water bath to count aerobic spore forming bacteria. Thereafter, a 0.1mL appropriate dilution was spread-plated in duplicate on predried surfaces of Plate Count Agar plates. Inoculated plates have incubated at 30°C–37°C for 24–48hrs. For microbial counts, plates with colonies between 30 and 300 were considered. Total coliforms and fecal coliforms were enumerated by multiple tube fermentation tests as described by (APHA, 2012). The results were expressed as MPN 100 mL^{-1}.

4.21 Procedures followed for microbial identification

3.21.1 Determination of Total Aerobic Mesophilic Bacterial Count

Total Aerobic Mesophilic Bacterial Count was done by pour plate technique. 1ml aliquot was added to each of 10^{-1} to 10^{-6} to make serial dilution. 0.1ml of 10^{-3} to 10^{-5} dilutions were transferred aseptically into sterile Petri dishes arranged in triplicates using sterile pipettes. The aliquots in the plates were then flooded with 15- 20ml of sterile nutrient agar containing 50mg/100ml nystatin to suppress the growth of fungi. The plates were rocked to ensure even

distribution of the inoculums. The plates were allowed to solidify and then were incubated for 24h at 37˚C. Colonies that developed were there after counted and expressed as colony forming unit per gram (CFU/g) (Onuorah et al., 2014).

4.21.2 Determination of Total Coliform Count

Total coliform counts of the water, soil and vegetable samples were determined by direct plate count method as described by (Vural et al,2013). Direct plate count was done using Mac Conkey Agar. Tenfold serial dilutions of the samples were made in sterile distilled water. 1ml of each of the dilution (10^{-3} to 10^{-5}) was introduced and spread on MacConkey agar in triplicates. The plates were incubated for 48h at 37˚C. Pinkish colonies indicating lactose fermenters were counted.

4.21.3 Determination of Fecal Coliform

Total fecal coliform count of all the samples were determined by plate count as described by (Vural et al,2013) using Eosin methylene blue agar. Serial dilutions of the samples were made in sterile distilled water. 1ml of each dilution was plated out using pour plate on Eosin methylene blue agar in triplicate. Colonies having green metallic sheen were counted as fecal coliforms. (Onuorah et al., 2014).

4.21.4 Isolation and detection of bacteria

Standard enrichment and selective culture procedures were used to determine the presence of bacteria in the water, soil and vegetable samples. Pure cultures of the isolates were identified following the methods described by (*Cheesbrough*, 2006). Tests carried out include Gram reaction, catalase test, citrate utilization test, motility test, methyl red, Indole test, gas production test and sugar fermentation test.

Figure 6 : vegetable sample preparation and spreading

4.22 Data Analysis

Statistical analysis Data of heavy metal concentrations in vegetables was checked for homogeneity of variance and normality. The data of heavy metal concentrations in all analyzed vegetables across the various sample sites was subjected to non-parametric analysis to assess the significance differences in heavy metal concentrations by site, vegetable type and their interaction. Pearson correlation analyses was carried out to assess the relationships of soil and vegetable metal concentrations. All statistical analyses were computed with SPSS software version 20. (Woldetsadik et al., 2017). The bacteriological sample analysis was performed by SPSS statistical software version 20 and the significance difference between sample point and sample type was made. Bacterial counts were calculated as colony forming units per gram (CFU g^{-1}) and colony forming units per milliliter (cfu mL^{-1}) and converted into log 10 values. (Woldegzina & Mulleta, 2016).

$$CFU/ml = \frac{No.of\ colonies\ X\ dilution\ factor}{volume\ of\ inoculums} \quad \ldots\ldots\ldots\ldots\ldots \text{ Equation 6 } \textit{(APHA, 2012)}$$

4.23 Ethical Consideration

The permission letter from Jimma university post graduate school was given to the concerned bodies. The purpose of the study was explained to the town agriculture offices and kebele leaders and permission was asked from them with written letter. In addition, the farmers were asked for permission during sample taking from the irrigation site.

4.24 Dissemination of the results

The results of the finding were disseminated for Jimma Town Administration, Jimma town urban agricultural office and Jimma University to be avail in library and for further action and benefits of the community.

CHAPTER FIVE: RESULTS

5.1 Evaluation of the analytical method

In this study, the method validation was made by a spiking experiment in which known quantities of the metal standard solution were added to the samples to be studied. Percentage recovery values, method detection limit, limit of quantification and instrumental detection limit for individual analysis for soil, irrigation water and vegetable samples are presented in table 3 below. The percentage recovery values of the metals for soil, irrigation water, and vegetable samples were found to be within the range of 88.6–96.5%, 101.2%, and 90.2–97.6%, respectively. These ranges are within the acceptable range (APHA, 2012) which recommends percent recovery no less than 75% and not more than 125% for spiked samples and the result confirmed the validity of the method utilized in the current study. The value for instrumental detection limit was less than the method detection limit and these confirms the method applied was acceptable since MDL (method detection limit) greater than IDL (Instrumental detection limit).

Table 3 Method detection limit, limit of quantification, instrumental detection limit and % recovery

Sample	Metal	MDL	LOQ	IDL	% Recovery
Water	Cd	0.007	0.023	0.005	101.2
	Pb	0.003	0.01	0.001	101.2
Soil	Cd	0.007	0.023	0.005	88.6
	Pb	0.003	0.01	0.001	96.5
Carrot	Cd	0.007	0.023	0.005	94.5
	Pb	0.003	0.01	0.001	90.2
Tomato	Cd	0.007	0.023	0.005	90.2
	Pb	0.003	0.01	0.001	97.6
Cabbage	Cd	0.007	0.023	0.005	95
	Pb	0.003	0.01	0.001	97.2

5.2 Physico chemical and trace metal constituents of the water, soil and vegetables

5.2.1 Physico-chemical constituents of water sample

The results of the pH and EC of irrigation water samples was presented in Table 4 below. The pH values of the water samples from three sampling points (upper, middle and lower stream) were 8±0.02, 7.5±0.03, and 7.3±0.02, respectively. The electrical conductivities (EC) of water samples from the upper stream, middle stream and lower stream were 58.5±0.04, 77.9±0.045 and 111.8±0.03µS/cm, respectively.

5.2.2 Heavy metal concentration in irrigation water

The concentration of heavy metal in irrigation water sample from upper, middle and lower stream was presented in table 4. The concentration of Cd in the irrigation water sample were 0.076±0.004 $mgkg^{-1}$ for the sample collected from upper stream, 0.085±0.003 $mgkg^{-1}$ for sample collected from middle stream, 0.092±0.002 mgkg-1 for sample collected from downstream. The concentration of Pb in the irrigation water sample was 0.011±0.001 $mgkg^{-1}$ for the sample collected from upper stream, 0.013±0.002 $mgkg^{-1}$ for sample collected from middle stream, 0.05±00 mgkg-1 for sample collected from downstream. The distribution of lead in irrigation water along different site has significant difference at 95% CI with P-value of 0.015.

Table 4: Physico-chemical and trace metal constituents of irrigation water samples

Site	pH	EC(µs/cm)	Cd (mg/kg)	Pb (mg/kg)
Upper stream	8±0.02	58.5±0.04	0.076±0.004	0.011±0.001
Middle stream	7.5±0.03	77.9±0.045	0.085±0.003	0.013±0.002
Lower stream	7.3±0.02	111.8±0.03	0.092±0.002	0.05±00
Mean	7.6±0.36	82.73±26.98	0.08±0.01	0.02±0.02
SD	0.36	26.98	0.01	0.02
p-value			0.051	0.015
Control	6.9±0.01	23±7.3	0.011±00	0.013±00
FAO,1985	6.5-8.4	700	-	5
WHO,2001	-	-	0.01	-
WHO,2006			0.003	0.05

5.2.3 Physico chemical composition of soil in Awetu river irrigated site

The electrical conductivity and pH of the soil on which vegetable grown was measured and the result was shown in table 5 below. In this study, electrical conductivities of the soil samples collected from Awetu river irrigated farmlands were found to be 62, 61 and 65µS/cm, for upper site, middle site and lower site respectively. The pH value of the soils for upper site, middle site and lower site of the soil were 6.8, 7.30 and 7.10 respectively.

5.2.4 Heavy metal concentration of soil in Awetu river irrigated site

The concentration of heavy metals (Cd and Pb) investigated in soil sample from the farming site was shown in table 5 below. The concentration of Pb from the upper site, middle site and lower site were 1.312±0.003 mgkg-1, 1.328±0.003 mgkg-1 and 1.344±0.003 mgkg-1. The concentration of Cd in the studied soil sample in the upper site, middle site and lower were 0.207±0.002 mgkg-1, 0.213±0.002 mgkg-1 and 0.234±0.002 mgkg-1 respectively. The distribution of heavy metals in soil along different site was statistically different with significance p-value of 0.015 for both cd and Pb at confidence interval of 95%.

Table 5: pH, EC and heavy metal concentration in Awetu river irrigated soil

site code	pH	EC(µS/cm)	Cd (mg/kg)	Pb (mg/kg)
Upper site	6.8±0.34	62±1.78	0.207±0.002	1.312±0.003
Middle site	7.30±0.32	61±1.81	0.213±0.002	1.328±0.003
Lower site	7.10±0.40	65±1.75	0.234±0.002	1.344±0.003
Mean	7.07±0.25	62.67±2.08	0.218±0.014	1.328±0.016
SD	0.25	2.08	0.014	0.016
p-value			0.015	0.015
Control	6.32±0.27	21±1.56	0.123±0.002	0.612±0.003
WHO/ FAO,2001			3	100
USEPA,2002			3	300
EU,2002			3	300

5.2.5 Heavy metal concentration in vegetable

The concentration of heavy metals (Cd and Pb) in the studied vegetables (carrot, tomato and cabbage) was given in table 6 below. The heavy metal concentration in carrot were found to be 0.106±0.012 $mgkg^{-1}$,0.103±0.012$mgkg^{-1}$ and 0.099±0.012$mgkg^{-1}$ for cadmium and 0.769±0.046 $mgkg^{-1}$,0.757±0.046 $mgkg^{-1}$ and 0.846±0.046 $mgkg^{-1}$ for Pb in the upper site, middle site and lower site, respectively. The concentration of heavy metals in cabbage was 0.128±0.012mgkg-1 ,0.134±0.012$mgkg^{-1}$ and 0.105±0.012$mgkg^{-1}$ for cadmium and 0.824±0.046 $mgkg^{-1}$,0.784±0.046 $mgkg^{-1}$ and 0.893±0.046$mgkg^{-1}$ for lead in the upper site, middle site and lower site, respectively. The concentration of heavy metals in tomato was found to be 0.108±0.012$mgkg^{-1}$, 0.112±0.012 $mgkg^{-1}$ and 0.102±0.012$mgkg^{-1}$ for cadmium and 0.788±0.046 $mgkg^{-1}$, 0.769±0.046 $mgkg^{-1}$ and 0.846±0.046 $mgkg^{-1}$ for lead in the upper site, middle site and lower site, respectively.

The heavy metal concentration in carrot, tomato and cabbage along the upper, middle and lower site was statistically different with p-value < 0.05 at specific p-value shown in distribution table 8 below at 95% confidence interval.

Table 6: Heavy metal concentration in vegetables

Sites	Vegetables	Heavy metal concentration in (mg/Kg)	
		Cd	Pb
Upper site	Carrot	0.106±0.012	0.769±0.046
	Tomato	0.108±0.012	0.788±0.046
	Cabbage	0.128±0.012	0.824±0.046
Middle site	Carrot	0.103±0.012	0.757±0.046
	Tomato	0.112±0.012	0.769±0.046
	Cabbage	0.134±0.012	0.784±0.046
Lower site	Carrot	0.099±0.012	0.846±0.046
	Tomato	0.102±0.012	0.846±0.046
	Cabbage	0.105±0.012	0.893±0.046
	P-value	0.027	0.027
Mean		0.111	0.808
SD		0.012	0.046

5.2.6 Comparisons of heavy metal in vegetable with different study

The heavy metal concentration in different vegetable from the present study was compared with other results conducted on edible parts of vegetables and the result were presented in table 7 below.

Table 7: comparison of metal with different study on edible vegetables

Vegetables	Concentration of metal in vegetables		References
	Cd	Pb	
Carrot	5.22	59.92	(A. Khan et al., 2013)
	0.07	716	(Aschale, 2015)
	0.023	0.029	(Shaheen, Nourin, & Islam, 2016)
	1.342	2.181	(Roy & Gupta, 2016)
	0.114	0.794	Present study
Cabbage	2.97	13.01	(Pradesh et al., 2013)
	0.04	0.32	(Aschale, 2015)
	1.9	12	(Deribachew B, 2015)
	0.102	0.862	Present study
Tomato	4.42	41.94	(A. Khan et al., 2013)
	2.36	12.20	(Pradesh et al., 2013)
	0.056	0.005	(Shaheen et al., 2016)
	0.739	3.713	(Roy & Gupta, 2016)
	0.116	0.770	Present study
Total vegetable guideline	0.20	0.30	FAO&WHO, 2001)
	1.5	2.5	India/Awashthi,2000

5.3 Transfer factor of heavy metal from soil to vegetable

As it was shown in table 8 below, the transfer factor of heavy metal from soil to vegetable ranged from 0.50 to 0.62. The metal concentrations in the extracts of the soils and plants were calculated on the basis of dry weight. If the ratios >1, the plants have accumulated elements, the ratios around 1 indicate that the plants are not influenced by the elements, and ratios <1 show that plants exclude the elements from the uptake. (Agic et al., 2015).

According to (Agic et al., 2015) if the transfer coefficient of a metal is greater than 0.5, the plant will have a greater chance of the metal contamination by anthropogenic activities. Heavy metal accumulation capacities of vegetable indicated that these vegetables can be used as possible bio indicators of Pb pollution. (Agic et al., 2015).

$$TF = \frac{Conc.in\ edible\ vegetable}{conc.in\ soil} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots. \quad Equation\ 7$$

Where TF= Transfer factor, c_v= concentration of metal in edible part of vegetable and $C_{s=}$ concentration of metal in soil. Where, $C_{vegetable}$ and C_{soil} represent the concentration of heavy metals in extracts of vegetables and soils on dry weight (DW) basis, respectively.(Ratul et al., 2018).

Table 8: Transfer factor of heavy metal from soil to vegetables

Site name	vegetables	Heavy meatal transfer factor	
		Cd TF	Pb TF
Site1	Carrot	0.51	0.59
	Tomato	0.51	0.59
	Cabbage	0.55	0.61
Site2	Carrot	0.50	0.58
	Tomato	0.53	0.58
	Cabbage	0.57	0.58
Site3	Carrot	0.48	0.64
	Tomato	0.48	0.64
	Cabbage	0.45	0.66
Mean TF		0.51	0.61

5.4 Comparison of Heavy metal in soil and vegetables

The concentration of heavy metals in vegetables and their respective soil was shown in table 9 below and the concentrations of heavy metals were higher in soils than vegetables grown on respective soil. This indicates that only a small portion of soil metals is transferred to the vegetables and the root acts as a barrier to the translocation of heavy metals within plant (Mohammed et al, 2011). This may reveal that the main source of metal contents of vegetables is from their corresponding soil content which might be affected by urban waste, the environmental interferences like pesticides, fertilizers and other additives that farmers use. Variations in transfer factor among the different vegetables may be attributed to differences in the concentration of metals in the soil and differences in element up taken by different vegetables.

Table 9: Comparison of heavy metal in vegetable and soil

vegetables	Cd_{veg}	Cd_{soil}	Pb_{veg}	Pb_{soil}
site1	0.10	0.21	0.79	1.31
site2	0.11	0.21	0.8	1.33
site3	0.12	0.23	0.83	1.34
mean	0.11	0.22	0.81	1.32
control	0.07	0.12	0.74	0.61

4.5 Distribution of Heavy metal along site from the three different sources

Distribution of heavy metal in irrigation water, soil, carrot and tomato were significantly different at p-value <0.05 for both Cd and Pb but the distribution of Pb in cabbage were similar at P-value 0.061 with confidence interval of 95%; see table 10 below.

Table 10: Distribution of heavy metal in water, soil and vegetables across different sites

Metals	Distribution in water	Distribution in soil	Distribution in carrot	Distribution in tomato	Distribution in cabbage
Cd	0.027**	0.026**	0.038**	0.027**	0.038**
Pb	0.050**	0.027**	0.027**	0.027**	0.061*

* The distribution is similar across site with P-value of 0.05

** The distribution is different with p-value of 0.05

A) Distribution of cadmium along site in water, soil, carrot, tomato and cabbage

Distributions of cadmium in irrigation water, soil, carrot, tomato and cabbage was shown in the following figure and it shows differences as we go from the upper site to the lower site of Awetu river based irrigation.

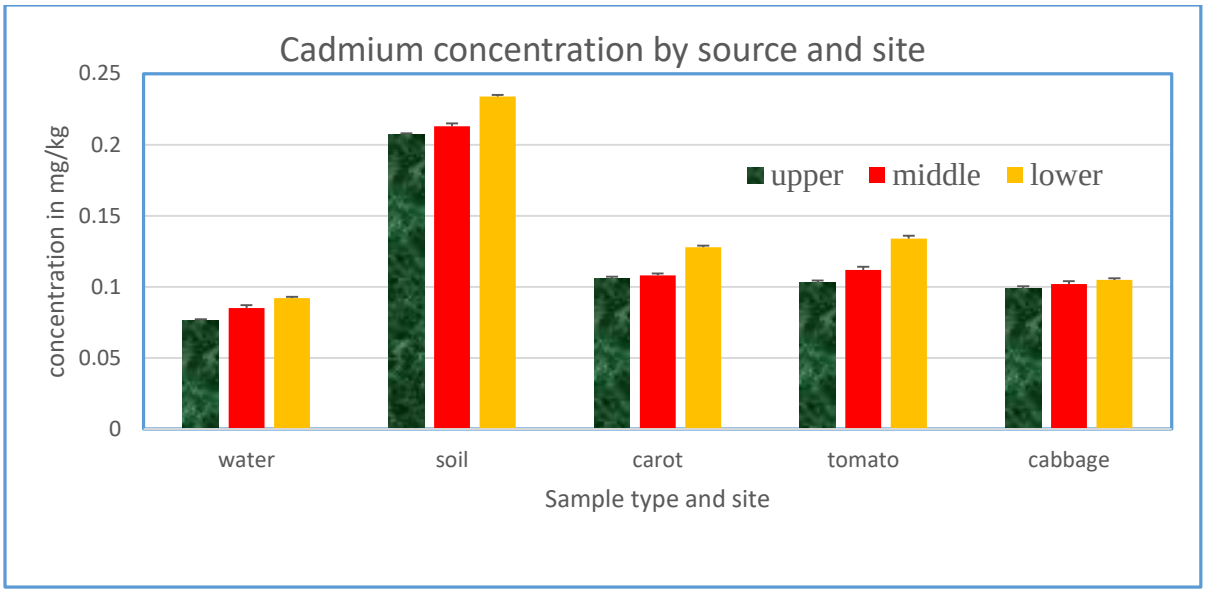

Figure 7 Cadmium distribution along site and source

Distributions of lead in irrigation water, soil, carrot, tomato and cabbage was shown in the following figure and it shows significant differences as we go from the upper site to the lower site of Awetu river based irrigation.

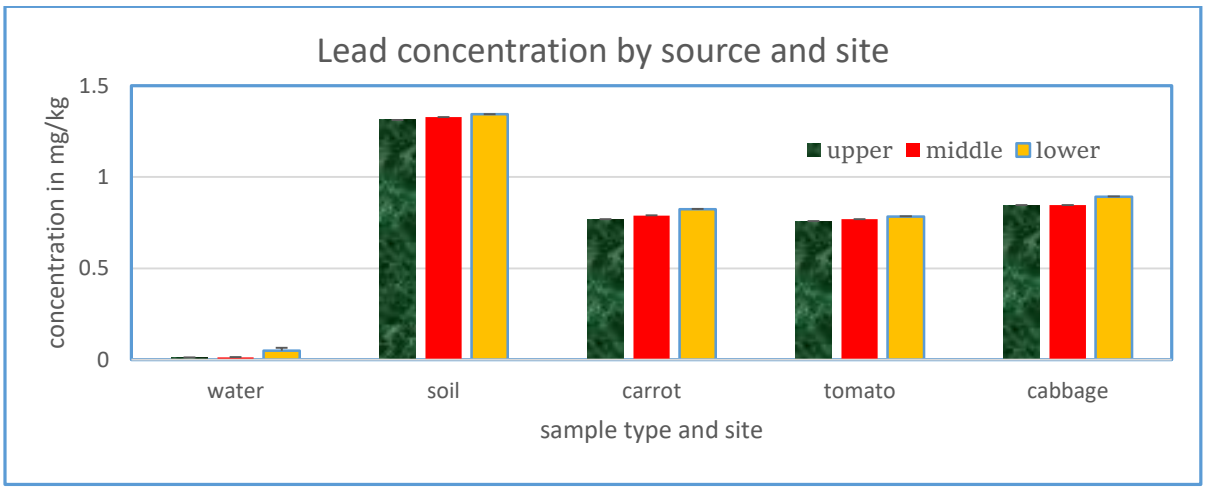

Figure 8 : Lead distribution along site and source

5.6 Correlation of heavy metal in soil and vegetable

The relationship between heavy metal contaminants in soil and vegetable were analyzed by Pearson's correlation coefficient. The correlation analysis is a bivariate method which is applied to describe the relation between two different parameters. The high correlation coefficient (near +1 or −1) means a good relation between two variables, and correlation around zero means no relationship between them at a significant level of 0.05%, it is strongly correlated, if r > 0.7,

whereas r values between 0.5 and 0.7 show moderate correlation between two different parameters. (Alghobar & Suresha, 2015)

Hence the below table 11 shows us that correlation of tomato to soil, cabbage to soil for both Cd and Pb, and carrot to soil for Cd have strong correlation but carrot to soil for lead have moderate correlation.

Table 11: Correlation of Heavy metals in soil and vegetables

Vegetables	Heavy metals	R	R2	Strength
Carrot	Cd	0.998**	0.9969	Strong
	Pb	0.683*	0.4663	Moderate
Tomato	Cd	0.735**	0.5401	Strong
	Pb	0.926**	0.8575	Strong
Cabbage	Cd	0.9792**	0.9589	Strong
	Pb	0.941**	0.8846	Strong

** Correlation is significant at p- value 0.01 (2-tailed)

*correlation is significant at p-value 0.05 (2-tailed)

5.7 Daily intake of heavy metal

Daily intake of metals was calculated using the following equation.(Khan et al., 2013):

$$DMI = \frac{C\ metal * C\ factor * D\ food\ intake}{B\ average\ body\ weight} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad Equation\ 8$$

Where, C_{metal}, C_{factor}, $D_{\text{food intake}}$ and $B_{\text{average weight}}$ represent the heavy metals concentrations in plants (mg/kg), conversion factor, daily intake of vegetables and average body weight, respectively. The conversion factor 0.085 was used to convert fresh green vegetable weight to dry weight as described by (Rattan et al., 2005; Wang et al., 2013). The average daily vegetable intakes for adults and children were considered to be 0.0512 and 0.03443 kg/person/day (WHO,2015), respectively, while the average adult and child body weights were considered to be 53.057 and 31.04 kg. (Ratul,et.al., (2018) and (WHO,2015).

Exposure of consumers and related health risks are usually expressed as tolerable daily intake (DIM) as a reference value established by FAO/WHO codex alimentarius commission. (FAO/WHO, 2001). Table 12 represents the estimation of each heavy metal intake through consumption of studied food stuffs. The results of present study showed that the mean levels of Cd and Pb were 0.11and 0.81 mg/kg respectively. Therefore, the DI of Cd could be $9.02x10^{-6}$ and $1.03x10^{-5}$ mg per day respectively for adult and child and DI of Pb $1.64x10^{-5}$ and $7.63x10^{-5}$ mg per day respectively. (Table 12).

Table 12: Daily intake of heavy metal from vegetables

Site name	Vegetables	Category	Cd DIM	Pb DIM
Upper site	Carrot	Adult	8.69E-06	6.31E-05
		Child	9.99E-06	7.25E-05
	Tomato	Adult	8.45E-06	6.21E-05
		Child	9.71E-06	7.14E-05
	Cabbage	Adult	8.12E-06	6.15E-05
		Child	9.33E-06	7.07E-05
Middle site	Carrot	Adult	8.86E-06	6.46E-05
		Child	1.02E-05	7.43E-05
	Tomato	Adult	9.19E-06	6.31E-05
		Child	1.06E-05	7.25E-05
	Cabbage	Adult	8.37E-06	6.94E-05
		Child	9.62E-06	7.98E-05
Lower site	Carrot	Adult	1.05E-05	6.76E-05
		Child	1.21E-05	7.77E-05
	Tomato	Adult	1.10E-05	6.43E-05
		Child	1.26E-05	7.39E-05
	Cabbage	Adult	8.61E-06	7.32E-05
		Child	9.90E-06	8.42E-05

5.8 Health risk and total hazard quotient

$$HRI = \frac{DMI}{RfD}$$ …… Equation 9

Where, DMI is the daily intake of metals and RfD is the oral reference dose(Khan et al., 2013) . The RfD values for Cd and Pb are 0.001, 0.004 mg/kg bw/day, respectively. (Badawy et al., 2013)

$$THQ = HRI(Cd) + HRI(Pb) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{ Equation 10}$$

Where, THQ=total hazard quotient, HRI(Cd)= health risk of cadmium and HRI(Pb)= health risk of lead. (Shaheen et al., 2016)

Table 13: Hazard quotient and health risks

Site name	Vegetables	Category	Cd HRI	Pb HRI	THQ
Upper site	Carrot	Adult	8.69E-03	1.58E-02	2.45E-02
		Child	9.99E-03	1.81E-02	2.81E-02
	Tomato	Adult	8.45E-03	1.55E-02	2.40E-02
		Child	9.71E-03	1.79E-02	2.76E-02
	Cabbage	Adult	8.12E-03	1.54E-02	2.35E-02
		Child	9.33E-03	1.77E-02	2.70E-02
Middle site	Carrot	Adult	8.86E-03	1.62E-02	2.50E-02
		Child	1.02E-02	1.86E-02	2.88E-02
	Tomato	Adult	9.19E-03	1.58E-02	2.50E-02
		Child	1.06E-02	1.81E-02	2.87E-02
	Cabbage	Adult	8.37E-03	1.74E-02	2.57E-02
		Child	9.62E-03	2.00E-02	2.96E-02
Lower site	Carrot	Adult	1.05E-02	1.69E-02	2.74E-02
		Child	1.21E-02	1.94E-02	3.15E-02
	Tomato	Adult	1.10E-02	1.61E-02	2.71E-02
		Child	1.26E-02	1.85E-02	3.11E-02
	Cabbage	Adult	8.61E-03	1.83E-02	2.69E-02
		Child	9.90E-03	2.11E-02	3.10E-02
Mean		Adult	9.09E-03	1.64E-02	2.55E-02
		Child	1.05E-02	1.88E-02	2.93E-02

5.9 Microbial count in irrigation water, soil and vegetables

5.9.1 Microbial count in irrigation water sample

The bacterial count in soil from irrigation site of Awetu river was shown in appendix table 15 and the mean log cfu/ml value for aerobic mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, staphylococci, total coliform and fecal coliform were 9.40cfu/ml, 8.83fcu/ml, 6.69fcu/ml, 3.87fcu/ml, 3.73fcu/gram and 3.57fcu/ml. The distribution of microbial

contaminants of the irrigation water were statistically different along site with p-value of 0.000 at 95% confidence interval.

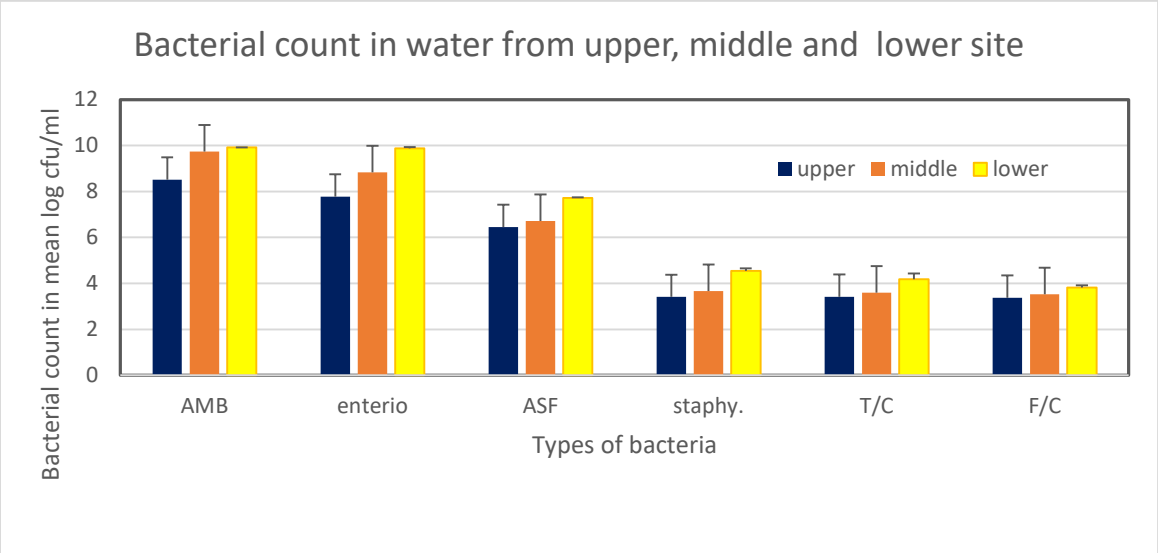

Figure 9 : Bacterial count in water sample

The distribution of microbial contaminants was significantly different across site with p-value of <0.05.

Where, AMB= Bacteria=aerobic mesophilic bacteria), Enterio= Enterobacteriaceae, ASF= aerobic spore former, staphy = staphylococci, T/C = total coliform and F/C =fecal coliform.

5.9.2 Microbial count in soil sample

The result for bacteriological analysis of soil sample was shown in appendix table 15. The mean log cfu/gram value for aerobic mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, staphylococci, total coliform and fecal coliform was 8.91cfu/gram, 7.91fcu/gram, 6.07fcu/gram, 4.16fcu/gram, 3.53fcu/gram and 3.26fcu/gram.

The distribution of microbial contaminants of soil sample along the study site was significantly different with statistical significance value of p<0.05 at confidence interval of 95%.

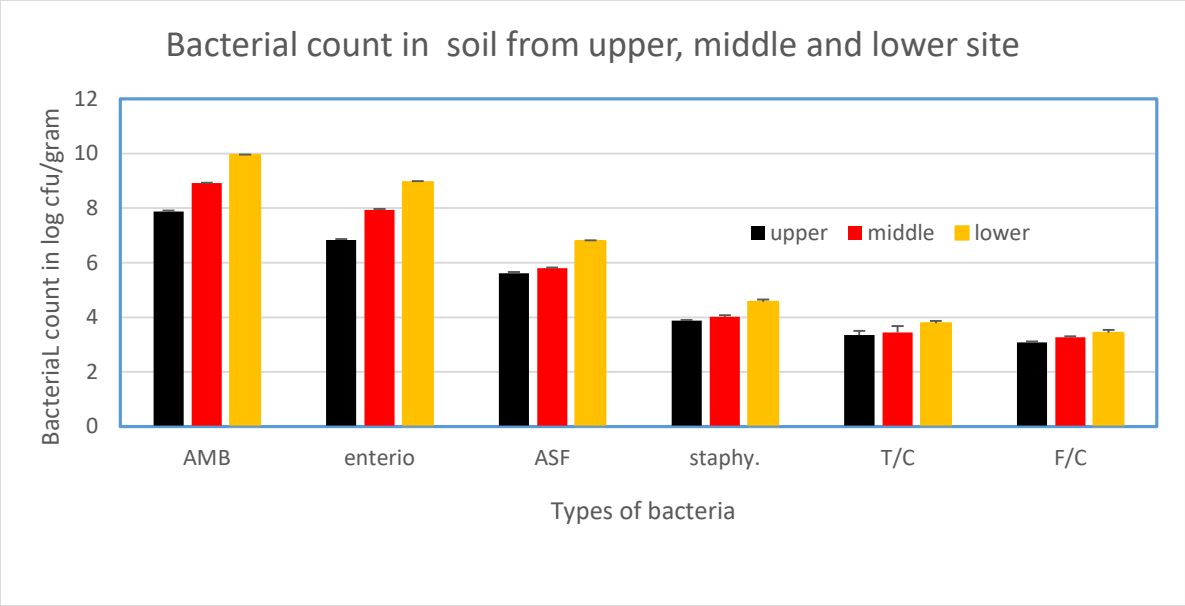

Figure 10 : Bacteria account in soil sample

Where, AMB=aerobic mesophilic bacteria, Enterio= Enterobacteriaceae, ASF = aerobic spore former, staphy = staphylococci, T/C= total coliform and F/C =fecal coliform.

5.9.3 Microbial analysis of vegetables samples

The bacteriological count in vegetables were identified by serial dilution and the result was shown in appendix table 15. The mean bacterial counts (log cfu/gram) of Aerobic mesophilic bacteria, Enterobacteriaceae, Aerobic spore formers, staphylococci, total coliform and fecal coliform were 8.70,7.78,6.02, 3.98, 3.43 and 3.25 log cfu g-1for carrot; 8.64,7.74,6.11, 3.87, 3.25 and 3.08 log cfu g-1 for cabbage, 8.58, 7.70, 5.95, 3.88, 3.11 and 2.93 log cfu g-1 for tomato respectively.

The distribution of microbial contaminants in carrot were different along site with p-value of 0.05 except for total coliform (p-value 0.056) and fecal coliform (p-value 0.078) which were statistically similar along site with confidence interval 95%. The distribution of microbial contaminants in cabbage were different along site with p-value of 0.05 except for total coliform (p-value 0.267) which were statistically similar along site with confidence interval of 95%. The distribution of microbial contaminants in tomato were different along site with p-value 0.05 except for aerobic spore formers (p-value 0.096) which were statistically similar along site with confidence interval of 95%.

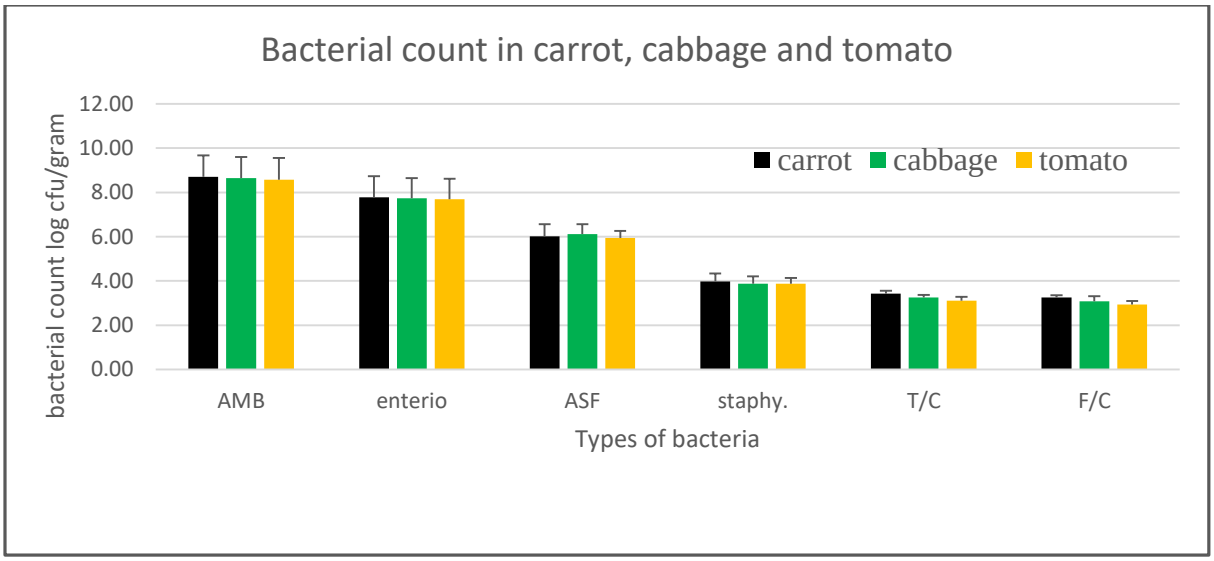

Figure 11 : Bacterial count in vegetable

Where, AMB=aerobic mesophilic bacteria, Enterio. = Enterobacteriaceae, ASF = aerobic spore former, staphy = staphylococci, T/C =total coliform and F/C= fecal coliform.

5.10 Distribution of bacterial count along upper, middle and lower site in water, soil and vegetables

Distribution of different bacteria in water, soil and vegetable was studied and the distribution were statistically different along the upper, middle and lower site with p-value < 0.05 except, total coliform p=0.056 and fecal coliform p=0.078 in carrot, total coliform p=0.267 in cabbage and aerobic spore formers p=0.096 in tomato; which was statistically similar across site with p-value >0.05 at confidence interval of 95%.

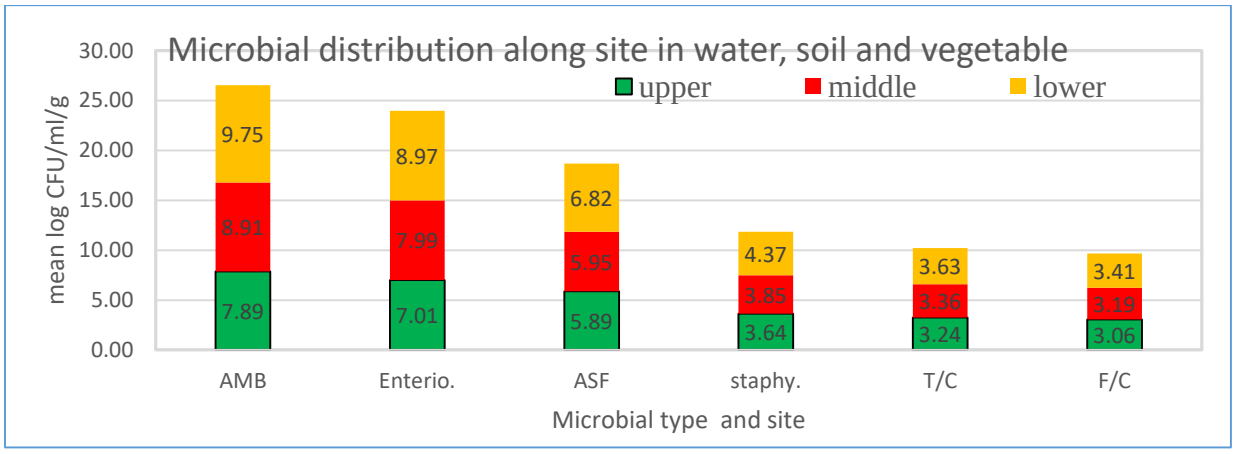

Figure 12 : Distribution of bacterial count along site in water, soil and vegetables

5.11 Microbial contaminants in control samples

The microbial count in the control sample of irrigation water, soil and vegetable was analyzed and the result were presented in table 14 below. The mean bacteriological count for aerobic mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, staphylococci, total coliform and fecal coliform were, 5.38, 4.35, 4.49, 2.58, 2.87 and 2.76 log cfu/ml in water, 4.78, 3.53, 3.66, 2.15, 2.98 and 2.89 log cfu/gram in soil, 4.50, 3.56, 2.39, 2.20, 2.79 and 2.72 log cfu/gram in carrot, 4.40, 3.44, 2.35, 2.16, 2.76 and 2.71 log cfu/gram in cabbage and 4.54, 3.49, 2.34, 2.12, 2.71 and 2.65 log cfu/gram in tomato respectively.

Table 14: Microbial contaminants of irrigation water, soil and vegetable samples

Sample type	Log of bacterial count in CFU/ml or CFU/gram					
	AMB	Enterio.	ASF	Staphy.	T/C	F/C
Water control	5.38±0.38	4.35±0.03	4.49±0.02	2.58±0.04	2.87±0.03	2.76±0.03
Soil control	4.78±0.15	3.53±0.10	3.66±0.16	2.15±0.05	2.98±0.02	2.89±0.02
Carrot control	4.50±0.09	3.56±0.21	2.39±0.07	2.20±0.08	2.79±0.02	2.72±0.03
Cabbage control	4.40±0.08	3.44±0.10	2.35±0.05	2.16±0.04	2.76±0.03	2.71±0.02
Tomato control	4.54±0.26	3.49±0.26	2.34±0.17	2.12±0.08	2.71±0.01	2.65±0.04

Where, AMB=aerobic mesophilic bacteria, Enterio. = Enterobacteriaceae, ASF = aerobic spore former, staphy = staphylococci, T/C = total coliform and F/C =fecal coliform.

5.12 Biochemical and morphological test results of microbial analysis

Total coliform from irrigation water, soil and vegetables were grown on the respective media and the rod-shaped, gram negative, oxidase negative, lactose fermenting and gas producing was counted as total coliform bacteria. The thermo-tolerant coliform with specific characteristics of lactose fermenter, indole, gas and acid producer, oxidase negative was counted as thermos-tolerant bacteria. Those which utilizes citrate as carbon source was count as Enterobacteriaceae, coagulase positive, road shaped gram positive, catalase positive was count as staphylococci and the sample of the three sources were exposed to heat at 80 ^{0}C for 10 minutes and the growth seen on respective media were counted as aerobic spore formers. The result for biochemical and morphological test for different bacteria was shown in the following figures.

a) Bacterial growth on selected media

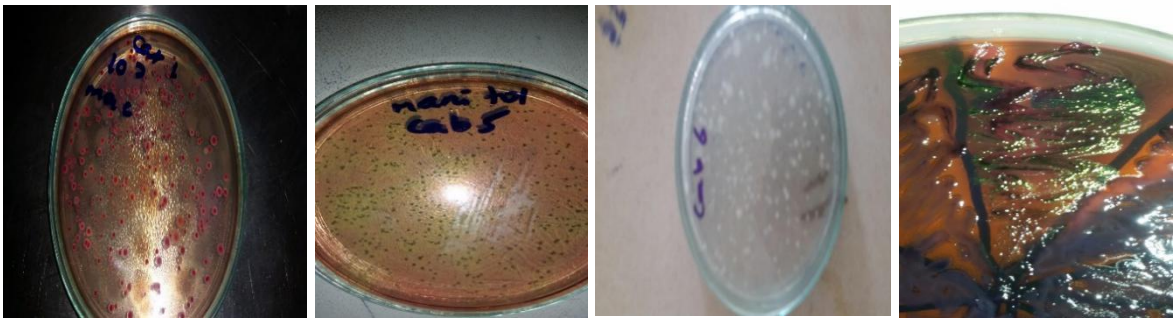

Figure 13 : colony forming unit on mac ckonkey agar; Figure 14 : colony forming unit on mannitol salt agar and plate count agar respectively; Figure 15 : green metallic sheen on ethylene methylene blue media confirmation for E. coli

Bio chemical test results

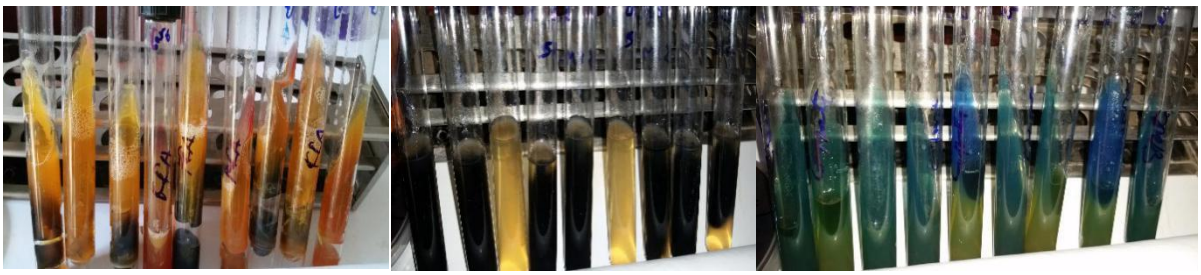

Figure 16 : KIA, SIM AND Citrate utilization test respectively

b) Gram staining

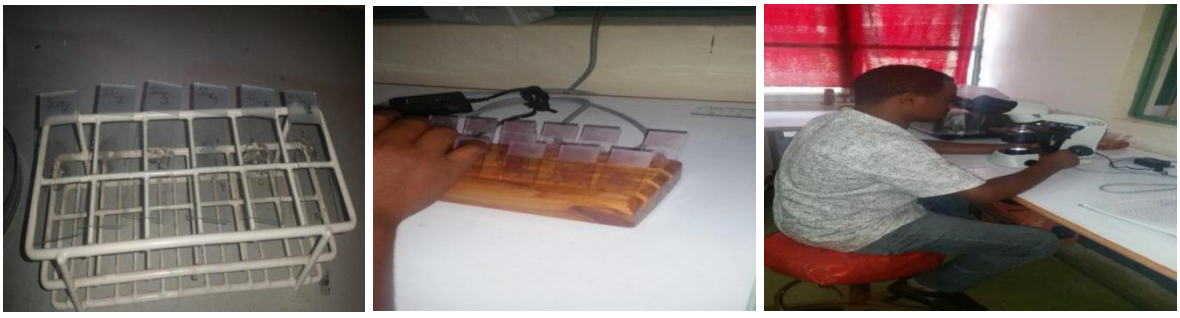

Figure 17 : Gram staining and morphological identification

CHAPTER SIX: DISCUSSION

6.1 Physico chemical constituents of water and soil samples

6.1.1 Physico chemical constituents of soil samples

In this study, conductivities of the soil samples collected from Awetu river irrigated farmlands were determined at 25^0C. Electrical conductivity and pH were determined in soil with ratio of 1:1 soil water ratio and the result revealed that electrical conductivity in the collected soil samples were found to be 62, 61 and 65µS/cm, respectively. The EC of the soil in the present study was less than the FAO/1985 recommended safe limit value for soil used for irrigation water which was 700 µS/cm. The pH value of the soils ranged were 6.8, 7.30 and 7.10 (Table 5). According to (Hizkeal ,2012) soils with pH range of 6.6-7.4 are neutral. Based on this classification, soil samples collected from vegetable growing areas around Awetu river with the value of the range 6.8 to 7.3 were neutral. pH is one of the factors which influence the bioavailability and the transport of heavy metal in the soil and heavy metal mobility decreases with increasing soil pH due to precipitation of hydroxides, carbonates or formation of insoluble organic complexes (Uduma, 2013). The amount of heavy metals mobilized in soil is a function of pH, properties of metals, redox conditions, soil chemistry, organic matter content, clay content, cation exchange capacity and other soil properties (Uduma, 2013).

According the current result, the pH of the soil sample has no effect in the mobilization of heavy metal from one point to another and the metal in the soil will maintained to its original place and it can be up taken by the respective vegetable grown on it. The result of pH was in line the WHO guidelines which was 6.5-8.4 for soil.

6.1.2 Physico chemical constituents of irrigation water samples

The pH and EC of irrigation water was measured by using digital pH meter and EC measuring portable probe on site and the result was recorded and shown in table 5. The pH was 8, 7.5 and 7.3 for upper stream, middle stream and bottom stream which was in line with FAO,1985 safe limit ranged 6.5-8.4 for water used for irrigation. The EC of irrigation water were measured and the result shows us that 58.5, 77.9 and 111.8 for upper stream, middle stream and bottom/lower stream point and it was in line with FAO,1985 guidelines which was 700 µS/cm.

According to the present result the pH and EC of the irrigation water cannot affect the metal concentration in the irrigation water since the acidity and alkalinity of the water was in the neutral condition and ions in the water was very low when we see their EC.

6.2 Heavy metal in irrigation water, soil, vegetables and control samples

The distribution of heavy metals in irrigation water, soil, carrot, tomato and cabbage along different site (upper, middle and lower) was tested by non-parametric kruskal Wallis test and their distribution was different statistically with p-value of 0.05 except distribution of Pb in cabbage which was similar across site with p-value of 0.061.

6.2.1 Heavy metals in irrigation water

The mean cadmium concentration in the water sample from this study 0.08 $mgL^{-1}$was in line with 0.06 mgL^{-1} (Deribachew et al., 2015) and greater than WHO and FAO/2001 which recommended the maximum permissible level 0.01 mgL^{-1}, but less than 0.10 mgL^{-1}which reported by (Khan et al., 2013) . The mean lead concentration in the water sample from the present study 0.02 mgL^{-1} was greater than study conducted by (Aschale, 2015)which reported 1.6 μgL^{-1} ,but much less than the WHO and FAO/2001 guidelines for safe limit which was 5 mgL^{-1} in water used for irrigation.

The distribution of heavy metals in irrigation water was tested with non-parametric kruskal-Wallis test and the result shows us that it has statistical significant difference across site category with p-value < 0.05 at confidence interval of 95%.

The metal concentration was different in the upper, middle and lower stream and the concentration of heavy metal was in order of Lower stream > middle stream > upper stream this may due the additional contaminant entrance in to the river as we go from the upper to downs. The low level of these metals with reference to recommended guidelines from the irrigation water may due to absence of point source such as industrial waste in the area, but these does not mean that the water cannot be polluted in the future by these metals due to the fast rate town expansion, presence of higher institution such as Jimma University and Jimma University Specialized Hospital and currently Jimma Industrial park was under construction and it can be another source of contamination if not managed and treated properly.

6.2.2 Heavy metal concentration in soil

The mean cadmium concentration in soil sample in the present study 0.218 mg kg^{-1} was in line with study conducted in Addis Ababa which reported 0.17 mg kg^{-1} (Aschale, 2015) , but the result was smaller than the (WHO/FAO,2001) which recommended the cadmium concentration in irrigation soil 3-6 mg kg^{-1}. The present study result for mean concentration of lead in soil from the irrigation site 1.33 mg kg^{-1} was less than the WHO/FAO,2001 recommended guidelines which recommended 100 mg kg^{-1} for lead in soil from irrigation site used for vegetable production.

The concentration of heavy metals in irrigated soil sample could be due to chemical fertilizers and pesticides used in agricultural land of the catchments, emission of Pb from vehicles, discharge of motor oil, grease, fuel burning and a battery from the road side and near the bridge. (Gebreyohannes and Gebrekidan, 2018)

The distribution of heavy metals in soil samples was tested with non-parametric kruskal-Wallis test and the result shows us that the distribution was statistically different with level of significances 0.05. The variation of heavy metals from the upper to lower site (concentration in the lower > middle > upper) was due to additional entrance of contaminants as we go down river from different sources. The reason why the amount of heavy metals in soil become lower according to guidelines was due to absence of point sources such as industries. But these does not indicate the soil was free of risk since the town was expanding and the waste from the town was directly released in to the surrounding river and agricultural land without any treatment and it can be accumulated in the soil if not managed properly.

6.2.3 Heavy metal concentration in Vegetables

The mean concentration of cadmium in carrot 0.114 mg kg^{-1} was greater the report 0.07 and 0.023 mg kg^{-1} (Aschale,2015) and (Shaheen et al., 2016) respectively, but the result was less than the report 5.22 and 1.342 mg kg^{-1} by (A. Khan et al., 2013) and (Roy & Gupta, 2016) respectively. The cadmium concentration in carrot 0.114 mg kg^{-1} was less than (WHO/FAO,2001) which recommended 0.2 mg kg^{-1}. The result for cadmium concentration in cabbage from the present study 0.102 mg kg^{-1} was greater than 0.04 mg kg^{-1} reported by (Aschale, 2015), but less than 2.97 and 1.9 mg kg^{-1} by (Pradesh et al., 2013),and (Deribachew et al.,2015)respectively. This result for cadmium was less than the WHO/FAO,2001 guidelines which recommended the maximum guidelines of 0.2 mg kg^{-1}. The mean concentration of

cadmium in tomato 0.116 mg kg^{-1} was greater than the study report 0.056 mg kg^{-1} by (Shaheen et al., 2016), but less than 4.62, 2.36 and 0.739 mg kg^{-1} which reported by (A. Khan et al., 2013), (Pradesh et al., 2013) and (Roy & Gupta, 2016) respectively. This result 0.116 mg kg^{-1} of cadmium concentration from tomato was less than the (WHO/FAO,2001) safe limit which recommends tolerable value of 0.2 mg/kg for Cd in vegetables.

The cadmium concentration in the three vegetables under the current study were greater than the WHO/FAO recommended guidelines of tolerable metal consumption from vegetables and it needs great attention to reduce the sources of cadmium contaminants such as dumping of car battery, car wash, direct drainage of sewer and fertilizers from entrance to the irrigation water and consequently to the farming the soil.

The mean concentration of lead in carrot from the present study 0.794 mg kg^{-1} was greater than the report 0.029 mg kg^{-1} by (Pradesh et al., 2013), but less than 55.92, 716, 2.181 mg kg^{-1} by (Khan et al., 2013), (Aschale, 2015) and (Roy & Gupta, 2016) respectively. The result 0.794 mg kg^{-1} lead concentration in carrot from the present study was greater than the (WHO/FAO, 2001) recommended guidelines which was 0.1 mg kg^{-1}. The mean lead concentration in cabbage 0.862 mg kg^{-1} was greater than 0.32 mg kg^{-1} by (Aschale, 2015) , but less than 13.01 and 12 mg kg^{-1} by (Pradesh et al., 2013) and (Deribachew et al.,2015) respectively. The mean lead concentration in tomato from current study 0.77 mg kg^{-1} was greater than 0.005 mg kg^{-1} reported by (Pradesh et al., 2013), but less than 41.94, 12.20 and 3.71 mg kg^{-1} by (A. Khan et al., 2013), (Pradesh et al., 2013) and (Roy & Gupta, 2016) respectively. The concentration of lead in both cabbage and tomato was greater than 0.3 mg kg^{-1} (WHO/FAO, 2001) recommended for vegetables.

The heavy metal concentration in all the studied vegetable were higher than the WHO/FAO safe limit and these was due to accumulation of heavy metals from soil, irrigation water and atmospheric deposition of these metals to the vegetables.

The distribution of heavy metals in carrot, tomato and cabbage samples were tested by non-parametric kruskal-Wallis test and the result shows us that the distribution was statistically different with level of significances 0.05 in both studied heavy metals (Cd and Pb) except the distribution of lead in cabbage which was similar along site with significance value of 0.061 at 95% confidence interval.

The heavy metal concentration in the studied vegetables from the upper, middle and lower site was different and it increases as we go from the upper site to the lower site and these may be due to additional contaminant entrance from different sources like application of fertilizers and pesticides, irrigation water contaminated with heavy metals from urban contaminated water and from the soil on which vegetables grown due to additional source entrance.

The concentration of heavy metals was different in different vegetable types studied and the concentration of heavy metals in root edible vegetable (carrot) was greater than fruit edible part (tomato) and fruit edible part was greater than leaf edible parts (cabbage) these difference was due to accumulation capacity difference of the studied vegetables.

6.2.4 Heavy metal in control samples of irrigation water, soil and vegetables

The mean heavy metal in control sample was lower than the test result, but at all site and source of study both Cd and Pb were identified and this may be due to agricultural implementation of fertilizers, pesticides, herbicides and runoff accumulated soil from different points of the farming site and the availability of those minerals in the natural soil of the area.

6.3 Microbial contaminants of irrigation water, soil and vegetables

6.3.1 Microbial contaminants of irrigation water

The present study shows that total and fecal coliform from water samples were 3.73 and 3.57 log cfu ml^{-1} respectively which was in line with (Gatta et al., 2015) which reports 3.74 and 3.58 log cfu ml^{-1} respectively for total and fecal coliforms in water used for irrigation, but the present result was higher than (Blumenthal et al., 2000), (WHO, 2011) guidelines which recommends 3 log cfu ml^{-1} for total coliform and < 3 log cfu ml^{-1} for fecal coliform.

The microbial contaminants in irrigation water was higher than the recommended microbial quality of water for irrigation purpose and this high value was due to absence of wastewater treatment in the town and direct sewage and sewer lines, runoff, wastewater from slaughter house and different institutions, animal waste and direct waste dump in to the river.

The mean distribution of (log cfu mL^{-1}) aerobic mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, staphylococcus, total coliform and fecal coliform along the three study site was statistically different with p-value < 0.05 for irrigation water samples. The microbial contaminants count was different in distribution from the upper stream to the bottom stream and it was in increasing order from top to down and these was due to additional entrance of

contaminants from different sources as the river crosses the town. The quality of irrigation water in the studied area was not good for cultivation of row eaten vegetables.

6.3.2 Microbial contaminants of soil

The total and fecal coliform from soil irrigated with contaminated water were 3.53 and 3.26 log cfu g^{-1} for total and fecal coliform respectively which was higher than study by (Badawy et al., 2013) which reports 2.91 and 2.18 log cfu g^{-1} for total and fecal coliform respectively but lower than (Gatta et al., 2015) which reports 6.60 and 3.31 log cfu g-1 for total coliform and fecal coliform respectively.

Microbial contaminant in soil sample was high as it was indicated in table 17 and this high value was due to application of microbial contaminated water for irrigation, fecal matter, application of manure, open field defecation on cultivation land, animal grazing area and cow dung. The mean distribution of (log cfu g^{-1}) aerobic mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, staphylococcus, total coliform and fecal coliform along the three study site was statistically different with p-value < 0.05 for soil samples.

The microbial contaminant in the bottom site was higher than the upper site and this may be due to additional contaminant entrance as the river crosses the town from upper to bottom and waste from sewage and sewer contaminated by the fecal matter enters to cultivation land.

6.3.3 Microbial contaminants of vegetables

The overall mean aerobic mesophilic bacterial count observed in this study ranged from 7.58 in tomato to 7.70 log cfu g^{-1} in carrot were in line with (Weldezgina & Muleta, 2016) which reported (7.71), but higher than previous report from by (Gatta et al., 2015) (4.02), the high AMB in the present study may be due to the absence of any treatment applied in the current study compared to the value reported. But the current result was lower than report by (Benti et al., 2014) (8.20), The result for AMB from the present study was above the ICMSF (International Commission on Microbiological Specifications for Foods) guidelines which recommend $4.9x10^6$ for raw eaten vegetables.

The overall all mean counts of Enterobacteriaceae in the present study ranged from 7.70 to 7.78 log cfu g^{-1} .This was higher than the other studies conducted on vegetables 5.08 and 6.60 log cfu g-1 by (Ashenafi, 2015) and (Weldezgina & Muleta, 2016) in Addis Ababa and Jimma in

Ethiopia respectively this high level of contamination may be due to fecal matter contaminant and direct sewage line connected to the river used for irrigating the vegetables.

According to (Weldezgina & Muleta, 2016) and guidelines recommended for fresh fruit and vegetables in London, over all mean counts (log cfu g^{-1}) of Enterobacteriaceae in carrot (7.78, cabbage (7.74) and tomato (7.70) reveled unsatisfactory level ($\geq$4 log cfu g^{-1}).

In the present study, over all mean count of staphylococci from vegetable samples ranged from 3.87 in tomato to 3.98 log cfu g-1 in carrot. This was higher than the study performed on similar vegetable in Jimma town reported by (Weldezgina & Muleta, 2016) who reported 2.71 in carrot to 2.76 in cabbage this high value from the present study as compared to similar study can be additional source of contaminants due to increment of urbanization and low waste management practice in the area. The present study result was lower than the study conducted in Addis Ababa supermarket performed by (Ashenafi, 2015) on microbial containments of lettuce and green pepper which reported 4.55 and 4.97 log cfu g-1 , Higher count from supermarket may be due to skin contact and environmental factor as compared to the present study as the current study conducted by taking sample at the point of cultivation.

In case of aerobic spore formers, the overall mean counts ranged from 5.95 in tomato in to 6.11 log cfu g-1 in cabbage. In all vegetables the counts were higher compared with report by (Ashenafi, 2015) where the count ranged from between 3.47 and 3.50 log cfu g-1 in green pepper and lettuce Addis Ababa and also greater than reports by (Weldezgina & Muleta, 2016) where the counts ranged from 5.71 and 6.54 log cfu g-1 in tomato and carrot, respectively from Jimma. This may be due to waste from municipal and institutional as well as human and animal fecal matter which contribute the addition of the organism to vegetables.

The overall mean counts of total coliform ranged from 3.11 to 3.43 and fecal coliform ranged from 2.93 to 3.25 log cfu g-1 from vegetable samples in the present study were relatively lower than the (Benti et al., 2014) which reported 6.5 and 5.57 log cfu g-1 for total coliform and fecal coliform in cabbage respectively. But The present study was greater than study conducted by (Weldezgina & Muleta, 2016) which reported 2.94 to 3.02 log cfu g-1 total coliform and 2.72 to 2.85 log cfu g-1 fecal coliform in tomato and carrot respectively. The difference in counts of total and fecal coliform can be from the degree of original contamination, sources of contamination. (Weldezgina & Muleta, 2016).

Pathogenic organisms are one of the main health risks when wastewater is used for irrigation. There is ample evidence indicating the presence of excreted pathogens on the surfaces of vegetables that were irrigated or fertilized with fecal products. (Gemmell & Schmidt, 2010).

Different bacterial types were identified from the vegetables studied from different sites. The presence of bacterial type in different sources and sites was due to irrigation water from urban polluted sources, sewage and sewer line, contamination sources from human and animal fecal matter, application of manure as fertilizers.

The mean count distribution of (log cfu g-1) aerobic mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, staphylococcus, total coliform and fecal coliform along the three study site was statistically different with p-value < 0.05 for carrot, tomato and cabbage sample except carrot for total coliform (p-value 0.056) and fecal coliform (p-value 0.078) , cabbage for total coliform (p-value 0.267) and tomato for aerobic spore formers (p-value 0.096) which was similar with p-value > 0.05 across the site. The exception for total and fecal coliform in carrot and cabbage which shows similarity across site may be due to high fecal matter contamination at middle and lower parts of the study area.

The Microbial contaminant found in all vegetable under present study were higher than guidelines sated by (ICMSF, 2001) which recommend Enterobacteriaceae < 10^4 , Staphylococcus < 10^4 , Total coliform < 10^4 , and fecal coliform < 10^3 .

The microbial contamination levels were in order of upper site < middle site < lower/bottom site this shows as that us we go from the upper to the lower; additional contaminants were entering to the farming plot from the town sewage and sewer line containing fecal matter and from another different sources containing microbial load due to poor waste handling and management of the town. The bacterial count in different vegetable was different. The bacterial count in carrot > bacterial count in tomato > bacteria count in Cabbage this was due to irrigation system in the area which uses water from line opened by the community and enters in to the vegetable from soil through the root systems and then to stem and other parts of the vegetables. As the study result shows us the average count in root edible(carrot) was greater than fruit edible (tomato) greater than leaf edible (cabbage).

Highest fecal coliform counts were observed in carrots similar to the report by (Woldezgina and Muleta, 2016) and (Francis et al., 2018) and Carrots being a root crop could have received contamination from the soil, irrigation water, animal wastes used as fertilizer and runoff.

The presence of pathogenic micro flora on the surface of fresh fruits and vegetables indicates the necessity for observing hygienic conditions during production, because such type of contamination can occur from water, soil, waste and humans. (Shobha, 2014)

The present study was conducted on vegetables growing plots and the sample was taken at the farming site, the test result shows us that it was much greater than the recommended guidelines for microbial contaminants of fresh vegetable and fecal coliform levels more than the $1x10^3$ per 100 g wet weight hence can be classified as undesirable for consumption according to the International Commission on Microbiological Specifications for Food (ICMSF, 2001) guidelines. Those bacterial species were commonly found in soil and may contaminate fruits and vegetables during harvesting and also the presence of this pathogen in fresh fruits and vegetables may cause diarrhea (due to enterotoxins) or vomiting (Emetic toxin). (Rai and kaur, 2015). The vegetable under present study was those eaten raw in most of the community and it can further be contaminated through washing, transportation and handling, therefore it needs great attention during handling, proper washing and cooking is necessary before eating since it was not safe.

6.3.4 Microbial contaminants in control irrigation water, soil and vegetables

Microbial contaminants were found in the control sample of irrigation water, soil and vegetables and the results of control sample for bacteriological test as shown in table 16. The result shows us that it was lower than the microbial count in study test results but it was positive for all tested bacteria's under the present study, this may be due to open defecation in the farming area and cattle grazing site being around the farm, application of manure as fertilizers by the local community and runoff entering in to site from the upper catchment which may contain human and animal wastes.

6.4 Transfer factor (TF) from soil to vegetables

Metal transfer factor from soil to vegetable was a key module of human exposure to heavy metals via food chain. Transfer factor of metals is essential to investigate the human health risk index (Agic et al., 2015).Table14 summarizes the TF values for selected heavy metals in selected vegetables collected from the study areas. The range of TF values for vegetables irrigated with

contaminated river water were from 0.50 to 0.52 and 0.60 to 0.62 for Cd and Pb and respectively. The presents study shows that the transfer factor of heavy metal was greater than the study conducted by (Baggs et al., 2001) which reported 0.227 and 0.222 for cadmium and lead and also greater than 0.1 and 0.05 which reported by (Agic et al., 2015) for cadmium and lead respectively. The present studies result for TF was lower than 1.46 for cadmium but greater than 0.45 for lead which was reported by (Deribachew et al,2015). The trend of TF for heavy metals in the vegetable samples was in order of Pb >Cd. The value for transfer factor < 1 indicates that the metals was less accumulated in vegetable than soil.

The transfer factor and variation of metal contents in vegetables depend on the physical and chemical nature of soil and absorption capacity of each metals by plants, which is altered by environmental and human factors and nature of the plants.(Singh et al., 2012).The results showed that there were significant variations in the levels of these metal amount in the examined vegetables across site by non-parametric test by kruskal Wallis tests $p<0.05$.

Heavy metal concentrations varied among the test vegetables, which reflect the differences in their uptake capabilities and their further translocation to edible portion of the plants. (Aweng et al., 2015)

The concentrations of metals in vegetables were generally lower than that of the corresponding soils. This might be attributed to the roots, which seems to act as a barrier to the translocation of metals. (Chao et al., 2007)

The concentration of both Cd and Pb was found to be higher in the soil samples than in the vegetables. This may be due to low metal availability in the soil and short life of studied vegetable to accumulate more metals from soil to their edible parts. The result shows us that the main sources of metal contents of vegetables from their corresponding soil contents which might be affected by urban wastewater from sewage and sewerage connected to the river used for irrigation, environmental interferences like pesticides, fertilizers and other wastes from small scale industries and garages. Variations in transfer factors among the different vegetables may be attributed to differences in element uptake by different vegetables. (Deribachew et al,2015)

The accumulation of the toxic metals in the edible parts of vegetables with high concentration could have a direct impact on the health risks of consumers. They are non-degradable, long

biological half-life, less visible, non- metabolized and can accumulate in the human body causing damage to the nervous systems, dysfunction of kidney, liver, lung, bladder, high blood pressures, prevalence of cancer, skin disorder and reproductive systems. (Jumbe & Nandini, 2009) Therefore, the concentration of toxic and potentially toxic element contaminants in vegetables are of concern as vegetables produced from these farms were mostly consumed by residents of the town. Prolonged and frequent human consumption of vegetables contaminated by heavy metals such as Pb and Cd, even at low levels, presents potential human health risks.

6.5 Daily intake of metals (DIM)

The estimated DIM through the consumption of vegetables for adults and children was presented in Table 12. The daily intake of metals (DIM) were calculated to averagely estimate the daily metal loading into the body system of a specified body weight of a consumer (Ratul et al,, 2018). DIM may be the realistic estimate for the average intake of metals from vegetables. DIM for Cd, and Pb ranged from 8.12E-06 to 1.1E-05 and 6.15E-05 to 7.32 E-05 mg/kg bw/day, respectively, for adults, while 9.33E-06 to 1.26E-05 and 7.07E-05 to 8.4E-05 mg/kg bw/day respectively, for children. Trend of the metal intake from all vegetables were Pb > Cd. The DIM values suggested that the consumption of vegetables grown in agricultural soils irrigated with polluted river water were nearly free of risks, as the provisional tolerable daily intake of metal is 0.06 mg and 0.214 mg for Cd and Pb respectively (Maleki *et al.*, 2014) and oral reference dose for Cd and Pb were 0.001 and 0.004 mg/kg bw/day, respectively (Ratul et al., 2018). This low DIM may be due to low consumption pattern of vegetable in the area.

6.6 Health risk index (HRI)

To assess the human health risk of heavy metals, it is necessary to calculate the level of human exposure to that metal by tracing the route of exposure of pollutant to human body. There are many exposures routes for heavy metals that depend upon a contaminated media of soil and vegetables on the recipients. Receptor population use the vegetables enriched with higher concentration of heavy metals which enters the human body leading to health risks (Khan et al., 2013). The estimated HRI for both adults and children for the consumption of vegetables for all measured heavy metals were given in Table 13. HRI for Cd and Pb ranged from 8.12E-03 to 1.10E-02 and 1.55E-02 to 1.83E-02, respectively, for adults, while ranged from 9.33E-03 to 1.26E-02 and 1.77E-02 to 2.11E-02, respectively, for children. The present study was much lower than the study conduct by (Ratul et al., 2018) which reported 0.14 and 0.17 in cadmium for

adults and child and 0.72 and 0.82 in lead for adults and child respectively. The study was also lower than the study conducted by (Shaheen et al., 2016) which reports HRI for 0.04 and 0.018for cadmium and lead respectively. Trend of health risk of heavy metals for the consumption of vegetables were Pb > Cd. The result revealed that HRI for both measured heavy metals for all studied vegetables was lower than 1. This may be due to low vegetable consumption pattern in the area, but the health risk may arise in long term consumption of the heavy metal contaminated vegetables by local community through accumulation in the body and it needs great attention for the future.

6.6 Limitation of the study

Environmental measurements are highly prone to variation in time and space change. But in this study the sample collection was conducted in during dry season and not collected in wet season in presence of runoff and rainfall which can lead to dilution and reduce contaminant levels in the sample.

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATION

7.1 CONCLUSION

The heavy metals concentrations in the studied vegetables were varied in the different sampling sites and among vegetable species reflecting contaminant increment as we go from upper stream to downward and the differences in uptake capabilities and their further translocation into edible parts. The mean concentrations of Cd and Pb was found higher than the safe limits by different organizations. This highest value may be due to the accumulation of lead contamination from small scale industries, environmental dust drops and heavy metals from fertilizers and pesticides. TF values for Cd and Pb for various vegetables were not significantly high since it was less than 1, but the correlation value for heavy metal between vegetable and soil shows us that there was strong correlation with p-value 0.01.

DIM values suggested that the consumption of vegetables grown in agricultural soils irrigated with polluted river water is nearly free of risks; since the oral reference dose for Cd and Pb are 0.001 and 0.004 mg/kg/day, respectively. Trends of health risk of heavy metals for the consumption of vegetables were Pb greater than Cd. The result revealed that HRI for both measured heavy metals were lower than 1 indicating safe for the consumers. But in the future the practice of disposing wastewater from painting of small scale industry, car wash, open river disposing of electronic waste and car battery may results in a long term accumulation of these metal and gradually increase the concentration in the environment and along the food chain and thus can cause serious health problems. Therefore, significant attention should be paid to prevent excessive build-up of heavy metals in the food chain by regular monitoring in agricultural soil and vegetables.

The result of microbial analysis for different bacterial type shows that all the bacteria under the present study was much higher than the recommended guidelines for consumption of raw eaten vegetables. The distribution of microbial contaminants was significantly different between the site and this indicate the effect of urban waste as we go down the town. The high level of microbial load in the study area may be due to urban sewage and sewer line entering to the river and corresponding soil under irrigation and vegetable produced on the site. Both irrigation water and fresh produce samples showed the presence of fecal coliforms and E. coli with the microbial load of river water typically several times higher than the values recommended by WHO guidelines for safe use of wastewater. This demonstrates that the transfer of river bound

microorganisms via irrigation water to fresh produce can take place and might therefore lead to fresh produce contamination at unacceptable levels. The elevated contamination levels of vegetables with bacteria may be due to human excreta, caw dung and application of manure. The microbial contaminants such as *E. coli*, Enterobacteriaceae and staphylococcus aureus leads to different human health risks. Microbial contaminants of vegetable indicate that all the vegetable samples were contaminated and none of them met the WHO maximum permissible level for raw eaten vegetable consumption. The contaminated river water used for irrigation contaminated the vegetable. Therefore, attention should be given to aware the community to use the vegetable by cooking and properly washing before eating the vegetables in raw and detail risk assessment should be conducted from production to consumption in order to provide complete intervention in reducing microbial diseases from vegetables.

7.2 RECOMMENDATION

1. To reduce heavy metal contamination of the vegetable; control of open field and river dumping of different wastes and waste separation should start in the town by the municipality.
2. Release of sewage and sewer lines in to the river should be avoided to reduce microbial contaminants in the vegetables by using appropriate waste management system such as wastewater treatment with collaboration of municipalities and the community through awareness and infrastructure building.
3. The Jimma town municipality should focus to prevent, regulate and monitor the waste from entering in to the river and prevent further contamination of the river from heavy metal and microbial contaminants.
4. The community consuming vegetable cultivated in these area should wash the vegetables properly before consuming and cooking should be practiced before eating the vegetables.
5. Further study should have to under taken on both sources of heavy metal and microbial contaminants in vegetables and I recommend for the next researcher to conduct health risk assessment of microbial contaminants to the local community.

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ANNEX

Annex 1

Table 15: Bacterial count in water, soil and vegetables

S/type	site	Bacterial count in log cfu/ml or per gram					
		AMB	Enterio.	ASF	Staphy.	T.C	F.C
Water	US	8.52±0.07	7.77±0.04	6.45±0.05	3.41±0.12	3.42±0.06	3.37±0.01
	MS	9.75±0.01	8.83±0.01	6.72±0.10	3.67±0.29	3.60±0.02	3.52±0.04
	LS	9.93±0.06	9.88±0.02	7.72±0.12	4.54±0.25	4.18±0.10	3.82±0.06
	Mean	9.40±0.77	8.83±1.05	6.97±0.67	3.87±0.59	3.73±0.40	3.57±0.23
	p-value	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
soil	US	7.88±0.04	6.84±0.03	5.61±0.04	3.88±0.02	3.35±0.15	3.08±0.04
	MS	8.91±0.02	7.94±0.03	5.80±0.02	4.03±0.05	3.44±0.24	3.27±0.03
	LS	9.95±0.01	8.95±0.04	6.80±0.02	4.57±0.09	3.79±0.08	3.43±0.11
	Mean	8.91±1.04	7.91±1.06	6.07±0.64	4.16±0.36	3.53±0.23	3.26±0.18
	p-value	0.000*	0.000*	0.000*	0.000*	0.042*	0.003*
Carrot	Upper	7.73±0.03	6.82±0.05	5.75±0.17	3.66±0.17	3.34±0.10	3.21±0.04
	Middle	8.71±0.03	7.78±0.04	5.65±0.13	3.92±0.08	3.38±0.14	3.18±0.07
	Lower	9.66±0.13	8.74±0.04	6.65±0.13	4.37±0.16	3.58±0.04	3.37±0.14
	Mean	8.70±0.96	7.78±0.96	6.02±0.55	3.98±0.36	3.43±0.13	3.25±0.11
	p-value	0.000*	0.000*	0.000*	0.003*	0.056**	0.078**
cabbage	Upper	7.69±0.01	6.82±0.02	5.84±0.04	3.63±0.04	3.18±0.22	2.87±0.04
	Middle	8.61±0.04	7.74±0.03	5.86±0.11	3.73±0.17	3.19±0.13	3.04±0.08
	Lower	9.62±0.11	8.64±0.07	6.64±0.17	4.26±0.10	3.38±0.09	3.33±0.05
	Mean	8.64±0.96	7.74±0.11	6.11±0.46	3.87±0.34	3.25±0.11	3.08±0.23
	p-value	0.000*	0.000*	0.000*	0.001*	0.267**	0.000*
Tomato	Upper	7.60±0.02	6.80±0.02	5.81±0.03	3.61±0.03	2.91±0.02	2.77±0.03
	Middle	8.58±0.01	7.66±0.12	5.73±0.05	3.92±0.07	3.17±0.06	2.94±0.03
	Lower	9.56±0.14	8.63±0.05	6.31±0.50	4.11±0.15	3.24±0.00	3.09±0.16
	Mean	8.58±0.98	7.70±0.92	5.95±0.31	3.88±0.25	3.11±0.17	2.93±0.16
	p-value	0.000*	0.000*	0.096**	0.002*	0.000*	0.015*

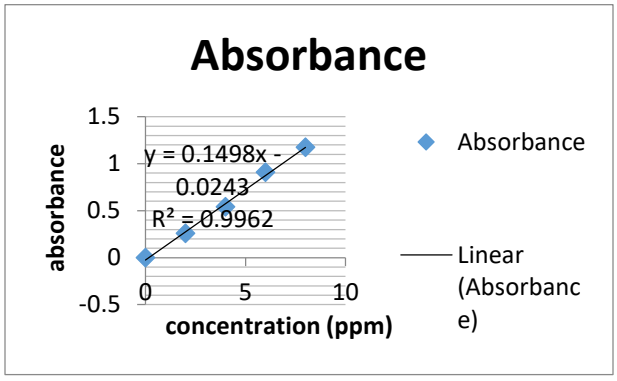
Annex 2

Table 16: Instrumental operation condition for the analysis of metal in sample of irrigation water, soil and vegetables

Metal	wave length	slit width(nm)	lamp current(Am)
Cd	228.9	0.7	2.1
Pb	283.2	0.7	1.9

Annex 3

Determination of calibration curve for heavy metals



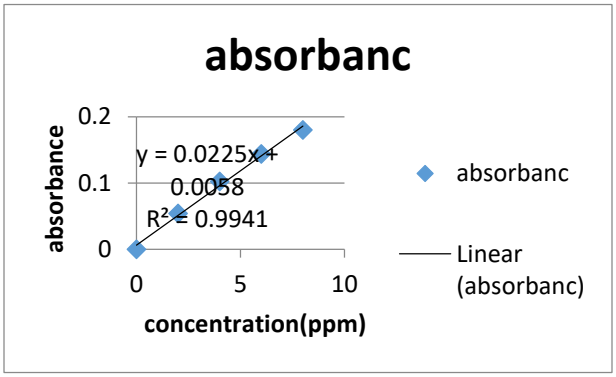
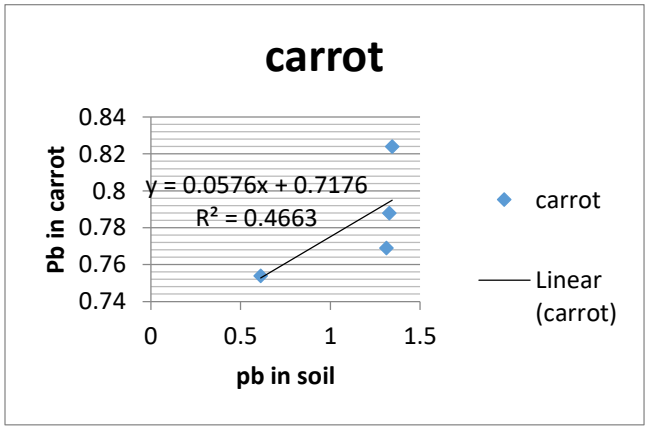


Figure 18 : Calibration curve of cadmium; Figure 19 : Calibration curve of lead

Annex 4

Correlation of Heavy metals in soil and vegetables

1. **Correlation of Lead in soil and carrot; soil and tomato**



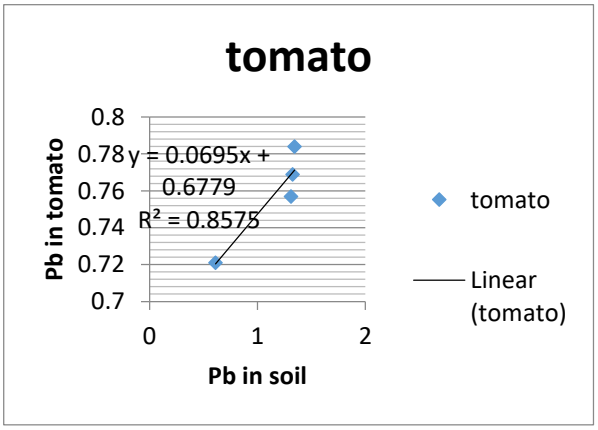


Figure 20 : Correlation of lead in soil and carrot; Figure 21 : Correlation of lead in soil and tomato

2. **Correlation of Lead in soil and cabbage; soil and carrot**

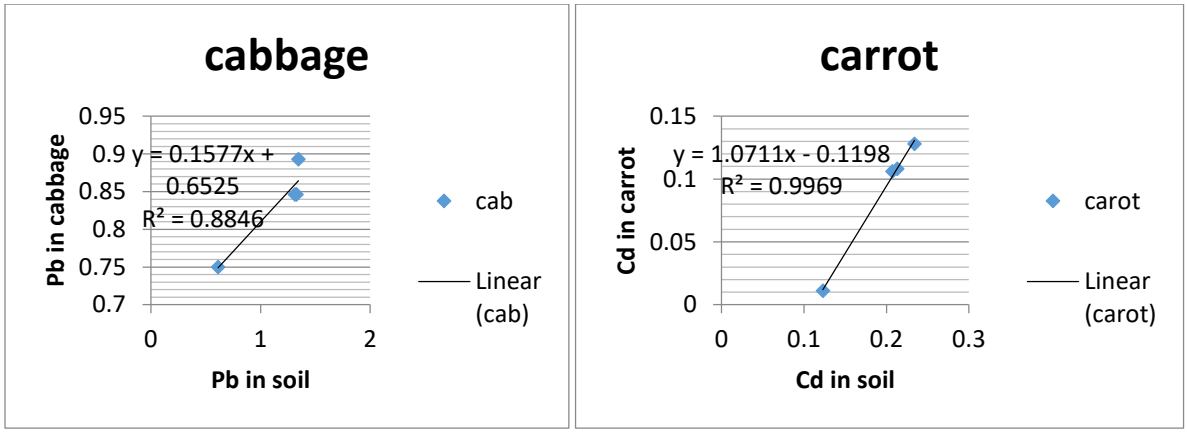


Figure 22 : Correlation of lead in soil and cabbage; Figure 23 Correlation of cadmium in soil and carrot

Correlation of cadmium in soil and tomato; soil and cabbage

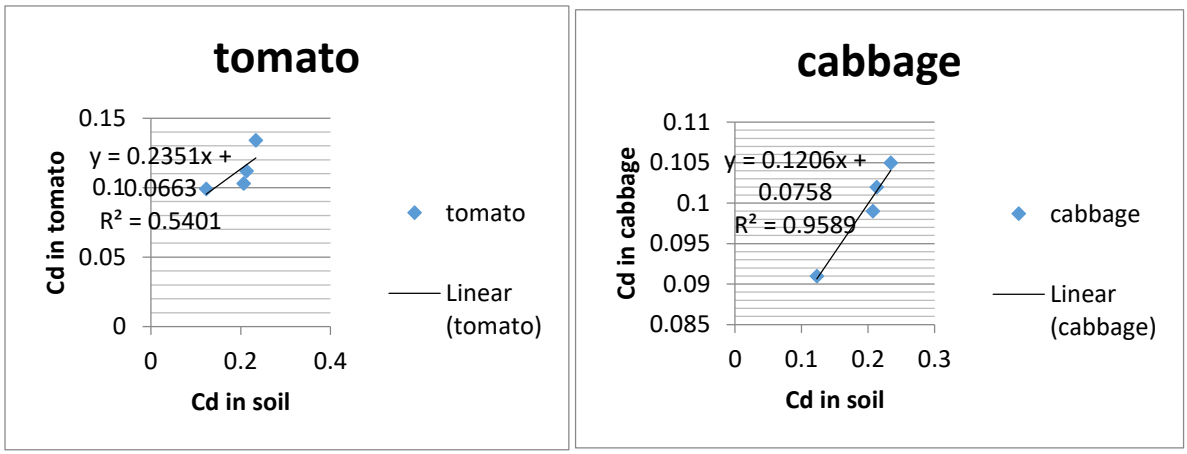


Figure 24 : Correlation of cadmium in soil and tomato; Figure 25: Correlation of cadmium in soil and cabbage

Annex 5

Procedure for bacteriological analysis of soil sample

A) Sampling soil

About 25 gram of soil sample was taken from each sampling point in pre sterilized poly ethylene bag and transported to laboratory.

Material and Reagents

- 25g fresh soil of each soil type
- One plastic cup for each soil type

- Benchtop balance (±0.01g)
- Weighing dishes
- Deionized water
- Plastic wrap
- Rubber bands
- Marking pens
- Dissecting probe
- Benchtop balance
- 1 sterile,95ml water blank for each soil type
- KIA
- Pre-prepared R2A agar plates pre-prepared glycerol-casein agar plates
- Citrate
- 4 sterile,9ml water blanks for each soil type
- 10 sterile,1ml pipettes for each soil type
- Pipette bulb
- 1 test tube
- Rack glass
- Hockey stick
- Spreader
- Ethyl alcohol for flame sterilization vortex gas burner
- SIM

Procedures

- 25g sample of each soil weighed into a labeled plastic cup.
- The samples were covered with plastic wrap to reduce moisture loss, and secured with a rubber band.
- The wrap was punctured several times with a probe to allow aeration without substantial moisture loss.
- The samples were weighed with the plastic wrap and rubber band and record the weights. To use the values to determine the final soil moisture content.
- The sample was incubated at room temperature for one week
- Preparation of the Plates
- Nutrient agar was prepared for dispensing the sample
- Each soil sample was reweighed to allow for soil moisture calculation at the time of plating.
- Dilution was prepared for each soil sample.
- For each sample 10g to a 95ml water blank and the suspension was shacked well.
- Before the soil settles in the bottle, 1ml of the suspension with a sterile pipette was removed and added in to a 9ml water blank and Vortex well.

- Repeat the procedures with a fresh 9ml water blank and sterile pipette. Vortex well. This resulted in dilutions of 10-1,10 -2,10 -3,10 -4, and 10-5g soil ml-1.
- Making Spread Plates for Bacteria
- Two to three plate for each dilution of 10 -3,10 -4, and 10-5 was made. After overtaxing, place a 0.1ml drop of each dilution (this increases your effective dilution by a factor of ten)
- Make a 10-fold dilution series:
- For one dilution (C), transfer 0.1 ml of suspension to each plate. After inoculating all replicate plates in one dilution.
- For each plate, sterilize a glass hockey stick spreader in a flame after dipping it in ethanol. Let the spreader cool briefly.
- The inoculum was speeded by moving the spreader in an arc on the surface of the agar while rotating the plate. Continued until the inoculum has been absorbed into the agar.
- Let the agar solidify, tape the plates together, and incubate them upside down for one week.

Counting Bacteria (after 1week incubation)

- Examine all of the bacteria plates carefully. Note differences in colony size and shape.
- Count the total number of bacterial colonies (CFUs) for each plate, including any actinomycetes. Average the totals for each dilution. Count only those plates of a dilution that are countable (30–200 colonies per plate).
- Calculate the sample mean of CFUs per gram of dry soil for each of your soils.

$$Bacteria\ per\ dry\ soil = \frac{Average\ count * dilution\ factor}{dry\ wt\ of\ 1\ gram\ moist\ soil}$$

Isolation of Pure Cultures

- ✓ Select colony that was separated from the neighboring colony
- ✓ Sterilize loop by dipping it in alcohol and flaming it.
- ✓ Remove small amount of a colony of interest onto the loop.
- ✓ The loop was Sterilized again and continued for others in similar way.

Gram Stain

- Bacteria was examined from each plates after one week. The colonies for uniformity of shape and size was observed.
- Small amount of drop of tap water to a slide with a wire inoculating loop. Use sterilized loop that flamed and small amount of culture was removed. The bacteria were mixed in the drop of water, spreaded on the size of the slide.
- The smear air-dried and then fixed the film by passing the slide through the Bunsen burner flame 2 or 3 times.
- 5 drops of crystal violet applied to the smear, allowing the dye to remain on the slide for 2 or 3 minutes.
- The slide rinsed with water and then with iodine solution. Covered with fresh iodine and let stands for two minutes. Rinsed with water, using a gentle stream.
- Decolorized with decolorizer. The decolorizer drop by drop to the smear with the slide held tilted. The decolorization was Continued until no more stain is seen to wash from the smear (30 seconds). Rinsed immediately in water.
- Counterstain for 30 seconds with safranin and the slide was rinsed with water.
- The was blotted carefully to hasten drying. Examined under oil using the oil immersion objective. Annex 7
- Procedures for vegetable sample analysis
- Media
- All the bacterial media used were procured from; Nutrient Agar, Nutrient Broth, MacConkey Agar, Eosin- methylene blue agar, Triple sugar Iron agar, Mannitol salt agar KIA, SIM, Simon citrate.

Annex 6

B) Procedure for bacteriological analysis of vegetable sample

Sample collection:

- Sample of fresh vegetables (carrot, tomato, cabbage) were analyzed and a total, five commonly consumed fresh vegetables namely tomato, carrot and cabbage were collected for the bacterial analysis

- Samples were collected in the sterile polythene zip bags to avoid any handling contamination and transported to laboratory for microbial analysis.
- The samples were collected in triplicate from the agricultural field from upper, middle and lower site. The samples were kept in the refrigerator at 4°C for later use.
- Sample processing:
- Twenty-five gram of each collected vegetable sample was weighed in sterile conditions and homogenized in sterile saline water using pestle and mortar for five minutes.
- The processed sample was added in to 225ml w: v peptone water.
- All the sterile conditions were maintained throughout the process. The homogenates were collected in sterile tubes and stored at -20ºC for further use.

a) Procedures

- Sample Collection
- 25 grams of each vegetable sample was weighed
- Homogenized by using pestle and mortar
- Ten-fold serial dilutions in sterile water
- Pure culture isolation and Maintenance
    - Morphological identification
    - Selective cum differential media based identification
    - Biochemical Identification

b) Isolation of Bacteria

- One ml of each sample was serially tenfold diluted in sterile water up to 10-5 dilution.
- The amount of 0.1 ml at 10-5 dilution was speeded over Nutrient agar media using sterile spreaders.
- The plates were incubated at 37 ºC for 12-24 hours for the appearance of colonies.
- Discrete colonies were sub-cultured in nutrient broth and streaked over different selective-cum-differential media agar plates i.e. MacConkey Agar, EMB agar and plate count Agar, mannitol salt agar and were incubated at 37ºC for 12-24 hours.
- The pure bacterial colonies obtained were primary identified using morphological analysis. Each isolated pure culture was maintained at 4ºC for further analysis
- Total Plate Count of Bacteria (CFU/ml) Microbial load in each vegetable sample was determined as CFU/ml and was calculated using formula.

$$\text{CFU/ml} = \frac{No.of\ colonies\ X\ dilution\ factor}{volume\ of\ inoculums}$$

c) Identification of Microorganisms

a) Morphological identification: The isolated bacteria were identified on the basis of negative staining and Gram's-staining

b) Selective media based identification: The pure isolated colonies were grown on media like, MacConkey agar, EMB agar, Mannitol Salt agar and were identified on the basis of characteristic growth appearance.

c) Biochemical Identification: The isolated bacterial colonies were confirmed by Biochemical test chemicals like KIA, SIM and Kligner iron agar.

Annex 7

C. Water sample analysis

a) Reagent and materials

- Equipment
  - Absorbent Pads
  - Autoclave
  - Balance
  - Culture Tubes
  - Forceps
  - Hot Air Sterilizing Oven
  - Total Coliform Incubator
  - Inoculating Equipment
  - Media Preparation Utensils
  - Membrane Filters
  - Membrane Filtration Units
  - Microscope and Lamp
  - Petri Dish Containers
  - Petri Dishes
  - pH Meter
  - Pipet Containers
  - Pipets
  - Refrigerator
  - Sample Containers
  - Thermometers
  - glucuronide (MUG).
  - Resistivity/Conductivity Meter
  - Equipment Timers
  - Fecal Coliform Incubator Ultraviolet (UV) Light

Reagents

- ✓ Mac ckonkey agar
- ✓ Nutrient agar
- ✓ Mannitol salt agar
- ✓ KIA
- ✓ SIM
- ✓ Simmons citrate
- ✓ Indole
- ✓ Kovac's reagent
- ✓ EMB
- ✓ Plate count agar

b) Sample collection

- The irrigation water sample was collected from the site by using pre sterilized sampling bottle of 500ml
- The sample was taken to laboratory by holding in cold box within three hours of collection and the sample maintained at 4^0 c for further analysis.

c) Sample analysis

- Microbiological analysis Fecal and total coliform counts were performed using the standard membrane filtration technique.
- The 100 ml water sample was filtered using 0.45 mm pore size, 47 mm diameter filter membrane as described by APHA (2005).
- Multiple tube technique was used for the enumeration of Most Probable Number of coliform bacteria.
- Serial dilution up to 10^{-5} was made and the sample was dispensed on the corresponding media in triplicate to count as MPN.
- Nutrient agar (NA) as a basal medium MacConkey agar as a differential medium and mannitol salt agar as a special medium were used to determine staphylococci, the sample was exposed to water bath for 10 minutes at 80^0 c for aerobic mesophilic bacteria, was enteric bacteria.
- The isolated pure culture was sub cultured on respective media to identify further specific organisms.
- Escherichia coli are isolated by inoculating the sample in ethylene methylene Blue. Enteric bacteria isolated on respective selective or differential media were identified on the basis of their colonial, morphological and Biochemical properties following Bergey's Manual of Determinative Bacteriology, 1994.

d) gram staining

- Gram staining was done to identify gram negative from gram positive.