INVESTIGATION OF BACTERIAL HAZARDS IN LOW QUALITY WATER USED FOR FOOD PRODUCTION IN URBAN AND PERI-URBAN AREAS OF MOROGORO, TANZANIA

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EXTENDED ABSTRACT

In Sub Saharan African countries, there is no or limited information on use of low quality water (LQW), hazards in foodstuffs produced using LQW and their associated health risks to humans, animals and the environment. The objective of this study was to investigate faecal bacterial hazards on low quality water; and foodstuffs produced using LQW in Morogoro, Tanzania. This study assessed the extent of *E. coli;* antimicrobial resistance (AMR) and virulence genes in Extended Spectrum beta lactamase (ESBL) producing strains; and *Salmonella* spp. contamination in LQW, tilapia and Chinese cabbage (*Brassica rapa* L. *chinensis*) irrigated using LQW. The 3M Petrifilm Select *E. coli* plates were used for enumeration and isolation of *E. coli* and ESBL producing *E. coli* at 44.4^{o}C. Isolation of *Salmonella* spp. was done as per ISO 6579:2002 (E) and its amendment of 2007, followed by; Pulsed Field Gel Electrophoresis (PFGE), genotyping, serotyping and antimicrobial susceptibility testing (AST). Levels of *E. coli*, ESBL producing *E. coli* and *Salmonella* spp. in wastewater were reduced from 5 - <1 log cfu/mL 4 - 0 log cfu/mL and 40 - <400 to <0.04 cfu/mL, respectively, following treatment in wastewater ponds. The ESBL producing *E. coli* isolates showed broad range of AMR genes in beta- and non-beta lactams antimicrobials including *bla*CTX-M1, *bla*CTX-M15, *bla*OXA-1; *aac*6, *aad*A4, *aac*6Ib, *str*A, *str*B; *dfr*A19, *dfr*A17, *dfr*A7; *cat*A1, *cat*B3; *qnr*A; *sul*1, *sul*2; *tet*A, *tet*B; and *mph*A, *mrx*; virulence genes (*hem*L, *iss*, *tir*, *iha*, *sat*, *sen*B); 13 *Salmonella* serovars including *S.* Kentucky, *S.* Chandans, *S.* Durban and *S.* Kiambu from LQW and tilapia. Occurrence of *E. coli* in fish flesh from wastewater was 0.05% and in Mindu dam was 0%;

while, in Chinese cabbage irrigated with river water was 86% and in treated wastewater was 10%. The concentration of *E. coli* in Morogoro river water was higher than the treated wastewater. Tilapia grown in wastewater treatment ponds and vegetables irrigated by treated wastewater were of good quality for human consumption. The quality of treated wastewater from the study sites complied with the WHO/FAO guidelines (1000 cfu/100 mL faecal coliforms) and can be recommended for use on food production.

DECLARATION

I, OFRED JONAS MHONGOLE do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work, done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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DEDICATION

To my late parents, Jonas Mhongole and Ledusina Gila, my spouse Getrude Migodela, my children (Helga, Walburga, Olga and Edelburga) for their endured expenses during a long journey of my education.

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LIST OF ABBREVIATIONS

CDC Centres for Disease Control

CLSI Clinical and Laboratory Standards Institute

CTX Cefotaxime

DHI Danish Hydraulic Institute

ECDC European Centre for Disease Prevention and Control

EFSA European Food Safety Authority

ESBL Extended Spectrum beta-lactamases

EUCAST European Committee on Antimicrobial Susceptibility Testing

FAO Food and Agriculture Organization

LQIW Low Quality Irrigation Water

LQW Low Quality Water

MIC Minimum inhibition concentration

PCR Polymerase chain reaction

PFGE Pulsed-field Gel Electrophoresis

SaWaFo Safe Water for Food

URT United Republic of Tanzania

WHO World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Low Quality Water in Urban and Peri-urban Areas

Water is an essential resource in supporting life of humans, animals, plants and other living systems on the earth. However, in arid and semi-arid regions of the world, water for agricultural and industrial development is a limiting resource (Nyilimbabazi *et al*., 2011; Al-Mashaqbeh *et al*., 2012; Babayan *et al*., 2012). In Sub-Saharan African countries, many people struggle to access limited clean and safe drinking water. Due to scarcity of clean and safe water, they opt to use low quality water (LQW) and is on the rise (Ensink *et al*., 2007). Although the use of wastewater in food production is recognized by farmers worldwide (Babayan *et al*., 2012), its use in Sub-Saharan African countries is informal. The term 'low quality water' used in this study covers domestic wastewater and grey water; polluted surface and ground water bodies linked to wastewater and storm water used for food production in urban and peri-urban areas of Morogoro, Tanzania. The LQW is not potable water and not recommended for domestic use.

Low quality water is widely used in food production such as agricultural irrigation and aquaculture. The use of wastewater for agricultural irrigation purposes is estimated 41% in Japan, 60% in USA (California), 25% in Palestine and 15% in Tunisia (Vigneswaran and Sundaravadivel, 2004). Fig. 1.1 shows that each continent has some information on wastewater, though data on the generation, treatment and use are limited or out-dated (Mateo-Sagasta *et al*., 2013; Sato *et al*., 2013). In Tanzania the information on the use of wastewater for food (aquaculture and horticultural) production are not available.

Figure 1.1: Number of countries with records on wastewater generation, treatment and use in the regional blocks of the world

Low quality water contains high concentrations of excretal pathogenic micro-organisms that can cause diseases in humans and animals (Hussain *et al*., 2002; Jiménez *et al*., 2010). The management of wastewater is therefore, an important requirement for sustainable development in the world (Valipour, 2013).

The World Health Organization (WHO) in 2006, prepared guidelines on safe use of wastewater, excreta and grey water to reduce health risks associated with LQW to farmers, consumers and communities around wastewater collection and treatment areas (World Health Organization, 2006a, b, c, d). As a strategy to meet WHO guidelines on safe use of LQW, the governments and stakeholders have to develop health risk preventive measures including wastewater treatment systems. If treatment of wastewater is properly done, wastewater treatment systems reduce more than 90% faecal bacteria in treated wastewater (Al-Sa'ed, 2007).

1.2 Drivers for Wastewater Use

1. High demand for fruits and vegetables in urban and peri urban areas has increased the use of LQW. Wastewater use is driven by physical and economical clean water scarcity, population growth and urbanization (World Health Organization, 2006b). In addition, as compared to potable water, LQW is freely available all year round (Jiménez, 2006; Mateo-Sagasta *et al*., 2015). In Sub Saharan Africa Countries, urban and peri urban farmers therefore, depend on LQW for food production. Low quality water use in food production in urban and peri-urban

communities is practiced globally (Wichelns *et al*., 2011; Mateo-Sagasta *et al*., 2015). However, the Regulatory Authorities in Sub-Saharan African countries lack guidelines on wastewater management and use including capacity to ensure collection and treatment of wastewater (Mateo-Sagasta *et al*., 2015). On the other hand, market demand is high and is characterized by a short distance which motivates them to transport even perishable vegetables without needs for packaging and cold chain facilities. Since, most consumers are not aware of foodstuffs and associated diseases from LQW use in food production; governments have to develop strategies to ensure proper treatment of wastewater and safe use of such water in food production.

2.

1.3 Food Safety and Health Risks Associated with Wastewater Use

1. Use of LQW for food production in urban and peri-urban areas has been implicated with outbreaks of various food and waterborne diseases. Antimicrobials are also used in human and animal medicine for treatment, infection prevention and as growth promoters (World Health Organization, 2011; Katakweba, 2014). There is therefore, concerns on emerging antimicrobial resistance from LQW and foodstuffs produced using LQW in humans and animals. Foodstuffs including meat, fish, fruits and vegetables, eggs and milk contaminated with faecal pathogens may become the main transmission route for pathogens that are resistant to antimicrobials and their resistance genes from food animals to humans (EFSA Panel on Biological Hazards (BIOHAZ), 2011; 2013). For instance foodstuffs contaminated with *E. coli* and *Salmonella* spp. with resistance genes from animals and water point sources can be transmitted to humans (Collignon, 2009). The presence of emerging ESBL producing bacteria from humans and animals suggests that food animals are among the potential

sources for transmission of antimicrobial resistant bacteria to humans and vice versa (EFSA Panel on Biological Hazards (BIOHAZ), 2011).

2.

3. Hazards are divided into three types; biological, chemical and physical; there presence in LQW and foodstuffs, can reasonably cause illness or injuries to consumers in the absence of control or reduction to acceptable levels. The biological (microbiological) hazards include pathogens such as *E. coli* pathotypes, *Salmonella* spp., *Listeria monocytogenes*, *Vibrio cholerae*, *Campylobacter jejuni* and *Clostridium* spp. (*C. botulinum* and *C. perfringes*) as well as protozoans and viruses. Outbreaks of food-borne diseases and infection caused by bacterial hazards from consumption of fresh fruits and vegetables have increased considerably (Mukherjee *et al*., 2007). Often, food-borne diseases of humans arise more from microbial than chemical hazards (Issa-zacharia *et al*., 2010). The major food-borne bacteria of concern in food industry include *E. coli* and *Salmonella* spp. (Newell *et al*., 2010).

4.

5. The antimicrobial resistance of zoonotic bacteria causing food-borne diseases is also on the rise due to the changing environmental conditions and inappropriate practices. Moreover, the susceptibility in humans population to such challenges and diseases is also on the rise due to the decline of acquired immunity, increased proportions of immune-compromised individuals and immunosuppressive treatments (Newell *et al*., 2010). However, studies on use of LQW for food production have been carried out in various countries, including: Pakistan, Cambodia, Vietnam and Ghana (Amoah *et al*., 2007; Anh *et al*., 2007; Ensink *et al*., 2007; Keraita *et al*., 2007). In these studies, faecal bacterial indicators ranged from <1 to 7 log cfu/g in vegetables.

6.

1.4 Problem Statement and Justification

In developing countries, many people struggle to access a limited clean and safe drinking water, as results waterborne infections and diseases outbreaks are common. In Sub Saharan African countries, information on use of LQW, foodstuffs produced using LQW and health risks associated with LQW in humans, animals and the environment are limited or not available (World Health Organization, 2014). In Tanzania, communities dwelling in urban and peri-urban areas experience inadequate supply of clean water for their use; as a consequence wastewater and polluted water from rivers and wells become main sources of water for food production (Mwang'onde *et al*., 2013). Increase in use of LQW in agriculture or aquaculture systems is therefore, due to inadequate supply of clean water, sanitation and wastewater treatment facilities (Ensink *et al*., 2007; Jiménez *et al*., 2010; Sato *et al*., 2013).

The use of LQW in crop irrigation and aquaculture is a potential source of faecal contamination in foodstuffs and the environment. Pathogens from LQW, including: *Escherichia coli* and *Salmonella* spp. are among major food-borne bacterial hazards in food industry (Mukherjee *et al*., 2007; Newell *et al*., 2010). Use of LQW in developing countries, urban and peri-urban areas is often unplanned (Ganoulis, 2012). Moreover, LQW is often freely available for use in crop irrigation and aquaculture. Despite all these, in Tanzania there are limited studies carried out to assess bacterial hazards associated with LQW, irrigated foodstuffs and fish grown in LQW fed aquaculture (Senzia *et al*., 2009; Mkali, 2014). The objective of this study was therefore; to investigate faecal bacterial hazards in LQW and food produced using LQW. The extent of *E. coli* and *Salmonella* spp. contamination, antimicrobial resistance and virulence genes in ESBL

producing *E. coli* in LQW, tilapia and Chinese cabbage (*Brassica rapa* L. *chinensis)* were assessed.

1.5 Study Objectives

1.5.1 Main objective

The main objective of this study was to investigate faecal bacterial hazards in low quality water and food produced using low quality water.

1.5.2 Specific objectives

The specific objectives were to:

- Assess the efficiency of wastewater treatment ponds in reduction of *E. coli* in urban and peri-urban areas of Morogoro, Tanzania.
- Characterize ESBL producing *E. coli* present in low quality water used for food production in urban and peri-urban areas of Morogoro, Tanzania.
- Isolate and characterize *Salmonella* spp. in wastewater and Tilapia in urban and peri-urban areas of Morogoro, Tanzania.
- Assess bacteriological quality of tilapia from wastewater treatment ponds in peri-urban areas of Morogoro, Tanzania.
- Quantify *E. coli* in *Brassica rapa* L. *chinensis* irrigated with treated wastewater in urban areas of Morogoro, Tanzania.

1.6 Study Area and Design

1.6.1 Study area

This study was conducted in Morogoro municipality and Mvomero district in Morogoro region during October, 2012 to February, 2015 (Mhongole *et al.,* 2016). Morogoro municipality lies at 06°49′20″S and 037°39′55″E and Mvomero district at 06°53′29″S and 37°33′37″E. According to United Republic of Tanzania (URT), 2012, population housing census; the population in Morogoro Municipality is 315 866 and Mvomero district is 312 109 people. Table 1.1 shows an average climatological data in Morogoro region from Tanzania Meteorological Agency (TMA) from 2000 to 2012. The Temperature ranged from 19.36 to 31.14^{o}C, and total rainfall ranged from 447.2 mm (2005) to 1182.1mm (2006) per year and the wind speed varied from 3.17 to 7.87 knots. The solar radiation ranged from 16.91 to 19.58 MJM^$^{-2}$ and evaporation rate ranged from 4.59 mm to 5.78 mm.

Table 1.1: Annual average of climatological conditions in Morogoro region from 2000 to 2012

Years	Max Temp ^{o}C	Min Temp^{o}C	RainfallL-(mm)/Yr	Evaportaion -(mm)	Wind speed (Knots)	Solar Radiation (MJM^ -2)
2000	30.41	19.36	789.3	5.42	5.38	17.75
2001	30.39	20.00	784.0	4.74	6.29	17.80
2002	30.14	20.10	950.8	4.80	6.61	17.71
2003	31.35	20.01	494.0	5.78	7.44	18.88
2004	30.53	19.86	765.3	4.98	7.16	17.61
2005	31.11	20.45	447.2	5.29	7.87	19.04
2006	30.32	20.46	1182.1	4.74	7.62	17.15
2007	30.58	19.99	835.7	4.65	6.70	17.56

2008	30.24	19.70	703.7	4.89	3.34	17.60
2009	30.94	19.75	637.3	5.24	4.03	18.20
2010	31.14	19.93	767.4	5.35	4.54	19.15
2011	30.34	20.02	784.7	4.59	3.17	16.91
2012	30.75	19.73	694.4	5.09	3.70	19.58

1.6.2 Study design

This study was carried out in five purposively selected sites. Namely, three wastewater treatment systems located in Mafisa, Mzumbe and Mazimbu (Fig. 1.2) as well as Mindu dam and Fungafunga areas along Morogoro river. Mindu dam is the main source of domestic water for urban and peri urban areas of Morogoro, and Fungafunga area is among the main areas for green leafy vegetables production in Morogoro Municipality. Samples of low quality water, tilapia and Chinese cabbage were collected from the study sites. The description of sample categories is shown in Table 1.2 and details in individual Chapters Two to Six. Fig. 1.3 shows the tilapia fish and Chinese cabbage used in the study. Fig. 1.4 shows plates for Fungafunga vegetables farm (L) and Mindu dam (R) study sites.

Samples were analysed for *E. coli,* cefotaxime (CTX) resistant ESBL producing *E. coli* and *Salmonella* species. In addition, antimicrobial resistance patterns for *Salmonella* spp. and ESBL producing *E. coli* isolates and their antimicrobial resistance and virulence genes in ESBL producing *E. coli* were screened.

Table 1.2: Samples of LQW, tilapia and Chinese cabbage from study sites

Objectives	Matrix	Samples (N)	Mafisa (n)	Mzumbe (n)	Mazimbu (n)	Fungafunga/ Morogoro river (n)	Mindu dam (n)
SPO1 – *E. coli*	LQW	125	85	40			
SPO 2 - ESBL	LQW	79	35	31	9	4	
SPO 3 - *Salmonella*	LQW	52	28	24			
	Tilapia	21			21		
SPO 4 - Tilapia	LQW	41	20				21
	Tilapia	61		41			20
SPO 5 – Chinese cabbage	LQW	37	12	12	10	3	
	Chine. Cabba.	69			48	21	
	Tatal	**485**	**179**	**148**	**89**	**28**	**41**

(N = Total samples and n= number of samples per specific objective/Chapter 2 to 6)

Figure 1.2: Study sites in Mafisa (a), Mzumbe (b) and Mazimbu (c) wastewater treatment ponds

Figure 1.3: Tilapia fish from Mzumbe (L) and Chinese cabbage from Mazimbu (R)

Figure 1.4: Fungafunga vegetables farm site (L) and Mindu dam (R)

1.7 Thesis Organization

This thesis is presented in accordance to the "Published papers format" of the Sokoine University of Agriculture, comprising of seven chapters. Chapter One introduces the entire study including a brief literature review, problem statement, study objectives as well as study area and design. Chapters Two to Six comprise of four original published papers and one manuscript. The papers and manuscript are presented and arranged according to the specific objective (s), namely:

- Removal of *Escherichia coli* in treated wastewater used for food production in Morogoro, Tanzania (specific objective I).

  Published: *African Journal of Microbiology Research*

- Antimicrobial resistance patterns of Extended-spectrum Beta-lactamase Producing *Escherichia coli* in Low Quality Water in Morogoro, Tanzania (specific objectives II).

  Manuscript intended to be submitted: *Journal of Comparative Immunology, Microbiology & Infectious Diseases*

- Characterization of *Salmonella* spp. from wastewater used for food production in Morogoro, Tanzania (specific objectives III).

  Published: *World Journal of Microbiology and Biotechnology*

- Bacteriological quality of Tilapia fish from treated wastewater in peri-urban areas of Morogoro, Tanzania (specific objective IV).

  Published: *Agriculture, Forest and Fisheries*

- Occurrence of *Escherichia coli* in *Brassica rapa* L. *chinensis* irrigated with treated wastewater in urban areas of Morogoro, Tanzania (specific objective V).

  Published: *African Journal of Biotechnology*

Chapter Seven contains general results and discussion, conclusions and recommendations.

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CHAPTER TWO

2.0 REMOVAL OF *ESCHERICHIA COLI* IN TREATED WASTEWATER USED FOR FOOD PRODUCTION IN MOROGORO, TANZANIA

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CHAPTER THREE

3.0 ANTIMICROBIAL RESISTANCE PATTERNS OF EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING *ESCHERICHIA COLI* IN LOW QUALITY WATER IN MOROGORO, TANZANIA

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ABSTRACT

The aim of this study was to isolate and characterize the ESBL producing *E. coli* from low quality water used for food production in urban and peri-urban areas of Morogoro, Tanzania. A total of 79 samples including untreated wastewater (28), intermediate (14), treated wastewater (27), treated wastewater downstream (6) and river water (4) were collected. Presumptive ESBL producing *E. coli* were screened using 0.25 mg/L cefotaxime. Thereafter, isolates were tested for AMR patterns by ESBL confirmatory MIC plates (ESB1F). One hundred and ten out of 117

isolates showed resistance to ampicillin (94%), cephalothin (93%); cefazolin, cefpodoxime, ceftazdime, ceftriaxone and cefotaxime (99%); ciprofloxacin (82%), gentamicin (59%) and cefepime (13%). Antimicrobial resistance and virulence genes were detected in 84 out of 110 isolates which showed AMR out of 117 isolates by Microarray *E. coli* genotyping combined assay. The ESBL producing *E. coli* isolates were resistant to beta-lactams (*bla*CTX-M1, *bla*CTX-M15, *bla*OXA), aminoglycoside (*aac*6, *aad*A4, *aac*6Ib, *str*A, *str*B), trimethoprim (*dfr*A19, *dfr*A17, *dfr*A7), chloramphenicol (*cat*A1, *cat*B3), quinolone (*qnr*A), sulfonamide (*sul*1, *sul*2), tetracycline (*tet*A, *tet*B) and macrolide (*mph*A, *mrx*). Virulence genes screened include *hem*L (74), *iss* (61), *tir* (58), *iha* (12), *nfa*E (6), *prf*B (14), *sep*A (6), *sat* (11) and *sen*B (9). One or both O-serotypes and H- flagellin antigens were screened in 84 isolates including the O91:H30, O86:H30, O15:H30 and O101:H21 serogroups. In this study, wastewater and surface water were contaminated with ESBL producing *E. coli* and their antimicrobial resistance and virulence genes, which are detrimental to humans and animals health.

Keywords: Antimicrobial resistance genes, health risk, low quality water, virulence genes, wastewater

1. 3.1 Introduction

Extended spectrum beta lactamases (ESBL) are defined as plasmid-encoded enzymes found in *Enterobacteriaceae*, frequently in *Escherichia coli* and *Klebsiella pneumoniae*. The enzymes confer resistance to a variety of beta-lactam antimicrobials, including penicillin; first to fourth generation cephalosporins and monobactam, but often not to carbapenem nor cephamycin [1]. Production of resistance genes by ESBL producing bacteria particularly the *Enterobacteriaceae*

is a significant resistance-mechanism and life threatening that constitutes a potential health risk to humans and animals [2–6].

Antimicrobials are either naturally found in the environment, in microorganisms, in animals and in plants or are synthetic compounds which are used for treatment of microbial infections [4,7]. Antimicrobial resistance is increasing due to misuse of antimicrobials in human and animal medicine as well as malpractices in agriculture and aquaculture [8–10]. Besides, antimicrobial residues entering into liquid wastes from hospitals, residential houses, business areas and animal farms are disseminated into wastewater and the environment [4]. Ultimately, antimicrobial resistant bacteria from the environment, water and foodstuffs transmitted to humans and animals cause infections that are difficult to treat by conventional antimicrobials [11–13]. Furthermore, resistant bacteria increase the chances of being transmitted between humans, particularly in hospital and community settings with high use of antimicrobials.

The *E. coli* pathotypes are among the pathogenic bacteria that cause infections in humans and animals including sepsis/meningitis, urinary tract infection (UTI) and diarrhoea. *E. coli* pathotypes include enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvassive (EIEC, enterohaemorhagic (EHEC), enteroaggregative (EAEC) and diffusely adherent *E. coli* (DAEC). The identification of *E. coli* serotypes is based on three antigens, the lipolysaccharide (O-antigen), flagellar (H- antigen) and capsular (K-antigen). However, only few laboratories have the capacity for typing K-antigens thus making the O- and H- antigens the major basis for serogroupings of Gram negative bacteria [14,15]. The O- and H- antigens are therefore, known as gold standard for typing pathogenic bacteria and clones of *E. coli* [14]. There are about 187 O-

and 53 H- known serogroups (antigens). A total of 44 out of 53 H- antigens are encoded by genes in *fliC* H- antigen allelles. The O-antigen defines the serogroups and the combination of the O- and H- antigens define the *E. coli* serotypes [14,16].

Low quality water (wastewater) is recognized as among the main sources of antimicrobial residues and faecal pathogens [4]. Altogether, they may contribute to development of multiple antimicrobial resistant bacteria in the environment [7]. Generally, wastewater use in food production systems in urban and peri-urban areas in Sub-Saharan African countries is on the rise due to increase in scarcity of clean water. However, data on the treatment and antimicrobial monitoring in wastewater used for crops irrigation and aquaculture in Sub Saharan African countries, including Tanzania are limited or not available [17]. Moreover, information on the extent of antimicrobial resistance in ESBL producing bacteria from wastewater used for food production is as well limited. Previous studies documented an increase in incidences of ESBL producing *E. coli* from hospitals and communities [18,19] as well as in food animals, environment and agricultural crops [1]. However, in Tanzania, there are few studies that have reported *bla*CTX-M-15 in hospital and community settings, livestock and the environment [20–22]. This study was therefore, designed to establish the prevalence, antimicrobial resistance- and virulence- genes patterns in ESBL producing *E. coli* from wastewater treatment ponds and river water used for food production in urban and peri-urban areas of Morogoro in Tanzania.

1. 3.2 Material and Methods

3.2.1 Study area and sampling

This study was carried out in urban and peri-urban areas in Morogoro Municipality and in Mvomero district between November, 2012 and April 2014. Four study sites were purposively selected comprising of three wastewater treatment systems (Fig. 3.1) and Morogoro river / Fungafunga area. Mafisa, Mazimbu and Morogoro river (Fungafunga) sites are located in urban Morogoro municipality, within 06°49′20″S and 037°39′55″E. The Mzumbe study site is located in Mvomero district of Morogoro region, within 06°53′29″S and 37°33′37″E. Wastewater at Mazimbu area is pumped to the wastewater treatment ponds while at Mafisa and Mzumbe, it flows by gravity. Mzumbe wastewater treatment ponds are located within Mzumbe University campus characterized with a short distance from students and staff housing, the hospital and other university facilities. Mafisa sewer canal is connected from different sources with a total length of 30 km; of which 9.7 km is the main sewer and 20.3 km are lateral sewers. Morogoro river flows from Uluguru Mountains and joins with several small rivers as well as treated wastewater at Mafisa.

First trial was carried out in November 2012, where 37 samples were collected from Mafisa (n=11), Mzumbe (n=13), Mazimbu (n=9) and Morogoro river/ Fungafunga (n=4) for five different days. Thereafter, Mafisa and Mzumbe wastewater treatment ponds were selected for a further study to ascertain removal efficiency of ESBL producing *E. coli*. These ponds were well operating while Mazimbu ponds were not well attended and operating to be used for the performance study. Second sampling was repeated in Mafisa and Mzumbe between October, 2013 and April 2014. A total of 42 samples of untreated wastewater, intermediate/partially and treated wastewater were collected from Mafisa (24) and Mzumbe (18) for seven days. Samples were collected from inlets and outlets of each pond (Fig. 3.1) as well as from Morogoro river at

Fungafunga area. Samples were collected by using sterile 50 mL falcon tubes with screw caps, and tightened with a rope. Samples were put in the cool box with ice cubes and immediately transported to the Pest Management Centre Laboratory, Sokoine University of Agriculture, and analysis was initiated on the same day.

3.2.2 Isolation of ESBL producing *E. coli*

Reduction of ESBL producing *E. coli* in wastewater treatment ponds was studied. Water samples were plated on petri-film select *E. coli* (SEC) plates with a chromogenic medium as per 3M Microbiology products protocol (St. Paul, USA). Water samples were serially diluted from 1:10 to 1:100,000 ten-fold as required. A volume of 0.05 mL of 0.25 mg/L Cefotaxime (CTX) solution was added into 1 mL of selected dilutions. Immediately, 1 mL was inoculated onto SEC plates and incubated at 44.4°C for 24 hours. After incubation, all colonies that grew on SEC plates and those which appeared blue were counted and were reported as CTX resistant *E. coli* (ESBL producing *E. coli*) according to the 3M Microbiology products protocol (St. Paul, USA).

One to four typical colonies from SEC plates were picked using a sterile loop and streaked onto MacConkey agar (MA) plates (Merck, Darmstadt, Germany). The MA plates were incubated at 37^{o}C overnight to confirm the *E. coli* phenotypic characteristics and pure culture colonies. From typical culture plates, one single colony was picked and inoculated into 5 mL Laury tryptose lactose broth (LTB), (Oxoid Limited, Hampshire, England) in the bijou bottles and incubated at 37°C for 24 hours. After incubation, 0.7 mL of the LTB culture growth was pipetted and transferred into 1 mL cryovials containing 0.3 mL of 70% sterile glycerol, to make a final concentration of 30% glycerol. The ESBL producing *E. coli* isolates were stored at -80°C for

molecular characterization. The isolates were shipped to Denmark at the Department of Veterinary Disease Biology, University of Copenhagen. All isolates were examined for antimicrobial resistance, virulence factors and genes, as well as O serotypes and H serogroups.

3.2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) of 117 ESBL producing isolates from plates with CTX was done by the minimum inhibition concentration (MIC) plates. The ESBL confirmatory MIC plate (ESB1F) panel with 16 antimicrobials in 96 wells were used (Trek Diagnostics System (TREKDS), East Grinstead, UK). The ESB1F contained the following antimicrobial agents: cefazolium (8-16), cefepime (1-16), cefoxitin (4-16), meropenem (1-18), cephalothin (8-16), cefpodoxime (0.5-64), ceftriaxone (1-128), ciprofloxacin (1-2), gentamicin (4-16) and ampicillin (8-16). Others were imipenem (0-5-16), peracillin/tazobactam (4/4-64/4), ceftazidime (0.25 - 128), ceftazidime / clavulanic acid (0.25/4 - 128/4); cefotaxime (0.25 - 64) and cefotaxime / clavulanic acid (0.25/4 - 64/4) in µg/mL. About 3 to 4 colonies from the overnight Blood agar (Oxoid Limited, Hampshire, England) were picked by a sterile loop and emulsified in 5 mL sterile distilled water, then, mixed well by vortexing and turbidity checked with McFarland 0.5 with aid of nephelometer. A volume of 10 µL was inoculated into 10 mL Müller Hilnton (MH) II broth (Oxoid Limited, Hampshire, England) in test tube, then, mixed well and poured into sterile petri dish. By using a multichannel pipette, 50 µL of culture suspension was inoculated in all 96 wells ESB1F sensititre plates and incubated at 37^{o}C for 16 - 20 hours. Following incubation time, plates were visualized by Sensititre Vizion System (TREKDS). The MIC cut off schemes for *Enterobacteriaceae* (ESBL producing *E. coli*) were used to interpret the

AST results of TREKDS. Furthermore, MIC values for ESBL producing *E. coli* were interpreted according to ESBL1F; Clinical and Laboratory Standards Institute [23].

3.2.4 Molecular characterization

Molecular characterization of positive ESBL producing *E. coli* isolates was done at Alere Technologies GmbH in Germany. Microarray typing *E. coli* genotyping combined assay (Alere Technologies GmbH, Germany) was used. The *E. coli* genotyping combined assay allowed DNA-based serogenotyping of most known antigens (i.e. 23 – O/ 47- H antigens). The Microarray was also equipped with 102 probes for detecting antimicrobial resistant genes and 88 probes for detecting virulence genes. It simultaneously screened broad range of important antimicrobial resistance (i.e. *bla*OXA) and virulence (i.e. stxA/stxB) genes.

3.2.5 Statistical analysis

The concentrations of ESBL producing *E. coli* was entered in the Microsoft Excel and analysed using SPSS statistics software version 20.0 of 2011 (IBM, California, USA). Paired sample means concentrations of *E. coli* were compared between the study sites by the Student's *t*-test. The concentration of *E. coli* was presented in boxplots. Results for antimicrobial resistance genes, virulence genes and O- and H- antigens were presented in pivot Tables and Figures. Furthermore, the antimicrobial resistance and virulence genes in ESBL producing *E. coli* isolates were analysed by Pearson correlation test. The differences between groups were reported at $P<0.05$.

3.3 Results

3.3.1 Isolation of ESBL producing *E. coli*

A total of 117 ESBL producing *E. coli* were isolated from water in Mazimbu (n=49), Mafisa (n=18), Mzumbe (n=30) and Fungafunga area (n=20). The mean concentration of ESBL producing *E. coli* in untreated and treated wastewater; and river water were significant differently ($P<0.05$) with higher concentration in untreated wastewater. However, there was 100% reduction of ESBL producing *E. coli* from 3.82 log cfu/mL (Mafisa), 3.66 log cfu/mL (Mzumbe) and 4.97 log cfu/mL in untreated wastewater in Mazimbu (Fig. 3.2) to <1 log cfu/mL in treated wastewater.

3.3.2 Antimicrobial susceptibility testing

A total of 110 out of 117 CTX ESBL producing *E. coli* isolates screened showed resistant. Table 3.1 shows the antimicrobial resistance patterns of ESBL producing *E. coli*. The ESBL producing *E. coli* isolates showed resistance to 10 out of 16 antimicrobials. The isolates showed low resistant to the fourth generation cephalosporin (cefepime) and was high in aminoglycoside (gentamicin); penicillin (ampicillin); first generation cephalosporin (cefazolin and cephalothin); fluoroquinolone (ciprofloxacin); third generation cephalosporin (ceftriaxone), cefpodoxime, ceftazdime and cefotaxime. All isolates were susceptible to cephamycin (cefoxitin), carbapenem (meropenem and imipenem) and beta lactam inhibitors; cefotaxime / clavulanic acid, ceftazidine / clavulanic acid and piperacillin / tazobactam.

3.3.3 Antimicrobial resistance genes

Tables 3.2 – 3.4 and Fig. 3.3 show the distribution and frequency of ESBL producing *E. coli* isolates antimicrobial resistance genes. Table 3.2 shows a range of beta lactam resistance genes (numbers of frequent screened isolates in brackets) including *bla*CTX-M-1, *bla*CTX-M-15 (80); *bla*TEM (66); *bla*OXA-1 (60); *bla*ACT, *bla*CMY (41); *bla*VIM (24); *bla*MOX-CMY9 (6). Table 3.3 shows frequently screened aminoglycoside resistance genes including *str*A, *str*B (62), *aac*6, *aac*6Ib (60), *aadA*4 (50), *aad*A1 (15) and *aad*A2 (22); tetracycline and macrolide resistance genes including *tet*A, *tet*B, *tet*D; and *mph*A and *mrx*. Table 3.4 shows the Trimethoprim, sulphonamide, chloramphenicol and quinolone resistance genes, including *dfr*A1, *dfr*A7, *dfr*A12, *dfr*A13, *dfr*A14, *dfrA*17and *dfrA*19; *sul*1, *sul*2 and *sul*3; *cat*A1, *cat*B3, *cat*III, *flo*R and *CMIA*1; and *qnr*A1, *qnr*B and *qnr*S.

3.**3.4 Virulence factors and genes**

The miscellaneous virulence factors were frequently screened in all 84 ESBL producing *E. coli* isolates, followed by toxins and fimbriae (15), adhesins (13), SPATE (serin protease auto-transporter) (7) and one isolate in secretion system factor (Fig. 3.4). Table 3.5 shows virulence factors and genes, which include adhesins (*iha; iha, nfa*E), fimbrae (*prf*B), secretion systems, serin protease autotransporters (*sepA*), toxins (senB) and miscellaneous (*hemL, intl* 1*; hemL, iss, intl*1). The virulence genes *eea*-consensus; *esp*AC rodentium; *per*A; *senB, sat, cnf*1*; hemL, intl*2*, iss; hemL, iss, iroN;* and *iss, intl*1 combinations were detected once in all study sites; the *iss, ireA*; and *sat* were detected twice at Mazimbu. Virulence genes *tsh, mchF* and *hemL, iss, iroN* combinations were detected once in a treated wastewater downstream isolate.

3.3.5 The O- and H- Serogrouping

Table 3.6 shows O-serogroups and H- flagellin antigens as well as the combination of the O- and H- antigens which were screened from 84 AMR ESBL producing *E. coli* isolates, including the O91:H30, O86:H30, O15:H30; and O101:H21serogroups. These antigens were screened (number of isolates in bracket) from Mazimbu (35), Mzumbe (23), Mafisa (15) and Fungafunga area (11) isolates.

3.4 Discussion

Generally, *E. coli* is only bacteria present in faeces of humans and animals, remains a good indicator of faecal contamination in environment and foodstuffs [24]. This study has quantified ESBL producing *E. coli* in low quality water (untreated wastewater, treated wastewater and river water). The ESBL producing *E. coli* were isolated from untreated wastewater and treated wastewater downstream at an irrigation field, it was also previously reported in a comparable studies in The Netherlands [25, 26]. These studies reported antimicrobial resistance in ESBL producing *E. coli* in wastewater and surface water. Wastewater treatment systems play an important role in disseminating both antimicrobials and excretal pathogens [27], which finally enters in the environment to water bodies and soil. The presence of ESBL producing *E. coli* in low quality water implies that there is faecal pathogens contamination [8]. Isolation of ESBL producing bacteria in treated wastewater indicates that, bacteria may survive in wastewater treatment ponds and reactivate after the treatment process [4].

The use of wastewater contaminated with antimicrobial resistant bacteria and their resistance genes in food production may present health risk to humans, animals and the environment [4,20].

Some antimicrobial agents which are used by humans and animals are not completely degraded and are excreted unchanged as active compounds in faeces and urine [28], thereafter, they enter into low quality water and ultimately get disseminated in the environment. The antimicrobial resistance of ESBL producing bacteria to different antimicrobials has been documented in the environment [8,27] and in animal meat [29].

In this study, total of 84 isolates out of 110 resistant ESBL producing *E. coli* isolates showed multiple antimicrobial resistance patterns in all eight classes of antimicrobials (Table 3.1- 3.4; Fig. 3.3). The ESBL producing bacteria, in particular *Enterobacteriaceae* enable transfer of antimicrobial resistant bacteria from food animals to humans [1,29]. The spread of ESBL in a community has been reported in India [30], and in Tanzania [36], resulting from poor sanitation and hygiene. A previous study on raw vegetables in Korea [31], reported presence of *E. coli* that harboured tetracycline, aminoglycoside, beta- lactam and quinolone resistance genes (*tet*A; *str*A, *str*B; *bla*TEM and *qnr*S.

Beta-lactam are the most commonly used drugs for treatment of bacterial infections in humans [6] and may be associated with treatment failures in some bacterial infections [32].The enzymes, CTX-M type ESBLs are reported the most prevalent ESBLs in the world and *bla*CTX-M-15 genes are often found in *E. coli* which are associated with antimicrobial resistance on treatment of humans and animals in the hospitals and community settings [22, 33, 34]. Furthermore, the TEM-1 and OXA -1 beta lactamases have been reported in food animals in the European countries [6]. For example, OXA - 1 confers a reduced susceptibility to fourth-generation cephalosporins and exhibits high resistance to both beta - lactam and beta lactam inhibitor

combinations. Tetracycline resistance genes *tet*A, *tet*B and *tet*D detected in this study were also found in Korea [31], implying that humans and animals waste could be the main source of tetracycline resistance genes. Generally, tetracycline is among the antimicrobials widely used for treatment of diseases in animals but also as growth promoters [37]. Moreover, animals from the study sites are not restricted from entry and grazing around wastewater treatment ponds.

In the present study, the ESBL producing *E. coli* isolates were found with wide range of virulence factor encoding genes (Table 3.5), therefore, the microarray *E. coli* genotyping combined assay was relatively easy and fast for the detection of *E. coli* pathotypes [35]. High occurrence of these virulent genes, as observed in the study sites, in particular Mazimbu, may be of public health and food safety concern to farmers and consumers of irrigated foodstuffs. A range of screened serogroups presented in Table 3.6 were previously documented as pathotypes of *E. coli* [14, 16] including EHEC (O15 & O86), ETEC (O8 &O15), EPEC (O55, O86 & O91), EAEC O15 & O86; as well as the combination of the O- and H- antigens as the *E. coli* serotypes.

To conclude, emerging ESBL producing *E. coli* in the environment and foodstuffs is a potential risk to public health. The use of low quality water, in particular untreated wastewater, in the study sites for food production can cause health risk to humans, animals and the environment. Furthermore, wastewater and surface water demonstrated to be prone to the contamination with ESBL producing *E. coli* and their antimicrobial resistance genes, virulence factors and genes, which are detrimental to human health. In view of these findings, further research on

contamination of *E. coli* pathotypes in food production systems and the environment in the study sites and Tanzania as a whole is recommended.

Conflict of interest: The authors of this paper have no conflict of interest.

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Table 3.1: Antimicrobial resistance in ESBL producing *E. coli* isolates (n=117)

Antimicrobials/ Location	DST - Mzumbe	Morogoro River	Mafisa	Mazimbu	Mzumbe	Total
Ampicillin	1	13	18	49	29	110
Cefazolin	1	12	18	48	29	108
Cefepime		5	3	7		15
Cefotaxime	1	12	18	48	29	108
Cefpodoxime	1	12	18	48	29	108
Ceftazidime	1	12	18	48	29	108
Ceftriaxone	1	12	18	48	29	108
Cephalothin	1	13	18	48	29	109
Ciprofloxacin		6	15	46	29	96
Gentamicin		4	10	43	12	69
Imipenem						0
Cefoxitin						0
Meropenem						0
Ceftazidime/clavulanic acid						0
Cefotaxime/clavulanic acid						0
Piperacillin/ Tazobactam						0

Table 3.2: Beta lactam resistance genes in ESBL producing *E. coli* isolates (n=84) from LQW in urban and peri urban Morogoro.

Antimicrobials/ Location	Mazimbu	Mafisa	Mzumbe	DST-Mzum	Morogoro river	Total
Beta Lactam						
blaCTX-M1, blaCTX-M15, blaACT, blaOXA-1					1	1
blaCTX-M1, blaCTX-M15, blaACT, blaTEM	1					1
blaCTX-M1, blaCTX-M15, blaACT, blaTEM, blaOXA-1		1	2			3
blaCTX-M1, blaCTX-M15, blaCMY, blaACT, blaTEM	2				2	4
blaCTX-M1, blaCTX-M15, blaCMY, blaACT, blaTEM, blaOXA-1	6		2			8
blaCTX-M1, blaCTX-M15, blaCMY, blaOXA-1			1			1
blaCTX-M1, blaCTX-M15, blaCMY, blaTEM	1					1
blaCTX-M1, blaCTX-M15, blaCMY, blaTEM, blaOXA-1	3		1			4
blaCTX-M1, blaCTX-M15, blaOXA-1	4	3	4		3	14
blaCTX-M1, blaCTX-M15, blaTEM	3	4		1	1	9
blaCTX-M1, blaCTX-M15, blaTEM, blaOXA-1	5	5	1			11
blaCTX-M1, blaCTX-M15, blaVIM, blaACT, blaTEM, blaOXA-1		1				1
blaCTX-M1, blaCTX-M15, blaVIM, blaCMY, blaACT, blaTEM	2	1			2	5
blaCTX-M1, blaCTX-M15, blaVIM, blaCMY, blaACT, blaTEM, blaMOX-CMY9, blaOXA-1	4		2			6
blaCTX-M1, blaCTX-M15, blaVIM, blaCMY, blaACT, blaTEM, blaOXA-1	3		7		1	11
blaTEM	1					1
blaVIM, blaCMY, blaACT, blaTEM			1			1
Grand Total	**35**	**15**	**21**	**1**	**10**	**82**

Table 3.3: Antimicrobial resistance genes (Aminoglycosides, Tetracycline and Macrolide) in ESBL producing *E. coli* isolates (n=84), from LQW in urban and peri urban Morogoro.

Antimicrobials/ Location Aminoglycosid	Mazim-bu	Mafi-sa	Mzum-be	DST-Mzumbe	Morogoro-river	Total
strA, strB	4			1		5
aac6, aac6Ib	1					1
aac6, aac6Ib, strA, strB	1		2			3
aac6, aadA1, aadA2, rmtA, aac6Ib, strA, strB	1					1
aac6, aadA1, aadA2, aac6Ib, ant2, strA, strB	1					1
aac6, aadA1, aadA2, aac6Ib, strA, strB	7	1				8
aac6, aadA2, aac6Ib, strA, strB	2					2
aac6, aadA4, aac6Ib	1	1	3			5
aac6, aadA4, aac6Ib, strA, strB	11	8	13		5	37
aac6, aadA4, aac6Ib, aac3IVa			1			1
aac6, aadA4, aac6Ib, aac3IVa, strA, strB			1			1
aadA1, strA, strB		1			1	2
aadA1, aadA2	1					1
aadA1, aadA2, ant2, strA, strB					2	2
aadA1, aadA2, strA, strB					2	2
aadA2, strA, strB	4	1				5
aadA4			**1**			**1**
aadA4, strA, strB	1	2	1		1	5
Grand Total	**35**	**14**	**22**	**1**	**11**	**83**
Tetracycline						
tetA	3	2	1	1	5	12
tetA, tetB	8	2				10
tetB	15	10	20		1	46
tetB, tetD	1					1
tetD	7	1			5	13
Grand Total	**34**	**15**	**21**	**1**	**11**	**82**
Macrolide						
mphA			1			1
mphA, mrx	24	14	19		6	63
Grand Total	**24**	**14**	**20**		**6**	**64**

Table 3.4: Antimicrobial resistance genes (Trimethoprim, Chloramphenicol, Sulphonamides and Quinolones) in ESBL producing *E. coli* isolates (n=84)

Antimicrobials/ Location	Mazimbu	Mafisa	Mzumbe	DST Mzumbe	Morogoro- river	Total
Trimethoprim						
dfrA	7	2			2	11
dfrA, dfrA5, dfrA14	1					1
dfrA12	2	1				3
dfrA12, dfrA14	7	1				8
dfrA14			1	1		2
dfrA17, dfrA					2	2
dfrA17, dfrA12, dfrA14	1					1
dfrA17, dfrA15, dfrA14			1			1
dfrA17, dfrA7			2			2
dfrA17, dfrA7, dfrA					1	1
dfrA19, dfrA17, dfrA7	10	11	16		6	43
dfrA19, dfrA17, dfrA7, dfrA13			2			2
dfrA19, dfrA7	3					3
dfrA7, dfrA	1					1
Grand Total	**32**	**15**	**22**	**1**	**11**	**81**
Chloramphenicol						
catA1	10	3				13
catA1, catB3	11	2				13
catB3	9	2	9		5	25
cmlA1	1					1
floR	1	1				2
Grand Total	**32**	**8**	**9**		**5**	**54**
Sulphonamides						
sul1	1	2	5			8
sul1, sul2	22	11	15		6	54
sul2	11	2	2	1	5	21
sul3	1					1
Grand Total	**35**	**15**	**22**	**1**	**11**	**84**
Quinolones						
qnrA1	**12**		**5**		**7**	**24**
qnrA1, qnrS	**3**					**3**
qnrB, qnrS		**1**				**1**
qnrS	**5**					**5**
Grand Total	**20**	**1**	**5**		**7**	**33**

Table 3.5: Virulence factors and genes in ESBL producing *E. coli* isolates (n=84), from LQW in urban and peri urban Morogoro

Factors	Genes	Mazimbu	Mafisa	Mzumbe	DST Mzumbe	Morogoro river	Grand Total
Adhesins	eae - consensus	1					1
	iha	1	2	2		1	6
	iha, nfaE	2				4	6
	Grand Total	**4**	**2**	**2**		**5**	**13**
Fimbrae	perA			1			1
	prfB	5	2	2		5	14
	Grand Total	**5**	**2**	**3**		**5**	**15**
Secret-Systems	espA_C_rodentium	1					1
	Grand Total	**1**					**1**
SPATE	sepA	1				5	6
	tsh				1		1
	Grand Total	**1**			**1**	**5**	**7**
Toxins	astA	1	1				2
	cma	1					1
	mchF				1		1
	sat	2					2
	senB, sat		1	2		5	8
	senB, sat, cnf1	1					1
	Grand Total	**5**	**2**	**2**	**1**	**5**	**15**
Miscellanous	hemL, intI1	8	4	9		1	22
	hemL, intI2		1				1
	hemL, intI2, iss	7	1			5	13
	hemL, intI2, iss, intI1	1					1
	hemL, intI2, iss, tir	1					1
	hemL, iss	2	1				3
	hemL, iss, intI1	12	7	11			30
	hemL, iss, iroN				1		1
	hemL, iss, iroN, intI1	1					1
	iss		1	2		5	8
	iss, intI1	1					1
	iss, ireA	2					2
	Grand Total	**35**	**15**	**22**	**1**	**11**	**84**

Table 3.6: O- & H- Antigens – ESBL producing *E. coli* isolates (n=84), from LQW in urban and peri urban Morogoro.

O-serotyping	H-Flagellin	DST Mzumbe	Morogoro river	Mafi-sa	Mazimbu	Mzum-be	Total-Isolates
O91	H30				2		2
	H4		1				1
O86	H30				1	1	2
O8	H21				1		1
O55	H4		1				1
O15	H30			1	1		2
O121	H4					1	1
O113	H9			1			1
O101	H21				8		8
	H10			1	4		5
	fliC H10				1		1
- (cross hybridization)	H30				1		1
--	H30		1				1
-	H10			3	6	11	20
	H4		4	3	5	8	20
	H30		4	1	5	1	11
	H6			2			2
	H51	1					1
	H21			1			1
	H9			1			1
	H5			1			1
Grand Total		**1**	**11**	**15**	**35**	**22**	**84**

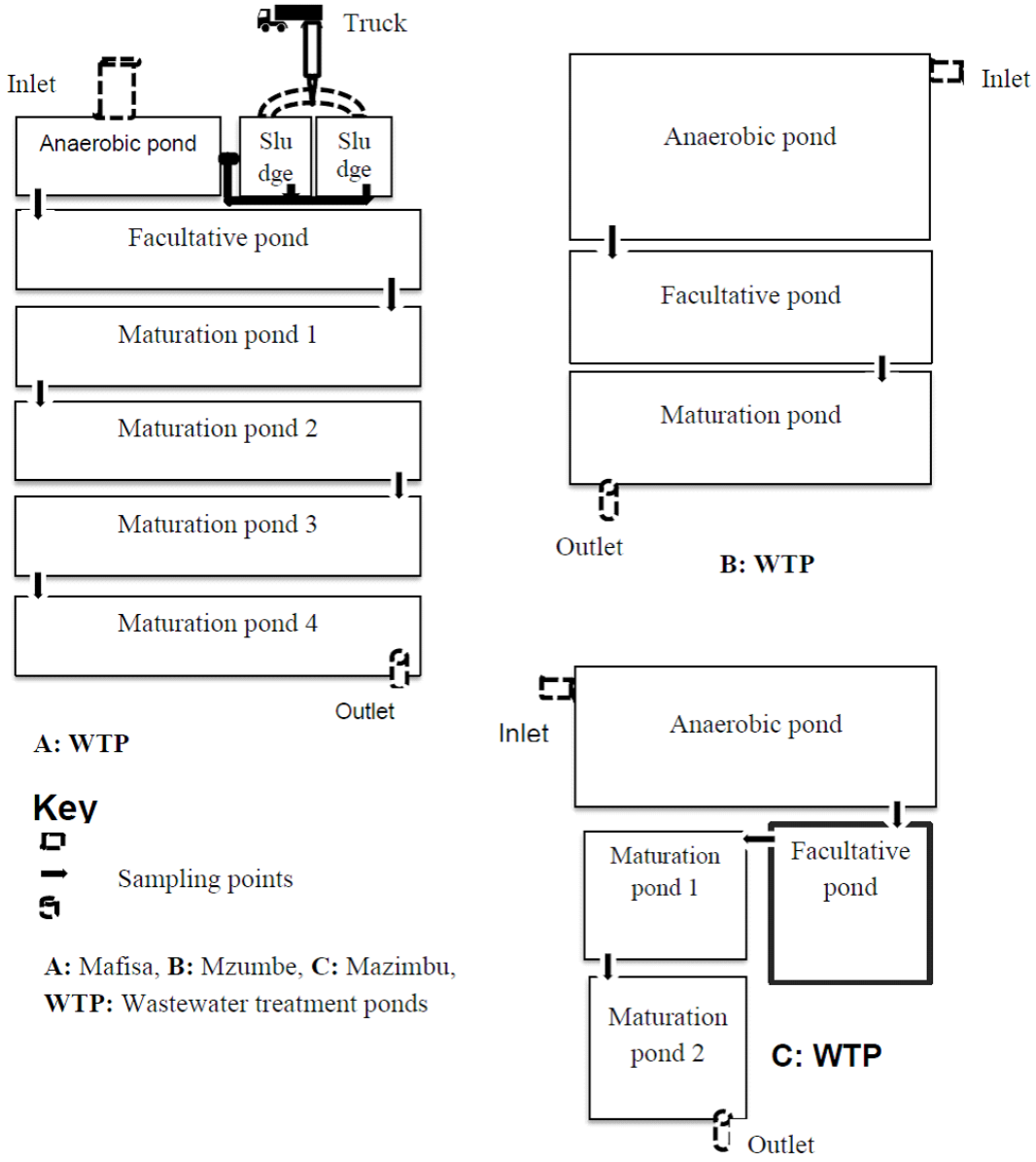

Figure 3.1: Schematic steps for Mafisa, Mzumbe and Mazimbu wastewater treatment ponds.

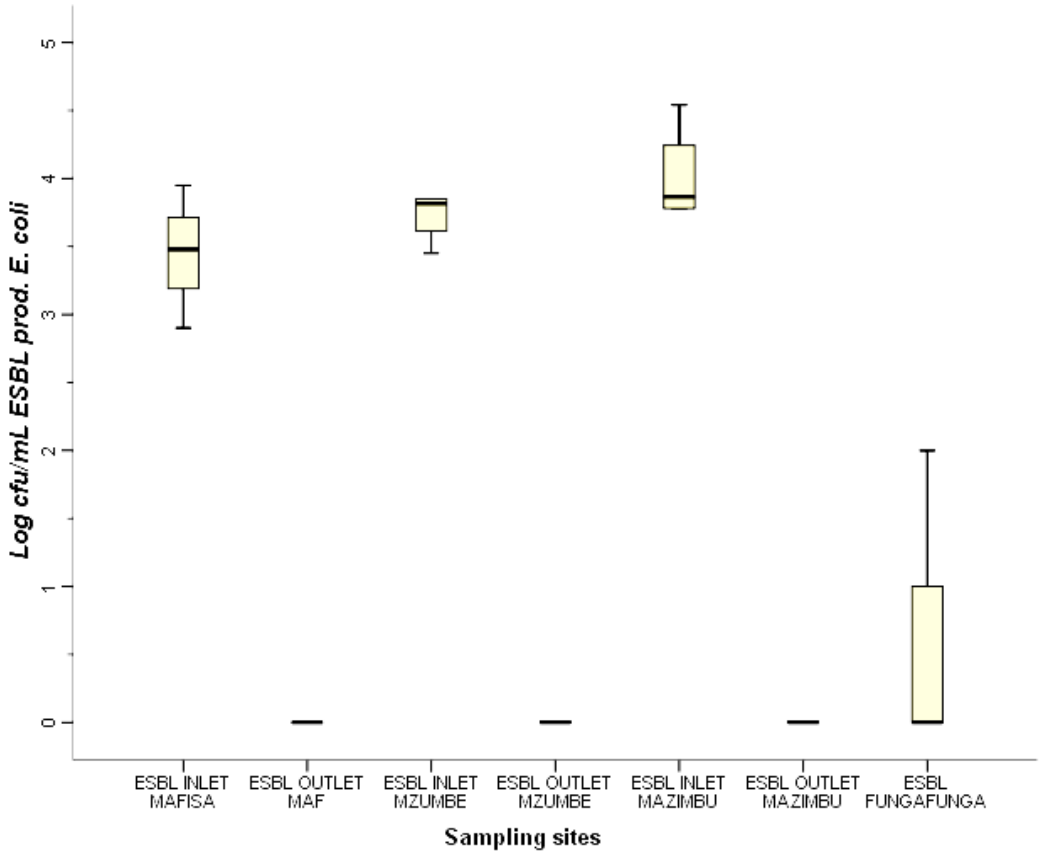

Figure 3.2: Counts of ESBL producing *E. coli* in different irrigation water samples from different study sites in Morogoro urban and peri urban areas.

Figure 3.3: Antimicrobial resistance of ESBL producing *E. coli* isolates

Figure 3.4: Virulence factors (VF) of ESBL producing *E. coli* isolates

SUPPLEMENT I

Microarray genotypying of ESBL producing *E. coli*

Materials and reagents used (Alere Technologies GmbH, Germany) include CM: Reference DNA from *E. coli* EDL933; A1: Lysis Buffer; A2: Lysis Enhancer; $B1^+$: Master Mix; B2: Labelling Enzyme (DNA polymerase); C1: Hybridization buffer; C2: Washing buffer 1: C3: Horseradish peroxidase (HRP) conjugate buffer 100x; C4: Conjugate buffer; C5: washing buffer 2; D1: HRP substrate.

Culturing and harvesting of bacterial cells and extraction of DNA

Isolates of ESBL producing *E. coli* and positive control (reference DNA from *E. coli* EDL933 (GenBank accession number NC_002655.2)) were sub-cultured onto BA and incubated overnight at 37°C to obtain a monoclonal culture of isolates. The monoclonal of the *E. coli* was transferred by using inoculating loop into 0.2 mL of 1 x PBS and mixed by vortexing. The standard *E. coli* combined assay of 2 µg c_{DNA} = 0.1- 0.4 µg / µL of intact genomic RNA free DNA from a single clone was used.

Amplification

The Master Mix was prepared by combining 3.9 µL of buffer plus labelling reagent ($B1^+$), 1 µL labelling - Primer-Mix (*E. coli*-PM1) and 0.1 µL of labelling enzyme (B2 - DNA polymerase) per sample. Five (5) µL of *E. coli* DNA (c_{DNA} = 0.2 µg/µL) was added to 5 µL of the prepared Master Mix ($B1^+$/B2). The amplification was done in a pre-programmed thermocycler, initially pre-heated to 105°C for 300 sec at 96°C. The thermocycler was run for 50 cycles with 20 sec at 50°C; 40 sec at 72°C; 60 sec at 96°C and allowed to cool and maintained at 4°C. A volume of 90

μL of hybridization buffer (C1) was added to 10 μL of DNA. All 100 μL was transferred to the array strips and hybridized at 55^{o}C at 550 rpm for 60 min. Thereafter was incubated twice, in 200 μL washing buffer 1 (C2) at 55^{o}C at 550 rpm for 12 min.

Conjugation

Horseradish peroxidase (HRP) conjugates buffer 100x / conjugate buffer (C3/C4 (1:100)), HRP substrate (D1) was pre-heated and dye precipitated at 25^{o}C. Washing buffer (C2) was discarded and the substrate incubated once in 100 μL C2/C4 conjugate at 30^{o}C at 550 rpm for 10 min. The conjugate buffer C3/C4 was discarded and incubated once in 200 μL washing buffer 2 (C5) at 30^{o}C at 550 rpm for 5 min. Buffer C5 was discarded and incubated with 100 μL substrate D1 at 25^{o}C for 10 min. For a positive reaction the dye was precipitated. After staining, substrate D1 was removed and discarded then scanned immediately. The arrayMate reader was then run. The worksheet /results file was named and saved in a memory stick.

A glossary of antimicrobial resistance genes, virulence factors and genes

Antimicrobial resistance genes		
Beta lactams	blaCTX-M1	class A extended-spectrum-beta-lactamase (X92506.1), including blaCTX-M15
	blaCTX-M15	class A extended-spectrum-beta-lactamase, including blaCTX-M15
	blaVIM	class B metallo beta-lactamase
	blaFOX	class C beta-lactamase blaFOX
	blaCMY	consensus sequence for blaCMY-
	blaACC	class C beta-lactamase blaACC-1/blaACC-2
	blaACT	class C beta-lactamase blaACT-1
	blaTEM	class A beta-lactamase - consensus sequence for blaTEM genes, including extended-spectrum beta-lactamases
	blaMOX-CMY9	class C extended-spectrum beta-lactamase precursor, associated with resistance to cephalosporins
	blaOXA-1	oxacillinase - class D beta-lactamase blaOXA-1
	blaOXA-2	oxacillinase - class D beta-lactamases blaOXA-2/blaOXA-15
Aminoglycosides	aac6	aminoglycoside 6'-N-acetyltransferase, associated with resistance to amikacin; dibekacin; isepamicin; netilmicin; sisomicin; tobramycin
	aadA1	aminoglycoside adenyltransferase; associated with resistance to streptomycin, spectinomycin
	aadA2	aminoglycoside adenyltransferase; associated with resistance to streptomycin, spectinomycin
	aadA4	aminoglycoside adenyltransferase; associated with resistance to streptomycin, spectinomycin
	aac6Ib	aminoglycoside 6'-N-acetyltransferase; associated with resistance to streptomycin, spectinomycin
	rmtA	16S rRNA methylase, associated with aminoglycoside resistance
	aac3IVa	3-N-aminoglycoside acetyltransferase; associated with resistance to apramycin; dibekacin; gentamicin; netilmicin; sisomicin; tobramycin
	ant2	aminoglycoside (2") adenylyltransferase; associated with resistance to dibekacin; gentamicin; kanamycin; sisomicin; tobramycin
	strA	aminoglycoside-3"-phosphotransferase (locus A); associated with resistance to streptomycin
	strB	aminoglycoside-6"-phosphotransferase; associated with resistance to streptomycin
	rmtD	16S rRNA methylase, associated with aminoglycoside resistance
	aadB	2"-aminoglycoside nucleotidyltransferase
	armA	16S rRNA methylase, associated with aminoglycoside resistance
Trimethoprim	dfrA	dihydrofolate reductase type 1
	dfrA5	dihydrofolate reductase type 5
	dfrA7	dihydrofolate reductase type 7
	dfrA12	dihydrofolate reductase type 12
	dfrA13	dihydrofolate reductase type 13
	dfrA14	dihydrofolate reductase type 14
	dfrA15	dihydrofolate reductase type 15
	dfrA17	dihydrofolate reductase type 17
	dfrA19	dihydrofolate reductase type 19
	Gene	
Quinolones	qnrA1	quinolone or fluoroquinolone resistance protein
	qnrS	quinolone or fluoroquinolone resistance protein

	qnrB	quinolone or fluoroquinolone resistance protein
		streptomycin resistance
Sulfonamides	sul1	dihydropteroate synthetase type 1
	sul2	dihydropteroate synthetase type 2
	sul3	dihydropteroate synthetase type 3
Chloramphenicol	catA1	chloramphenicol acetyltransferase (group A)
	catB3	chloramphenicol acetyltransferase (group B)
	catIII	chloramphenicol acetyltransferase
	cmlA1	chloramphenicol transporter
	floR	florfenicol export protein
Tetracyclines	tetA	tetracycline resistance protein A, tetracycline efflux protein
	tetB	tetracycline resistance protein A, class B
	tetD	tetracycline resistance protein A, class D
Macrolides	mphA	macrolide 2'-phosphotransferase
	mrx	member of macrolide inactivation gene cluster mphA-mrx-mphR
Virulence factors and genes		
Adhesins	eae - consensus	an outer membrane protein important for the attachment to host cells; pathogenesis factor
	iha	adherence-conferring protein
	nfaE	chaperone protein - required for the expression of aggregative adherence fimbria II
Miscellaneous	hemL	glutamate-1-semialdehyde aminotransferase
	intI2	class 2 integron integrase
	iss	increased serum survival
	intI1	class 1 integron integrase
	iroN	outer membrane siderophore receptor
	ireA	siderophore receptor - iron-regulated outer membrane protein
	tir	translocated intimin receptor (consensus)
Toxins/ SPATE	astA	chaperone protein - required for the expression of aggregative adherence fimbria II
	cma	colicin M activity protein
	sat	Sat serine protease
	senB,	enterotoxin
	cnf1	cytotoxic necrotizing factor type 1
	mchF	putative microcin L transport protein
Fimbriae	prfB	major pilu subunit operon regulatory protein
	perA	transcriptional activator
Secretion systems	espA_C_rodentium	EspA - protein (type III secretion system), associated with Citrobacter rodentium

SPATE	tsh	Tsh - hemoglobin-binding protease
	sepA	SepA - serine protease autotransporter of *Enterobacteriaceae*

CHAPTER FOUR

4.0 CHARACTERIZATION OF *SALMONELLA* SPP. FROM WASTEWATER USED FOR FOOD PRODUCTION IN MOROGORO, TANZANIA

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SUPPLEMENT II

Molecular characterization of *Salmonella* spp. isolates

The *Salmonella* spp. isolates were further confirmed by Polymerase Chain Reaction (PCR) genotyping. The forward and reverse *Salmonella* (genus specific) primers ST11 (5/-AGC CAA CCA TTG CTA AAT TGG CGC A-3/) and ST15 (5/-GGT AGA AAT TCC CAG CGG GTA CTG-3/) (TAG Copenhagen, Denmark). A volume of 6 µL of PCR products were loaded into wells of solidified 1% Agarose gel (BioReagent, USA) with a drop of Ethidium bromide (Sigma-Aldrich, USA). Agarose gel electrophoresis was run at 100^{o}C for 30 minutes. The gel image was captured by GelDoc EQ system with Quantity One Version 4.2.1 software (Bio-Rad Laboratories, Hercules, California, USA) using UV trans-illumination and images were saved in TIFF format (Fig. 4.1). Figure 2 illustrates the plasmid profiles of *Salmonella* serovars from the study sites.

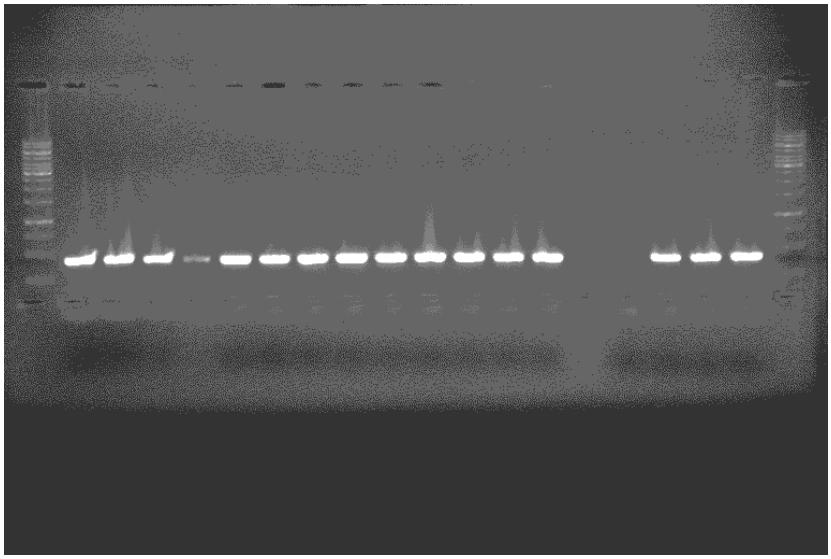

Figure 4.1: Agarose gel electrophoresis (1%) of PCR amplified products (429bp) using genus specific PCR primer sets. Lanes 1-16 are examined *Salmonella* spp. isolates. BL: blanks. Lane K: 1 Kb DNA marker.

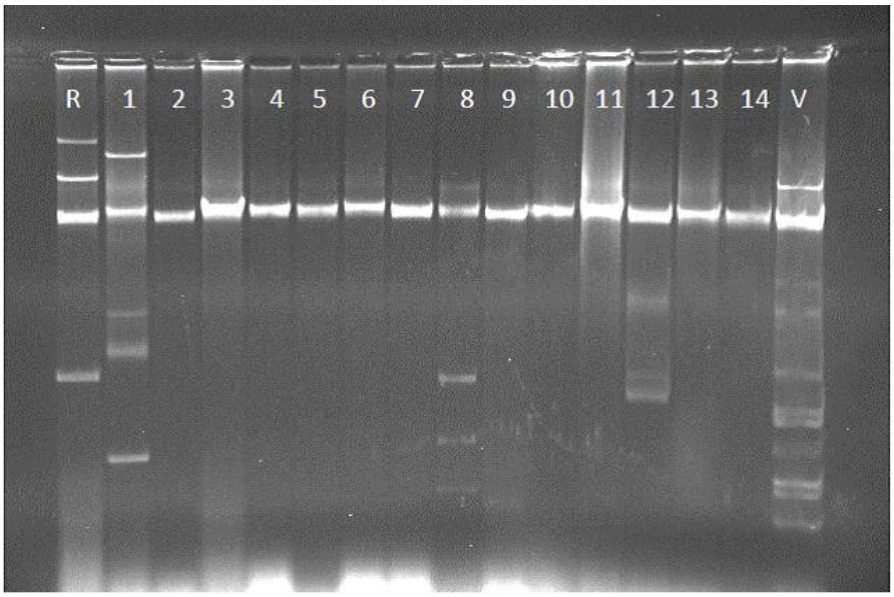

Figure 4.2: SeaKem gold agarose (0.8%) gel electrophoresis of plasmid profile of *Salmonella* serovars (Lanes 1-14) from wastewater and fish intestines. R & V - Marker: - 39R (*E. coli*) and V - V517 (*V. cholerae*) respectively. Chromosomes (Chrom)**; - plasmids (**Plasm**); Lanes 1-14 are *Salmonella* serovars:- Typhimurium, *Salmonella enterica* subsp. enterica 16:a:-, Kentucky, Orientalis, *Salmonella enterica* subsp. salamae 22:g,t:-, Jangwani, Senftenberg, Durban, Cerro, *Salmonella enterica* subsp. salamae 42:r:-, Kiambu, Chandans, Sandiego, *Salmonella typhimurium* (ATCC13311).**

CHAPTER FIVE

5.0 BACTERIOLOGICAL QUALITY OF TILAPIA FISH FROM TREATED WASTEWATER IN PERI-URBAN AREAS OF MOROGORO, TANZANIA

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CHAPTER SIX

6.0 OCCURRENCE OF *ESCHERICHIA COLI* IN *BRASSICA RAPA* L. *CHINENSIS* IRRIGATED WITH LOW QUALITY WATER IN URBAN AREAS OF MOROGORO, TANZANIA

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CHAPTER SEVEN

7.0 GENERAL RESULTS AND DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General Results and Discussion

This thesis reports the extent of bacterial contamination in low quality water, tilapia and Chinese cabbage (*Brassica rapa* L. *chinensis*). In the present study, levels of *E. coli* in untreated wastewater was 5 log cfu/mL (Mhongole *et al*., 2016a); the ESBL producing *E. coli* was 4 log cfu/mL (Chapter III) while *Salmonella* spp. was 40 to <400 cfu/mL (Mhongole *et al*., 2017) in untreated wastewater. The *E. coli*, ESBL producing *E. coli;* and *Salmonella* spp. were all reduced to <1 log cfu/mL and <0.04 cfu/mL respectively following treatment through wastewater treatment ponds. These findings were in agreement with those reported by Pescod (1992) and Mara (2000), that wastewater treatment ponds with maturation ponds are effective in removing faecal pathogens. Concentration of *E. coli* in water from Mindu dam ranged from 0 to 15 cfu/mL and in treated wastewater was <1 log cfu/mL (Mhongole *et al*., 2016b). These values were less than the concentration of *E. coli* (2.40 log cfu/mL) found in polluted Morogoro river

water (Mhongole *et al*., 2016c). This implies that river water is not free from direct and indirect contamination with domestic wastes, while treatment ponds are closed systems and if well controlled like Mzumbe wastewater treatment ponds, treated wastewater can as well be controlled. These findings agree with others (Mateo-Sagasta *et al*., 2013), they reported that the bacterial loads (*E. coli)* from irrigation water are in the order of untreated wastewater > river water > dam water and treated wastewater. Treated wastewater of the quality observed in this study conforms to the recommended WHO guidelines of 2006 and may be used for food production.

The presence of multiple antimicrobial resistance and virulence genes in ESBL producing *E. coli* signifies the potential health risk associated with handling and use of LQW. These findings are similar to those reported in The Netherlands (Bouki *et al*., 2013; Ojer-Usoz *et al*., 2014), on presence of antimicrobial resistance and virulence genes in ESBL producing *E. coli* from wastewater and surface water in urban areas. The presence of multiple antimicrobial resistant *Salmonella* serovars; *S.* Kentucky, *S.* Chandans, *S.* Darban and *S.* Kiambu in wastewater confirms their public health and food safety importance. Based to these findings, safe use of treated wastewater for food production, will not pose health risk to humans.

The concentration of *E. coli* in fish flesh from study sites was <1 log cfu/g (detection limit) and in fish intestines was about 2 log cfu /g and 3 log cfu /g for fish from Mindu dam and Mzumbe wastewater, respectively. Concentration of *E. coli* in fish intestines was significantly different ($P<0.001$), higher at Mzumbe wastewater than in Mindu dam water. The total plate count (TPC) on fish flesh from the two study sites were comparable ($P>0.05$), the mean density was < 3 log cfu/g. Often, use of LQW in food production is reported as the main source of foodstuffs

contamination (Abakpa *et al*., 2013; Cobbina *et al*., 2013; Alemayehu *et al*., 2015). However, in this study, the quality of tilapia from wastewater treatment – (maturation pond) was compared with those from Mindu dam (Mhongole *et al*., 2016b), implying that, treated wastewater may be used for aquaculture.

Escherichia coli contamination in Chinese cabbage irrigated with treated wastewater was 10% (n=48) and in Chinese cabbage irrigated with river water was 86% (n=21) (Mhongole *et al*., 2016c). The levels of *E. coli* in Chinese cabbage irrigated with river water ranged from 0.00 - 4.10 log cfu/g as compared to 0.00 - 1.36 log cfu/g in those irrigated with treated wastewater. These findings show that river water was contaminated than the treated wastewater, though, water from rivers is often regarded as of good quality than treated wastewater, and vegetables irrigated by treated wastewater are perceived of poor quality. However, risk of vegetables contamination with LQW increase in the order of potable / rain water, deep wells and shallow wells; surface water in proximity to animals, human habitation and associated wastes; and untreated or partially treated wastewater (Mateo-Sagasta *et al*., 2013).

In developing countries, in particular, Sub -Saharan African countries, there is limited or no data on wastewater treatment and use (Sato *et al*., 2013; Mateo-Sagasta *et al*., 2015). Unfortunately, consumers are uninformed on wastewater irrigated foodstuffs and most are unaware of the diseases associated with consumption of food contaminated with LQW. This, is therefore, underlines the need for educational programs to inform the public about the health risk associated with unsafe use of LQW for food production (Keraita and Akatse, 2012).

7.2 General Conclusions and Recommendations

This study has found that, if wastewater treatment facilities in the study sites in urban and peri urban areas of Morogoro are well operating and maintained, they have the capacity to reduce faecal pathogens of public health importance, to acceptable levels, in treated wastewater. These findings show that the quality of treated wastewater from the study sites complied with the WHO/FAO guidelines. However, if storm- and run-off water is not controlled from entry into the treatment ponds may lead to poor performance of wastewater treatment ponds, contamination of environment and receiving water bodies.

Since untreated wastewater and river water are contaminated with faecal bacteria (*E. coli* and *Salmonella* spp.), it's use in food production may pose health risk to humans (consumers and handlers) and animals on exposure to the contaminated water and foodstuffs. Moreover, this study informs the stakeholders on the need of environmental monitoring, which requires integrated interventions as well as increased awareness on waste management to individuals and national levels. Therefore, training of farmers and handlers on safe use of wastewater and good agricultural practices as well as implementing good pre- and post-harvest handling practices is indeed important.

This study showed that, wastewater and polluted river water are prone to contamination with ESBL producing *E. coli* and their AMR- and virulence- genes. Presence of AMR- and virulence-genes in ESBL producing *E. coli* in wastewater may pose health risk to humans and animals.

This study further showed that *Salmonella* spp. were only detected from the inlet wastewater but were not detected in treated wastewater from outlet. However, thirteen *Salmonella* serovars were identified in wastewater and fish (intestines) grown in wastewater; and their antimicrobial resistance patterns, while fish flesh was free from *E. coli* and *Salmonella* species.

Findings from this study further showed that, tilapia grown in treated wastewater were of good quality as compared to those from Mindu dam. Hence, health risk to consumers may be reduced given that preventive measures are well observed. On the other hand, Chinese cabbage irrigated with treated wastewater was of better quality than those irrigated with polluted river water. However, safe use of LQW depends on awareness, knowledge and hygiene practices by the farmers. This implies that, use of treated wastewater in food production could be safer than polluted river water.

Although this study has investigated *E. coli*, ESBL producing *E. coli* and *Salmonella* serovars and their antimicrobial resistance, further studies to characterize *E. coli* pathotypes, *Salmonella* serovars and other pathogens as well as others, including *Listeria monocytogenes*, *Campylobacter jejuni* and *Clostridia* spp. (*C. botulinum* and *C. perfringes*) associated with wastewater use in food production is needed. Also, microbial risk assessment for communities using such wastewater is highly recommended.

In order to draw broad conclusions on compliance of wastewater use with recommended standards, there is a need of conducting a study on broad spectrum of faecal microbial organisms (bacteria, protozoans and viruses) associated with wastewater. Studies involving different

geographical conditions and seasonal trends and their effects on the efficiency of wastewater treatment ponds of similar design are equally important.

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