ANTIBIOTICS IN GHANAIAN ENVIRONMENT: OCCURRENCE, UPTAKE, MODEL AND RISK ASSESSMENT OF VEGETABLES IRRIGATED WITH LOW QUALITY WATER

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in partial fulfilment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

in Analytical Chemistry

By:

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DECLARATION

I hereby declare that this thesis is my own work towards the PhD. degree and that, to the best of my knowledge and belief, it contains no material that has been accepted for the award of any other degree in any educational institution nor material previously published or written by another person, except where due reference is made in the text of the thesis.

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DEDICATION

This piece of work is dedicated to Mr. Godwin K. Tordzro

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ABSTRACT

Hospital wastewater and effluents from waste stabilization ponds in Kumasi, Ghana, are directly discharged as low quality water into nearby streams which are eventually used to irrigate vegetables. The presence of 12 commonly used antibiotics in Ghana (metronidazole, ciprofloxacin, erythromycin, trimethoprim, ampicillin, cefuroxime, sulfamethoxazole, amoxicillin, tetracycline, oxytetracycline, chlortetracycline and doxycycline) were investigated in the water samples. Greenhouse uptake studies of tetracycline and amoxicillin antibiotics by lettuce and carrot plants were performed and then used for modelling uptake of antibiotics using STELLA® software. Finally, the occurrence of these 12 antibiotics in lettuces irrigated with low quality water in Kumasi Ghana were investigated. Antibiotics in the water samples were extracted using solid phase extraction, the plants samples were extracted using accelerated solvent extraction followed by clean up on SPE. All samples were analyzed on HPLC-MS/MS. The total load of antibiotics discharged through the WSP effluents and hospital wastewater was up to 3.1 g/day. Low quality water used for vegetable irrigation considered for this study had antibiotics concentrations up to 0.2 ppb. Interestingly, the concentrations of antibiotics in irrigation water were not significantly different from that of the stream samples ($p = 0.03$). The concentrations of antibiotics determined in lettuce collected from vegetable farms and markets in Kumasi, Ghana ranged from 13.5 to 104.3 ng/kg. Seven out of 12 antibiotics investigated were detected in at least one sample. Estimated daily intakes of erythromycin and sulfamethoxazole for the consumption of lettuce were 6.4×10^{-7} and 2.0×10^{-7} μg/kg body weight/d respectively. These estimated daily intakes are several times lower than acceptable daily intakes of 0.5 and 50 μg/kg body weight/d respectively, implying no toxic effect to human consumption. The outcomes of this study suggest there could be indirect exposure of humans to antibiotics through vegetable consumption and drinking water in Ghana.

Although the levels found in lettuce plant could not cause toxic effect to human's further research needs to be investigated since low levels of antibiotics in food and low quality water could contribute to development of bacterial resistance.

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LIST OF ABBREVIATIONS

- Metronidazole (MET)
- Ciprofloxacin (CIP)
- Erthromycin (ERY)
- Trimethoprim (TRIM)
- Tetracycline (TC)
- Oxytetracycline (OTC)
- Cyclotetracycline (CTC)
- Doxycycline (DC)
- Amoxicillin (AMX)
- Ampicillin (AMP)
- Cefuroxime (CEF)

- Sulfamethoxazole (SUL)
- Waste stabilization ponds (WSP)
- Komfo Anokye Teaching Hospital (KATH)
- University Hospital (USTH)
- Internal standard mix (IS mix).
- Solid-phase extraction (SPE)

CHAPTER ONE

1. INTRODUCTION

1.1 BACKGROUND

Antibiotics, which have been identified as one of the groups of chemicals of emerging public health and environmental concern recently because microbes develop resistance to antibiotics even at sub minimum inhibitory concentrations (WHO, 2014). Since the introduction of antibiotics in the late 1930s, their usage in human and animals have increased significantly. Antibiotics are considered as "pseudo-persistent" because they are continuously being discharged into the environment which leads to their permanent presence. The major classes of antibiotics include; β-lactams, macrolides, fluoroquinolones, aminoglycosides, sulfonamide and tetracycline. The β-lactams (amoxicillin and penicillin) are the most used antibiotics for human therapy (Haung et al., 2001).

Excretion of incompletely metabolized antibiotics by humans and animals is the primary source of antibiotics in the environment (Li, 2014). Other sources may include the disposal of unused antibiotics and waste from pharmaceutical manufacturing processes (Kathryn et al., 2005). Residential (private residences, dormitories, and hotels) and commercial facilities (including hospitals) are known contributors of antibiotics to municipal wastewater (Alder et al., 2004). Other potential contributors of antibiotics to surface and groundwater are effluent from wastewater treatment plants (WTPs) (Alder et al., 2004) and industrial facilities (including pharmaceutical plants), and surface run-off from concentrated animal feeding operations (Hirsch et al., 1999). Antibiotics are widespread and well documented in various environmental compartments including municipal sewage (Castiglioni et al., 2006), wastewater stabilization ponds (WSP)

(Møller et al 2014), hospital sewage (Duong et al., 2008), groundwater (Hirsch et al., 1999) and surface water (Kolpin et al., 2002), usually at concentrations in the ng/L to a few µg/L range. A couple of studies (Boxall et al., 2006; Dolliver et al., 2007; Herklotz et al., 2010; Kumar et al., 2005) have recently demonstrated that plants can take up pharmaceutical compounds from the growth media via their roots.

The major concern surrounding antibiotic uptake by plants is contamination of the food supply and the associated health risks. Although human health implications of antibiotic residues in food crops are largely unknown, several potential adverse impacts including allergic/toxic reactions, chronic toxic effects as a result of prolonged low-level exposure (Phillips et al., 2004; Sarmah et al., 2006), development and spread of antibiotic-resistant bacteria (Gullberg et al., 2011; Kim and Aga, 2007; van den Bogaard, 2000), and disruption of digestive system functioning (Bedford, 2000; Schuijt et al., 2013) have been speculated. Hence, it is important to know the amounts of these antibiotics released in the aquatic environment to be able to properly evaluate the risks, the effects and the potential impacts of these products. At present, the understanding of the behaviour of veterinary and medical drugs in soils and plants is very sketchy.

Several investigators developed fugacity model to study the fate and transport of organic contaminants in plants (Paterson et al., 1994; Trapp et al., 1994, 1990). Presently, crop-specific models have been developed, i.e. specific models for roots (Trapp, 2002), potatoes (Trapp et al., 2007), leaves (Trapp and Matthies, 1995) and fruits (Trapp et al., 2003). These models are all based on the same physico-chemical principles and the actually occurring processes and their parameterization depend on the type of crop. The basic processes used in these models include; advective uptake into plants, diffusive uptake, chemical equilibrium, transport in xylem and phloem, dilution by growth, and particle deposition from soil and air.

In low and middle-income countries, antibiotics are widely available to the public, from a variety of sources, including hospitals and pharmacies, licensed medicine stalls and drugstores, roadside stalls and peddlers (Lerbech et al., 2014; Senah, 1997; Wolf-Gould et al., 1991). Despite prohibitive legislations in Ghana, antibiotics can be purchased without prescription (Okeke et al., 2007; Radyowijati and Haak, 2003; Seiter and Gyansa-lutterodt, 2009). This widespread availability has led to inappropriate use by patients and healthcare providers (Adu-Sarkodie, 1997; Radyowijati and Haak, 2003; Wolf-Gould et al., 1991).

1.2 HYPOTHESIS

Based on background information, the hypothesis of this project is that antibiotics do not occur in Ghanaian environment and are not likely to be taken in by vegetables irrigated with low quality water. If they do not occur in the environment, then they will not be detected in the potential sources (hospitals and WSP wastewater) and low quality water used for vegetable irrigations. If plants are not likely to take up antibiotics, then there would be no accumulation and plants could not be an indirect route on human exposure to antibiotics.

1.3 JUSTIFICATION

Antibiotics in urine have been reported in Ghana (Bekoe et al., 2014) suggesting the possibility of antibiotics in Ghanaian environment. Other studies have revealed the misuse of antibiotics in Ghana and mismanagement of antibiotics waste disposal, suggesting the possibility of antibiotics being present in Ghanaian environment (Sasu et al., 2012), Studies on environmental occurrence of antibiotics in Ghana is scanty and sketchy, however, low quality water, which refers to surface

water polluted with raw to diluted wastewater (Raschid-Sally and Jayakody, 2008) use for vegetable production is a common reality. This research work therefore would provide some data on occurrence of antibiotics as well as identify health hazards with the use of low quality water for vegetable production in Kumasi. The findings would enable meaningful decision to be made about the safe use of low quality water in vegetable production.

1.4 OBJECTIVES

The main objective of this project is to identify health hazards associated with the use of low quality water for vegetable production in Kumasi.

The following specific objectives were pursued:

- identify and quantify antibiotics in wastewater from 2 hospitals (Komfo Anokye Teaching Hospital and KNUST University Hospital) and 3 waste stabilization ponds (Ahensan WSP, Chirapatre WSP and Asafo WSP) in Kumasi.
- identify and quantify antibiotics in low quality water used for vegetable irrigation in Kumasi.
- perform greenhouse uptake studies of some antibiotics by lettuce and carrot plants.
- simulate the uptake of some antibiotics by plant using STELLA® software.
- identify and quantify antibiotics in lettuce plant exposed to irrigation with low quality water in Kumasi.
- identification of health risk associated with the use of low quality water and vegetable.

CHAPTER TWO

2 LITERATURE REVIEW 2.1 ANTIBIOTIC: SOURCES, PROPERTIES, OCCURRENCE AND FATE IN THE ENVIRONMENT

The Antibiotic refers to any substance of natural, synthetic, or semi-synthetic origin which at low concentration kills or inhibits the growth of microorganisms (usually bacteria and fungi) but causes little or no damage to the host. The terms antibiotic and antimicrobial agent are often used interchangeably. Since the introduction of penicillin during the Second World War, antibiotics have been viewed as miracle drugs to infectious diseases. Isolation of new antibiotics proceeded quickly and most of the major classes were isolated during the 1940s to 1960s (Walsh and Wright, 2005). Accelerated decline in deaths caused by infections was seen during the antibiotics era. For example, after the introduction of sulphadiazine, deaths from childbed fever caused by *Streptococcus pyogenes* decreased by 50 % in England and Wales (Cohen, 2000). However, today the miracle may be over due to increasing antibiotic resistance in bacteria, including multi-resistant bacteria, which threaten early effective treatment of bacterial infections.

2.1.1 Physicochemical Properties of Antibiotics

2.1.1.1 Tetracyclines

The tetracyclines (TCs) are broad-spectrum antibiotics widely used in veterinary medicine. They are active against a range of organisms such as Myco-plasma and Chlamydia, as well as a number of Gram-positive and Gram-negative bacteria. Tetracycline (TC), oxytetracycline (OTC) and chlortetracyclines (CTC) are widely used in animal feeds to maintain health and improve growth

efficiency in farm animals. These chemicals are characterized by a partially conjugated four-ring structure with a carboxyamide functional group (Mitscher, 1978). The molecule of tetracycline has several ionizable functional groups of a rather unusual type (Fig. 2.1) and the charge of the molecule depends on the solution pH.

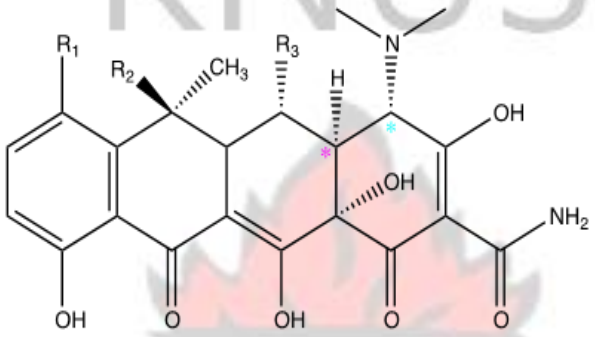


Fig 2.1 Basic structure of tetracyclines

An examination of their pKa values suggests that TC, OTC and CTC have similar pH dependent speciation, which is also consistent with their structural relationship. Therefore, assigning pKa in any one of the antibiotics, a similar relationship can be assumed for the other two (Stephens et al., 1956). There are three distinct acidic functional groups for tetracycline: tricarbonyl methane (pKa 3.3); dimethyl ammonium cation (pKa 9.6); and the phenolic diketone (pKa 7.7). The multiple ionizable functional groups present in TCs suggest that at environmentally relevant pH values, they may exist as a cation (+ 0 0), zwitterion (+ - 0), or as a net negatively charged ion (+ - -) (Sassman and Lee, 2005). Therefore, it can be envisaged from these ionization schemes that in the pH regime of environmental interest (pH 4–8), the antibiotics would be dominated by the zwitterionic species and would reach maximum concentration at pH 5.5. TCs are relatively stable in acidic media, but not in alkaline conditions, and form salts in both media (Halling-Sørensen et al., 2002). They have been found to form complexes with chelating agents such as divalent metal ions and β-diketones and strongly bind to proteins and silanol groups (Oka et al., 2000). In general,

these compounds are sparingly soluble in water (Florence and Attwood, 2006); however, solubility of the corresponding hydrochlorides is reported to be much greater (Thiele-Bruhn, 2003).

2.1.1.1.1 Uses of tetracyclines

Tetracyclines are generally used in the treatment of infections of the urinary tract, respiratory tract, and the intestines (Halling-Sørensen et al., 2002) and are also used in the treatment of chlamydia, especially in patients allergic to β-lactams and macrolides; however, their use for these indications is less popular than it once was due to widespread development of resistance in the causative organisms. Their most common current use is in the treatment of moderately severe acne and rosacea (tetracycline, oxytetracycline, doxycycline or minocycline). Doxycycline is also used as a prophylactic treatment for infection by Bacillus anthracis (anthrax) and is effective against Yersinia pestis, the infectious agent of bubonic plague. It is also used for malaria treatment and prophylaxis, as well as treating elephantiasis.

Tetracyclines remain the treatment of choice for infections caused by chlamydia (trachoma, psittacosis, salpingitis, urethritis and L. venereum infection), Rickettsia (typhus, Rocky Mountain spotted fever), brucellosis and spirochetal infections (borreliosis, syphilis and Lyme disease). In addition, they may be used to treat anthrax, plague, tularemia and Legionnaires' disease. They are also used in veterinary medicine (Chopra and Roberts, 2001).

2.1.1.1.2 Mode of action of tetracyclines

Tetracyclines are described as bacteriostatic (Byarugaba, 2010). Bacteriostatic antibiotics only inhibit the growth or multiplication of the bacteria giving the immune system of the host time to clear them from the system. Complete elimination of the bacteria in this case therefore is dependent

on the competence of the immune system. However, the mechanism of action is inhibition of protein synthesis based on the structure of the bacteria or the function that is affected by the agents.

2.1.1.2 Sulphonamides

The sulphonamides were the first effective chemotherapeutic agents to be employed systemically for the prevention and cure of bacterial infections in humans. The sulfonamide class contains a large number of antibacterial drugs, including sulfadiazine, sulfamethazine (sulfadimidine), sulfathiazole, sulfamethoxazole, and many more. Potentiated sulphonamides, in which a sulfonamide and an antibacterial diaminopyrimidine such as trimethoprim are combined, demonstrate improved efficacy compared with sulphonamides alone. This is attributed to numerous factors, including toxicological concerns associated with some sulphonamides and the lack of contemporary data to support the historical uses of other sulphonamides (Brayfield, 2011).

The sulphonamides are structural analogues of para aminobenzoic acid and competitively inhibit dihydropteroate synthetase, the enzyme that catalyzes the synthesis of dihydrofolic acid (folic acid). Organisms susceptible to sulphonamides must synthesize their own folic acid, unlike mammalian cells, which utilize preformed folic acid. The decreased synthesis of dihydrofolic acid, in turn, causes decreased synthesis of tetrahydrofolic acid (folinic acid), which is required for the synthesis of DNA.

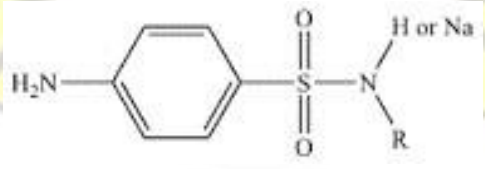


Fig. 2.2 Basic structure of sulphonamides

A variety of effects may result, including suppression of protein synthesis, impairment of metabolic processes, and inhibition of growth and multiplication in susceptible organisms.

Sulphonamides, which are not efficacious in the presence of purulent material, are bacteriostatic.

2.1.1.2.1 Uses of Sulphonamides

Sulphonamides are used to prevent acute systemic or local infections, including actinobacillosis, coccidiosis, mastitis, metritis, colibacillosis, pododermatitis, polyarthritis, respiratory infections, and toxoplasmosis. Sulphonamides are also used in the treatment of American foulbrood disease caused by Paenibacillus larvae and European foulbrood disease caused by Melissococcus pluton that affect honeybees. Sulphonamides in combination with pyrimethamine are used to treat protozoal diseases such as leishmaniasis and toxoplasmosis. Sulphonamides are most effective in the early stages of acute infections when organisms are rapidly multiplying (Brayfield, 2011; Reeves, 2012).

2.1.1.2.2 Mode of action of Sulphonamides

The sulphonamides (SAs) are synthetic bacteriostatic antibiotics (Byarugaba, 2010) with a wide spectrum against most Gram-positive and many Gram-negative organisms. Sulphonamides inhibit multiplication of bacteria by acting as competitive inhibitors of p-aminobenzoic acid in the folic acid metabolism cycle (O'Neil et al., 2001). The sulphonamides consist of a benzene ring, an amine moiety ($–NH_2$), and a sulfonamide group ($–SO_2NH_2$).

2.1.1.3 β-Lactams

β-lactam consists of penicillins and cephalosporins. Penicillins have their antibiotic effects directly connected to the β-lactam ring.

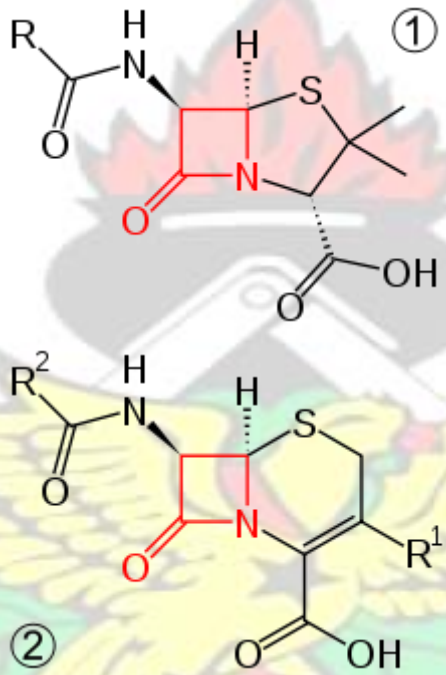


Fig. 2.3 Basic structures of beta-lactam
Penicillin (1) and cephalosporin (2) antibiotics.

In acidic and alkaline media, this ring is easily cleaved. Cephalosporins are derivatives of 7amino-cephalosporanic acid, condensed with a six-membered heterocycle in contrast to the fivemembered heterocycle of penicillins (Thiele-Bruhn, 2003).

The β-lactams generally are wholly ionized in plasma and have relatively small volumes of distribution and short half-lives. They do not cross biological membranes well but are widely

distributed in extracellular fluids. Elimination is generally through the kidneys. The penicillins are characterized by their 6- aminopenicillanic acid (6-APA) core. This is a thiazolidone ring linked to a β-lactam ring and a sidechain at position C6, which allows them to be distinguished from one another. Penicillins can be separated into six groups on the basis of their activity.

Benzylpenicillin (penicillin G) was the first β-lactam purified for clinical use from Penicillium cultures. Clinical limitations were soon recognized, with instability in the presence of gastric acids, susceptibility to β-lactamase enzymes, and ineffectiveness against many Gram-negative half-life of around 30–60 min.

Shortly after the development of benzypenicillin, cephalosporin C was isolated from the fungus Cephalosporium acremonium. Cephalosporins have a 7- aminocephalosporanic acid core that includes the β-lactam ring and were of early interest because of activity against Gram-negative bacteria. In addition, these antibiotics are less susceptible to the action of β-lactamases.

β-Lactam antibiotics are largely free of toxic effects, and the margin of safety is substantial. The major adverse effect is acute anaphylaxis, which is uncommon and associated mostly with penicillins; urticaria, angioneurotic edema, and fever occur more commonly.

2.1.1.3.1 Uses of β-Lactams

β-lactam antibiotics are indicated for the prophylaxis and treatment of bacterial infections caused by susceptible organisms. At first, β-lactam antibiotics were mainly active only against Grampositive bacteria, yet the recent development of broad-spectrum β-lactam antibiotics active against various Gram-negative organisms has increased their usefulness.

2.1.1.3.2 Mode of action of β-Lactams

β-Lactam antibiotics are bactericidal and act by disrupting peptidoglycan synthesis in actively multiplying bacteria (Lorrain, 1972). β-Lactams bind to proteins in the cell membrane [penicillinbinding proteins (PBPs)], which are enzymes that catalyze cross-linkages between the peptide chains on the N-acetylmuramic acid-N-acetylglucosamine backbone of the peptidoglycan molecule. Lack of cross-linkages results in the formation of a weak cell wall and can lead to lysis of growing cells (Lorrain, 1972).

2.1.1.4 Macrolides

Lactone structures with cycles greater than 10 C-atoms are termed macrolides.

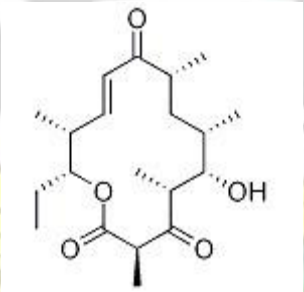


Fig. 2.4 Basic structure of macrolides

Many macrolides are weak bases and are unstable in acids. Their water solubility varies considerably between the different derivatives (Thiele-Bruhn, 2003). Erythromycin is a macrolide antibiotic produced by Streptomyces erythreus

2.1.1.4.1 Uses of Macrolides

Antibiotic macrolides are used to treat infections caused by Gram-positive (e.g., Streptococcus pneumoniae) and limited Gram-negative (e.g., Bordetella pertussis, Haemophilus influenzae) bacteria, and some respiratory tract and soft-tissue infections. The antimicrobial spectrum of macrolides is slightly wider than that of penicillin, and, therefore, macrolides are a common

substitute for patients with a penicillin allergy. Beta-hemolytic streptococci, pneumococci, staphylococci, and enterococci are usually susceptible to macrolides. Unlike penicillin, macrolides have been shown to be effective against Legionella pneumophila, mycoplasma, mycobacteria, some rickettsia, and chlamydia.

Macrolides are not to be used on non-ruminant herbivores, such as horses and rabbits. They rapidly produce a reaction causing fatal digestive disturbance. It can be used in horses less than one-yearold, but care must be taken that other horses (such as a foal's mother) do not come in contact with the macrolide treatment.

2.1.1.4.2 Mode of action of Macrolides

Erythromycin may be bacteriostatic or bactericidal depending on the organism and drug concentration. It inhibits bacterial protein synthesis by binding to bacterial 50S ribosomal subunits; binding inhibits peptidyl transferase activity and interferes with translocation of amino acids during translation and assembly of proteins (Byarugaba, 2010).

2.1.1.5 Fluoroquinolones

Most fluoquinolones (FQs), also known as quinolones, exhibit large chemical stability. The quinolones are a family of synthetic broad-spectrum antibiotic drugs. Quinolones, and derivatives, have also been isolated from natural sources (such as plants, animals and bacteria) and can act as natural antimicrobials and/or signalling molecules.

Quinolones exert their antibacterial effect by preventing bacterial DNA from unwinding and duplicating. The majority of quinolones in clinical use are fluoroquinolones, which have a fluorine

atom attached to the central ring system, typically at the 6-position or C-7 position. Most of them are named with the -oxacin suffix. They are insensitive to hydrolysis and increased temperatures, but are degraded by UV light (Thiele-Bruhn, 2003).

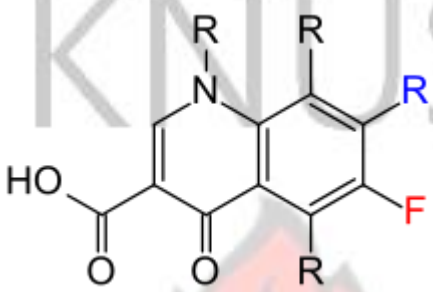


Fig. 2.5 Basic structure of fluoquinolones

2.1.1.5.1 Uses of Fluoroquinolones

The first quinolone to be used clinically for its antimicrobial activity was nalidixic acid in 1962; this drug is a derivative of chloroquine (Andersson and MacGowan, 2003). Today, naladixic acid and other first-generation quinolones such as flumequine and oxolinic acid are used primarily in aquaculture (Reeves, 2012). Successive generations of quinolones have a fluorine atom in the quinolone ring structure, typically at the C6 position. Several fluoroquinolones, including danofloxacin, difloxacin, enrofloxacin (which is deethylated to form ciprofloxacin), marbofloxacin, orbifloxacin, and sarofloxacin, are used in veterinary but not human medicine. Conversely, some fluoroquinolones that are important in human medicine are not labelled for animal use (Andersson and MacGowan, 2003).

The activity type of the fluoroquinolone antimicrobial drugs is concentration-dependent. Because quinolones accumulate in the cytosol of macrophages and neutrophils, they are often used to treat intracellular pathogens. The preponderance of macrophages and neutrophils in infected tissues

compared to healthy tissues may explain the higher concentrations of fluoroquinolones attained in infected tissues.

2.1.1.5.2 Mode of Action of Fluoroquinolones

The fluoroquinolones enter bacterial cells via porins and inhibit bacterial DNA gyrase in many Gram-negative bacteria, or topoisomerase IV in many Gram-positive bacteria; thereby inhibiting DNA replication and transcription. Fluoroquinolones also cause the cessation of cellular respiration and disruption of membrane integrity.

2.1.1.6 Nitroimidazoles

The chemical synthesis and biological testing of numerous nitroimidazoles occurred following the discovery in 1955 of azomycin, a 2-nitroimidazole compound, and the demonstration of its trichomonacidal properties a year later. The trichomonacidal activity of metronidazole, a 5nitroimidazole, was reported in 1960.

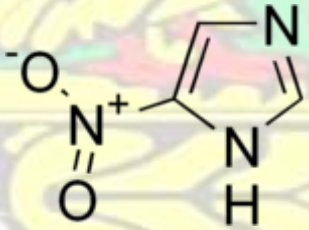


Fig. 2.6 Basic structure of nitroimidazoles

The chemical synthesis of other 5-nitroimidazole compounds, including dimetridazole, ipronidazole, ronidazole, and tinidazole, followed. In addition to antiprotozoal activity, these compounds display concentration-dependent activity against anaerobic bacteria. Both activities are utilized in human and veterinary medicine, although the use of nitroimidazoles in

foodproducing species is prohibited in Australia, Canada, the EU, and the United States. Although JECFA has not established Acceptable Daily Intake (ADI) values for metronidazole, dimetridazole, or ipronidazole, they did allocate a temporary ADI for ronidazole in 1989 but it was withdrawn in 1995 (Reeves, 2012).

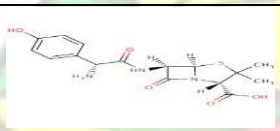
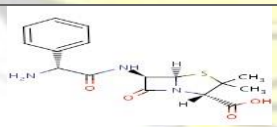
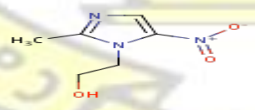
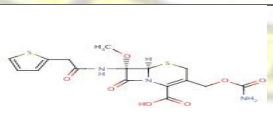
2.1.1.6.1 Uses of Nitroimidazoles

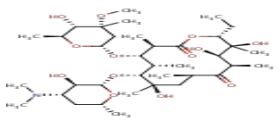
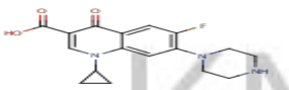
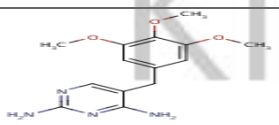
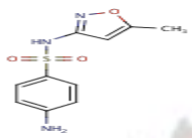
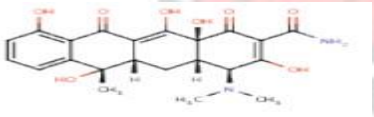
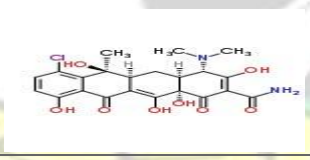
A nitroimidazole used to treat amebiasis; vaginitis; trichomonas infections; giardiasis; anaerobic bacteria; and treponemal infections. It has also been proposed as a radiation sensitizer for hypoxic cells. Metronidazole, a synthetic antibacterial and antiprotozoal agent of the nitroimidazole class, is used against protozoa such as *Trichomonas vaginalis*, amebiasis, and giardiasis. Metronidazole is extremely effective against anaerobic bacterial infections and is also used to treat Crohn's disease, antibiotic-associated diarrhea, and rosacea.

2.1.1.6.2 Mode of Action of Nitroimidazoles

Metronidazole is of the nitroimidazole class. It inhibits nucleic acid synthesis by disrupting the DNA of microbial cells. This function only occurs when metronidazole is partially reduced, and because this reduction usually happens only in anaerobic cells, it has relatively little effect upon human cells or aerobic bacteria.

Table 2.1. Chemical and physical properties of analytes of interest.

Name	Structure	Log Pow	Sw (mg/L)	pKa	Mw (g/mol)
Amoxicillin		0.87	3430	3.23	365.4
Ampicillin		1.35	$1.01x10^4$	3.24	349.4
Metronidazole		-0.02	9500	2.62	171.15
Cefuroxime		-0.02	$1.95x\ 10^4$	3.59	427.45

Erythromycin		3.06	1.44	8.88	733.92
Ciprofloxacin		0.28	3x 10^{-4}	6.09	331.34
Trimethoprim		0.91	400	7.12	290.3
Sulfamethoxazole		0.89	610	5.8-6.2	253.27
Tetracycline		-1.35	231	3.3	444.43
Chlorotetracycline					

2.1.2 Sources and Concentrations of Antibiotics in the Environment

The source of antibiotics can be divided into two; point source pollution and diffuse pollution. Point source pollution is a single identifiable source which originates from separate locations and can be calculated in mathematical modeling (Lapworth et al., 2012). For instance, industrial effluent, hospital effluent and sewage treatment plants as well as the septic tank are the major point sources to the soil and water resources. Antibiotics are mainly introduced into the environment through two routes, human or animal treatment. However, it is important to realize that the same or similar antibiotics can be used in both human and animal treatment. The antibiotics are dispersed in two ways, (1) through excretion (urine and faeces), or (2) direct disposal. A substantial part of all antibiotics consumed are not absorbed or metabolised by the body, but excreted in their active form in the urine and faeces (Li et al., 2014). Urine and faeces are transported to wastewater

treatment plants or can be used directly in manure. Direct disposal includes addition of food additives directly to the water in fish farms or treatment of crops. One major source is probably the disposal of outdated or remainders of antibiotics in household and farm drains. In Germany it has been estimated that 20 - 40% of all administered antibiotics are disposed into hospital effluent (Kümmerer, 2003). In most industrialised countries like Sweden and USA up to 85% of households are connected to municipal sewers. Wastewater treatment plants are therefore probably primary routes of entry for antibiotics into the environment in developed countries. Several studies have also described the occurrence of different antibiotics in both untreated and treated water (Kim and Aga, 2007). The majority of the studies describe lower concentrations in treated water, suggesting a partial removal in waste treatment plant (Andreozzi et al., 2004; Bendz et al., 2005; Heberer and Heberer, 2002; Khan and Ongerth, 2005). Recent studies concerning other pharmaceutical residues in the aquatic environment have clearly shown that elimination in municipal sewage treatment plants are often incomplete. Ternes (1998), determined elimination rates, generally ranging between 60 and 90%, for a variety of medium polar drugs during sewage treatment. The polar antibiotics may not be eliminated effectively, as a large part of elimination is achieved by absorption on activated sludge which is partly mediated through hydrophobic interactions. Hence, one can expect to find antibiotic substances in surface waters.

On the contrary, diffuse pollution is hard to be identified, because of the discrete location where it occurs over a broad geographical scales (Lapworth et al., 2012). One of the examples is the runoff including agricultural runoff from the animal waste and manure, urban runoff from domestic waste and the leakage from waste treatment systems (Bueno et al., 2012). Organic contaminants such as pharmaceuticals enter into the soil and water resource through different ways, with sewage sludge being one of the most important ways (Harrison et al., 2006). Applying the sewage sludge to the land surface is paramount diffuse source of the pharmaceuticals going to the soils and the

freshwater resources (Lapworth et al., 2012). Hoverstad et al. (1986), determined several antibiotics in human feces during 6 days of regular application. They found trimethoprim and doxycycline ranging from 3 to 40 mg/kg and erythromycin concentrations ranging from 200 to 300 mg/kg. Sulphonamides, like sulfadimethoxine, are sufficiently stable in manure to maintain significant residual activity until field manuring. Another diffuse source of pollution to the environment is the groundwater surface interface. The interface is the indirect route that the exchange of the surface water and the groundwater by the runoff and the downward migration due to attenuation mechanism in the soil and unsaturated zone (Lapworth et al., 2012). According to the review conducted by Pal et al. (2010), it found that there are different kinds of pharmaceuticals compounds with high concentration in the freshwater in river and canal.

Compare with point source pollution, diffuse pollution has generally lower environmental loading because it has higher potential for natural attenuation in the soil and subsurface (Murray et al., 2010). Fig 2.7, shows the pathways of pharmaceuticals discharged from the sources and then transferred to the receptors. There are six major sources namely, landfill, animal waste, freshwater aquaculture waste, hospital waste, industrial waste and domestic waste (Eggen et al., 2010; Heberer, 2002; Lapworth et al., 2012; Pal et al., 2010).

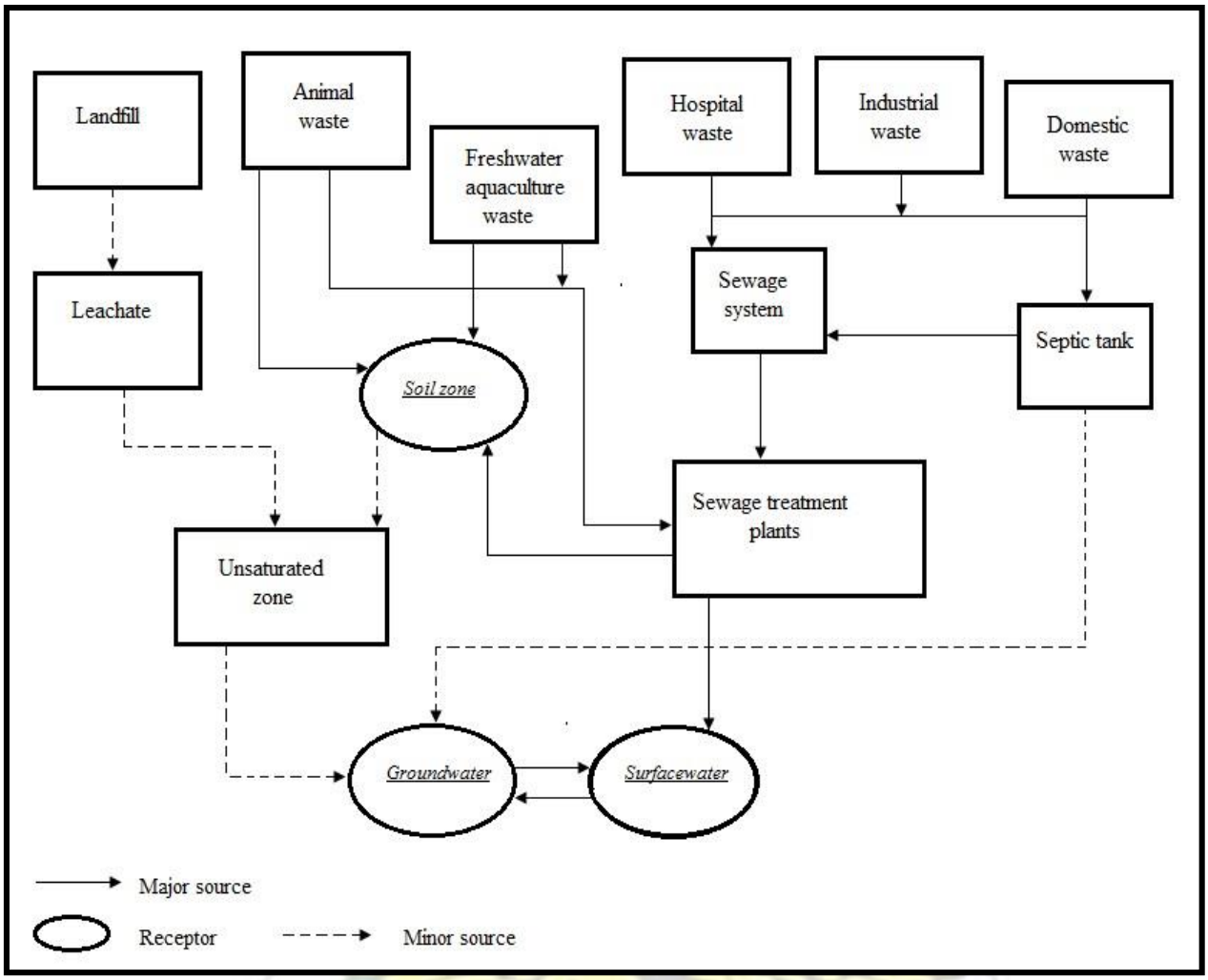


Fig. 2.7: Potential sources and pathways of antibiotic pollution Source: (Heberer, 2002; Lapworth et al., 2012).

The discussed sources of pharmaceuticals result in detectable residual concentrations in diverse environmental compartments (Hirsch et al., 1999). A nationwide survey of pharmaceutical compounds in USA, revealed a number of veterinary and human antibiotics in 139 river water samples with concentrations up to 0.7 μgL^{-1} (Kolpin et al., 2002). In England, representative single substances from the classes of macrolides, SAs, and TCs were determined in river water in concentrations close to 1 µg/L (Watts et al., 1982), a concentration that reduced aqueous microbial activity in biotests (Backhaus and Grimme, 1999).

Metronidazole were reported in two conventional activated sludge plants situated in the Po Valley, northern Italy, with concentration range of 9 - 21 ng/L (Aukidy et al., 2012). K'oreje et al. (2012), analysed effluent sample from the Dandora sewage plant, Kenya and detected no metronidazole. Ciprofloxacin, was not detected in effluent samples from New Mexico (Brown et al., 2006), but found up to 499 ng/L in two conventional activated sludge plants situated in the Po Valley, northern Italy, (Aukidy et al., 2012). Erythromycin concentrations were found in effluents of STP at Switzerland up to 287 ng/L (Giger et al., 2003), and 6000 ng/L in STP effluent at Germany (Hirsch et al., 1999).

2.1.4 Fate of Antibiotics in the Environment

a. Sorption and fixation in soil

Antibiotics of different structural classes vary considerably in their molecular structures and physicochemical properties. Some of them are hydrophobic or non-polar, whereas others are completely water soluble or dissociate at pH values typically found in soils. Thus, distribution coefficients (K_d) for the adsorption of antibiotics to soil materials and aquatic sediments vary for sulphonamides from 0.6 to 4.9, for tetracyclines from 290 to 1620, and for fluoroquinolones from 310 to 6310 (Tolls, 2001). From the extractability, Katz and Katz, (1983) deduced that the strength of sorption to soil increases in the following sequence: oxytetracycline = chlortetracycline < erythromycin. However, sorption of antibiotics to soil minerals is weaker than to soil organic matter. Sorption of pharmaceutical antibiotics is especially influenced by soil pH (Holten Lützhøft et al., 2000), soil organic matter (Gruber et al., 1990), and soil minerals (Batchelder, 1982). Pinck et al., (1961a) reported a much stronger adsorption of aminoglycosides and tetracycline to expandable three-layer clay minerals than to illite and kolinite. Correspondingly, nearly the

opposite sequence was found for desorption of these antibiotics, however, no release of aminoglycosides was determined (Pinck et al., 1961b). It is assumed that besides adsorption, diffusion into porous soil particles also contributed to the fixation. The strong adsorption of fluoroquinolones to soils, especially to clay minerals, was accompanied by an expansion of the spacing of montmorillonite (Nowara et al., 1997). The authors proposed coulombic interactions and the adsorption of anionic antibiotics via cation bridging to clay minerals as the main mechanism for fluoroquinolones adsorption. The deprotonated carboxylic group of fluoroquinolones-carboxylic acids is fixed to the clay minerals while the sorption of the decarboxylated derivative is much smaller.

Sorption and fixation of antibiotics is strongly governed by the property of antibiotics to ionize, which depends on the pH of the medium (Yeager and Halley, 1990). Octanol/water coefficients of ionizing antibiotics change considerably in pH range around the acid dissociation constant (Holten Lützhøft et al., 2000). Electrostatic forces mostly drive the sorption of these derivatives to charged surfaces of mineral and organic exchange sites (Holten Lützhøft et al., 2000). Adsorption coefficients (K_d) of sulphonamides increased from < 1 up to 30 when the soil pH decreased in the range of 8 to 4 (Boxall et al., 2002). This was related to the ionization of the amphoteric sulphonamides. Adsorption of antibiotics to soil organic matter is strong and depends on the quality and the composition of soil organic matter as well, as it has been shown for sulfapyridine. Adsorption of sulfapyridine was significantly correlated with the concentration of the lipids and lignin dimers in the soil organic matter of the particle fractions (Thiele, 2000). Adsorption of sulphonamides to the clay size fraction with stable organo-mineral complexes was about two times greater than to the sand size fraction, which was mostly characterized by particulate organic matter of plant origin (Thiele et al., 2002).

Correspondingly, the adsorption of oxytetracycline increased with increasing aromaticity of organic soil components (Suan and Dmitrenko, 1994a). As for soil minerals, the adsorption of oxytetracycline to humic acid varies significantly with pH (Sithole and Guy, 1987). The tetracyclines bind to humic acids and proteins especially via anionic functional groups. Since K_{oc} was sufficiently estimated from K_{ow} a major contribution of hydrophobic partitioning was concluded. However, the K_{oc} concept is not valid for the majority of polar antibiotics (Thiele et al., 2002). The much stronger sorption of tetracyclines to dissolved organic matter than expected from K_{ow} clearly stresses that sorption is not attributable to hydrophobic partitioning, but ionic interactions and hydrogen bonds (Tolls, 2001). In general, the adsorption of antibiotics like fluoroquinolones and sulphonamides to faeces that are rich in organic matter is strong (Marengo et al., 1997). However, distribution coefficients of oxytetracycline is smaller in manure than in soils (Loke et al., 2002). Accordingly, Boxall et al., (2002) observed decreasing K_d values for sulfachloropyridazine with an increasing proportion of manure in soil. This was not related to the mobilizing effect of dissolved organic matter, but to the pH effect of the alkalinic manure.

Adsorption of most antibiotics to soils is fast. Erythromycin, aminoglycoside, and SAs reach sorption equilibrium in soil after several hours (Thiele, 2000; Yeager and Halley, 1990).

Adsorption of TCs to various exchange sites is characterized by two processes of different kinetics (Sithole and Guy, 1987; Suan and Dmitrenko, 1994b) that can be interpreted as a fast initial adsorption to outer surfaces, followed by a penetration into interlayers of clay minerals and micropores. Adsorption mostly reduces the antibiotic potency of the compounds (Ingerslev and Halling-Sorensen, 2000). It is assumed that this is especially the case when the bioactive functionality associates with the exchange sites (Thiele, 2000). Correspondingly, desorption yields a re-activation of the antimicrobial potency (Halling-Sørensen et al., 2002). However, sorption or

fixation does not necessarily result in a complete elimination of the antimicrobial activity (HallingSørensen et al., 2003).

b. Mobility and transport

Field investigations revealed that point sources caused ground water contamination resulting from transport of antibiotics through soil as determined in the vicinity of manure lagoons (Campagnolo et al., 2002) and at a disposal site of a pharmaceutical plant (Holm et al., 1995). A diffused contamination of surface water by antibiotic leaching from agricultural soils has also been reported (Alder et al., 2004). In contrast, antibiotics were only detected in a small number of ground water samples from intensive livestock production in Germany (Hirsch et al., 1999).

However, to date, only a few systematic investigations related to the mobility and transport of antibiotics in soil exist. Numerous antibiotics have a low water solubility, they are relatively less polar, and strongly retarded in soils. It is assumed that significant transport of such antibiotics like tetracyclines is restricted to fast preferential and macropore flow or is facilitated by co-transport with mobile colloids like DOM. In submerged marine sediment, oxytetracycline transport within 220 days was restricted to 2 - 4 cm (Samuelsen et al., 1992).

Also, leaching of avermectin in soil columns was small but increased significantly with preferential flow within the cracks of a structured silt loam (Gruber et al., 1990). In accordance with results from sorption experiments, the weakly adsorbing olaquindox completely leached through soil columns, while the stronger adsorbing tylosin was retained in different depths depending on the soil properties (Rabølle and Spliid, 2000). No transport was found for oxytetracycline. Sulfachloropyridazine was not leached through a sandy soil, while for structured clay soil, rapid

preferential transport of the SA into drainage water was observed within 7 days (Boxall et al., 2002).

c. Degradation and inactivation

A number of antibiotics such as fluoroquinolones, sulphonamides, and tetracyclines are susceptible to photodegradation (Halling-Sørensen et al., 2003). Accordingly, chlortetracycline was degraded on the surface of a Chernozem (black soil containing up to 15% humus) at a DT_{50} of 5.8 days (Thiele-Bruhn et al., 2003). However, under similar conditions no significant abiotic degradation of fenbendazole and sulfapyridine was determined. Thus, photodegradation has no significant effect on the concentration of antibiotics in soils, especially when they are spread onto soils as contaminants in sludge or slurry.

As competing processes, fixation to and penetration into voids of the soil solids protect antibiotics from photodecomposition. Hydrolysis, another major abiotic process, also yields transformation of antibiotics (Halling-Sørensen, 2000). The concentration of chlortetracycline aged in sterile soil possibly declined due to this process, while the extractable concentrations of fenbendazole and sulfapyridine did not change after an initial strong fixation (Thiele-Bruhn et al., 2003).
Photodecomposition in water declines with increasing water depth and turbidity (Lunestad et al., 1995).

Degradation of xenobiotics in soils is mainly driven by microbial processes and numerous antibiotics are susceptible to enzymatic transformation reactions like oxidative decarboxylation and hydroxylation (Chen et al., 1997). In mammals, antibiotics are mostly metabolized by a biphasic mechanism. First, functional groups are coupled to the molecule by monooxygenases,

reductases, and hydrolases, followed by a covalent conjugation in the second phase, rendering the molecule more hydrophilic, excretable (Daughton and Ternes, 1999). The conjugation reactions are reversible and parent compounds have been observed in the environment (Halling-Sørensen et al., 2002). However, antibiotics usually degrade further in dung, manure, and soil (B HallingSørensen et al., 2000; Ingerslev and Halling-Sorensen, 2000). Additions of manure or sludge, containing a large number of microorganisms, mostly result in increased biodegradation of antibiotics in soil (Ingerslev and Halling-Sørensen, 2001; Ingerslev et al., 2001). Degradation of antibiotics is governed by their molecular composition. Macrolides and penicillins are targets for fast degradation in soil (Gavalchin and Katz, 1994).

Generally, the degradation of most xenobiotics is faster and more complete under aerobic as compared to anaerobic conditions. Correspondingly, degradation of oxytetracylcine, tylosin, sulfadiazine, streptomycin, metroniadazole, and olaquindox in activated sludge, soil and surface water was similar or slightly lower under anaerobic as compared to aerobic conditions, while ciprofloxacin was not degraded under anaerobic conditions (Halling-Sørensen et al., 2003; Ingerslev et al., 2001).

2.1.5 Effect of Antibiotics on Environmental Species

a. Effects on soil organisms and plants

Pharmaceutical antibiotics are designed to affect mainly microorganisms. Hence, the toxic dose to pathogens are often several magnitudes smaller than for humans (Wollenberger et al., 2000). Accordingly, dose related effects on soil microorganisms have been determined (Herron et al., 1998). Antibiotics such as streptomycin and cycloheximine are generally used to selectively inhibit growth of bacteria and fungi in soil experiments. Consequently, other pharmaceutical antibiotics cause changes in the composition of the indigenous soil microbial population as well (Ingham and

Coleman, 1984). In contrast, species of soil fauna were not affected by even excessive doses of antibiotics (Herron et al., 1998). The effects of antibiotics on organisms are essentially influenced by their bioavailability that depends on the soil properties, availability of nutrients, and presence of root exudates (Herron et al., 1998). Multivalent cations inhibit the antibiotic potential of tetracyclines and fluoroquinolones (Froehner et al., 2000). Degradation products of sulfadiazine, streptomycin, ciprofloxacin, and olaquindox showed no significant potency in a soil bacterial assay (Halling-Sørensen et al., 2003). However, a transformation of pharmaceuticals does not necessarily result in the decline in their antibiotic potential. Various metabolites of tetracyclines (such as anhydrotetracycline and 4-epianhydrotetracycline) still exhibited bacterial toxicity in sewage sludge and soil (Halling-Sørensen et al., 2002).

Antibiotic potential can increase with time from what may be due to bioaccumulation (Migliore et al., 1993). Bioaccumulation of 15 FQs was determined in E. coli, S. aureus and P. Aeruginosa. However, the resulting intra-corporal concentrations were not correlated with the antibiotic effects of the pharmaceuticals (Asuquo and Piddock, 1993).

b. Antibiotic resistance

Antimicrobial resistance is the ability of a bacteria to survive and reproduce despite the presence of an antimicrobial agent that would normally inhibit the growth of or cause death to a susceptible population of that bacteria. Bacteria may be resistant due to intrinsic resistance to one or more classes of antibiotics, such as gram-negative bacteria to vancomycin, or acquire resistance by de novo mutation or resistance-coding genes from other organisms (Kolár et al., 2001).

There are three common mechanisms by which bacterial resistance to antibiotics is acquired. An organism may acquire gene-encoding enzymes which enable the bacteria to destroy the antibacterial agent before it can have desired effect. An example of this is beta-lactamase

producing bacteria and their consequent resistance to most penicillins. Second, an organism may develop efflux pumps, enabling it to extrude the antibiotic from the cell. This simply keeps the antibiotic from reaching its target site. Third, several genes may be acquired by an organism for a metabolic pathway, producing an altered bacterial cell wall that no longer contain the specific binding site for an antibiotic agent to bind (Andersson and Hughes, 2010).

Susceptible populations of bacteria may become resistant by one of these three mechanisms through either genetic mutation or selection, or by acquiring genetic information from other bacteria which codes for resistance, through conjugation, transformation, or transduction. Conjugation occurs when the cell surface of a resistant bacteria and a recipient come into contact and transfer DNA plasmids that contain genes coding for resistance. Transformation occurs when bacteria take up DNA from dead bacteria in close proximity and incorporate the new genetic material, which may have advantageous genes such as resistance, into their own DNA.

Transduction occurs when genes contained in the head of the virus are injected into a bacteria that the virus subsequently attacks (Andersson and Hughes, 2010; Murray and Baron, 2007).

Since bacteria can acquire resistance by both genetic mutation and by genetic transfer from other bacteria and virus, they may become resistant to multiple classes of antibiotic, resulting in multiple-drug resistance. Most resistance is obtained by gene transfer from a resistant bacteria to a susceptible bacteria through horizontal gene transfer (Arias and Murray, 2009). This type of gene transfer, and consequent spread of resistance, can easily occur on the skin surface or in the gut, where different bacteria mix. Høiby et al., (1997), demonstrated that antibiotics may be excreted via sweat glands, and an increase in resistant S. epidermidis was observed after antibiotics were consumed.

Antibiotics released into the environment can provoke the formation of resistance in microorganisms (Al-Ahmad et al., 1999). The initiation of resistance is promoted by exposure of the microbes to a sublethal doses of antibiotics (Gavalchin and Katz, 1994). Antibiotic residues in the environment are suspected to induce resistances in bacterial strains causing a serious threat for public health as more and more infections can no longer be treated with the presently known antibiotics. Stelzer et al. (1985), investigated Klebsiellae isolates of a sewage treatment plant in which 90% exhibited an insensitivity against ampicillin and 6% showed multiple resistances. Interestingly, investigated drinking waters contained resistant bacteria, which was explained by an assumed faecal contamination (Pandey and Musarrat, 1993; Pathak et al., 1993).

In Ghana, antibiotic resistance surveillance has revealed an escalation antibiotics drug resistance to pathogens such as Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeuroginosa and pathogenic E. coli (Newman et al., 2011; Sackey et al., 2001). A nationwide study on bacterial resistance in humans showed high resistance for various antibiotics (Newman et al., 2011), some of which are considered to be used in animal husbandry.

A study performed on Gram-negative bacteria isolated from hospitalized patients showed high prevalences of resistance to tetracycline (82%), ampicillin (75%), chloramphenicol (75%), and cotrimox- azole (72%) (Newman et al., 2011). In addition, more than 80% of Escherichia coli strains that were isolated from stools of healthy volunteers in Ghana exhibited resistance to ampicillin, tetracycline, chloramphenicol and co-trimoxazole (Nys et al., 2004). These studies do not only indicate a widespread prevalence of antimicrobial resistance in the most important bacterial pathogens in Ghana (Nys et al., 2004), but also that resistance is a serious problem both for community-acquired as well as for nosocomial infections (hospital-acquired).

c. Antibiotic uptake in and effects on plants

The uptake and effects of antibiotics on plants varies considerably between reports and depends on the antibiotic substance and plant species (Migliore et al., 1995; Patten et al., 1980). Yields and nutrient uptake by radish, wheat, and corn increased in the presence of 160 mg/kg of TCs (Batchelder, 1982). In contrast, performance of pinto beans (Phaseolus vulgaris) significantly reduced in a sandy loam, but not in clay loam that is most likely due to the inhibition of root nodulation by rhizobia (Batchelder, 1982).

The effects of antibiotics on plants reviewed by Jjemba, (2002) indicated that negative impacts of antibiotics on plants were mostly determined by *in-vitro* experiments at concentrations that are unlikely to occur in fields soils. Negative impacts of contaminated manure on field soils were most likely related to excessive nitrogen or heavy metals, but not antibiotics. Accordingly, the plant growth inhibiting effect of sulfadimethoxine was smaller in vivo (Migliore et al., 1996). This was related to a slow bioaccumulation of the antibiotic from the nutrient solution into the plant (Migliore et al., 1996) that may have been suppressed in soil by aging of the antibiotics.

Ahmed et al. (2015), found the distributions of TCs and SAs accumulated in plant to be roots > leaves > fruits. Migliore et al. (1996), also determined that the concentration of antibiotics in the roots was higher than other parts of wheat and corn. Similarly, Liu et al., (2013), concluded that the distribution of all antibiotics in the wetland plant (Phragmites australis) followed the sequence root > leaf > stem. On the other hand, Hu et al. (2010), found that the distribution of antibiotics in various tissues of the vegetables was leaves > stems > roots. Hu et al. (2010) also insisted that the types and growth stages of vegetables would affect the distribution of antibiotics. The differences of antibiotics uptake and the distribution in plant are due to the limited understanding of the

interactions of antibiotic concentrations in manure/soil, specific crops, plant growth stage, and plant physiology (Dolliver et al., 2007).

2.1.6 Guidelines of Antibiotics in the Environment

According to the research conducted by Lapworth et al. (2012), scanty information about the toxicity, impact and monitoring data of majority of antibiotics in the aquatic environment.

Therefore, absence of regulatory guidelines for antibiotics in drinking water and the groundwater. For instance, the EU Drinking Water Directive does not have the standards for antibiotics (Lapworth et al., 2012).

Maximum residue levels (MRL) have been established for antibiotics (JECFA, 2011). Although MRLs have been set for various animal-based food products, limits have not been established for plant-based products. In general MRL for antibiotics in animal tissues are below 1 mg/ kg fresh weight (JECFA, 2011).

Acceptable daily intake (ADI) values have been established for some antibiotics (JECFA, 2011). The ADI value indicates the level of a chemical that can be ingested daily over a lifetime without health risk. For antibiotic compounds with ADI, the values are less than 50 mg/kg body weight per day (JECFA, 2011).

2.1.7 Environmental Risk Assessment

General principles and guidelines for environmental risk assessment (ERA) of new and existing chemicals have been introduced by European Medicines Evaluation Agency (EMEA) and the Food

and Drug Administration (FDA), employing similar tiered system. Both are based on the comparison between the predicted environmental concentrations (PEC) or measured environmental concentration (MEC) and the worst-case predicted no effect concentrations (PNEC) estimated from standard toxicity assays (EMEA, 2006; FDA, 1998).

An assessment of the environmental risk associated with the use of a specific chemical and a specific process gives industries the possibility of making the right selection of materials, chemicals, and processes to the benefit for the economy of the enterprise and the quality of the environment. Similarly, society needs to know the environmental risks of all chemicals used in society so as to phase out the most environmentally threatening chemicals and set standards for the use of all other chemicals.

Modern abatement of pollution therefore includes environmental risk assessment, ERA, which is defined as the process of assigning magnitudes and probabilities to the adverse effects of human activities. The process involves identification of hazards such as the release of toxic chemicals to the environment by quantifying the relationship between an activity associated with an emission to the environment and its effects. The entire ecological hierarchy is considered in this context including the effects on the cellular (biochemical) level, on the level of organs, on the organism level, on the population level, on the ecosystem level and on the entire ecosphere.

The application of environmental risk assessment is rooted in the recognition that: 1) the elimination cost of all environmental effects is impossibly high, 2) practical environmental management decisions must always be made on the basis of incomplete information.

Uncertainty plays an important role in risk assessment (Suter, 1993). Risk is the probability that a specified harmful effect will occur or in the case of a graded effect, the relationship between the magnitude of the effect and its probability of occurrence. Risk assessment has emphasized risks to

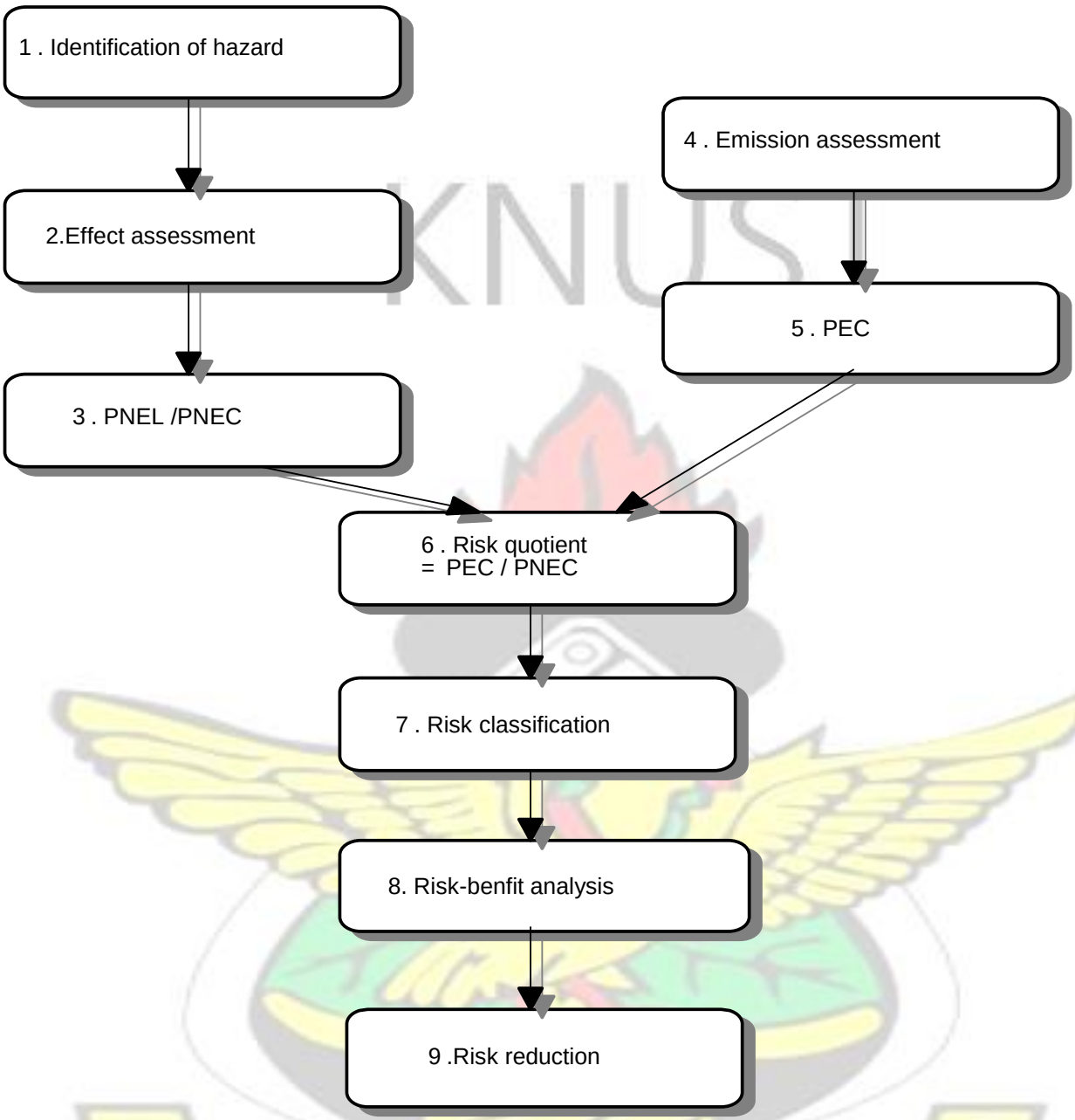
human health and has to a certain extent ignored ecological effects. However, some chemicals that have no or only little risk to human health cause severe effects on ecosystems such as aquatic organisms. Organisms interact directly with the environment and it is organisms that are exposed to toxic chemicals. Uncertainty is addressed using an assessment (safety) factor from 10 to 1000. The choice of assessment factor depends on the quantity and quality of toxicity data. Other relationships than the uncertainties originating from randomness, errors and lack of knowledge may be considered when the assessment factors are selected, for instance cost-benefit. This implies that the assessment factors for drugs and pesticides may be given a lower value due to their possible benefits.

Lack of knowledge results in undefined uncertainty that cannot be described or quantified. It is a result of practical constraints on our ability to accurately describe, count, measure or quantify everything that pertains to a risk estimate. Clear examples are the inability to test all toxicological responses of all species exposed to a pollutant and the simplifications needed in the model used to predict the expected environmental concentration. The most important feature distinguishing risk assessment from impact assessment is the emphasis in risk assessment on characterizing and quantifying uncertainty. Therefore, it is of particular interest in risk assessment to analyze and estimate the analyzable uncertainties. Such as natural stochasticity, parameter errors, and model errors. Statistical methods may provide direct estimates of uncertainties. They are widely used in model development.

The use of statistics to quantify uncertainty is complicated in practice by the needs to consider errors in both the dependent and independent variables and to combine errors when multiple extrapolations should be made. Monte Carlo analysis is often used in the development of models to overcome these difficulties (Bartell et al., 1992).

2.1.7.1 Procedures in Environmental Risk Assessment

Chemical risk assessment may be divided into nine steps (Jørgensen et al., 2015) which are shown in Fig. 2.8. The nine steps correspond to questions which the risk assessment attempts to answer to quantify the risk associated with the use of a chemical. The nine steps are presented in detail below with reference to Fig. 2.8



PNEL - Predicted no effect level, PNEC- Predicted no-effect concentration, and PEC- Predicted environmental concentration

Fig. 2.8 Nine steps procedure of risk assessment

Which hazards are associated with the application of the chemical? This involves gathering data on the types of hazards - possible environmental damage and human health effects. The health effects include congenital, neurological, mutagenic, endocrine disruption (so called estrogen) and

carcinogenic effects. It may also include characterization of the behavior of the chemical within the body (interactions with organs, cells or genetic material). What is the possible environmental damage including lethal effects and sub-lethal effects on growth and reproduction of various populations? As an attempt to quantify the potential danger posed by chemicals, a variety of toxicity tests has been devised. Some of the recommended tests involve experiments with subsets of natural systems for instance microcosms or with entire ecosystems. The majority of testing new chemicals for possible effects has, however, been confined to studies in the laboratory on a limited number of test species. Results from these laboratory assays provide useful information for quantification of the relative toxicity of different chemicals. They are used to forecast effects in natural systems, although their justification has been seriously questioned (Cairns et al., 1987).

What is the relation between dose and responses of the type defined in step 1? It implies knowledge of NEC (non-effect concentration), LDx- (the dose which is lethal to x% of the organisms considered), LCy- (the concentration which is lethal to y% of the organisms considered) and ECzvalues (the concentration giving the indicated effect to z% of the considered organisms) where x, y and z express a probability of harm. The answer can be found by laboratory examination or we may use estimation methods. Based upon these answers a most probable level of no effect, NEL, is assessed. Data needed for steps 1 and 2 can be obtained directly from scientific libraries, but are increasingly found via on-line data searches in bibliographic and factual databases. Data gaps should be filled with estimated data. It is very difficult to get complete knowledge about the effect of a chemical on all levels from cells to ecosystem. Some effects are associated with very small concentrations, such as the estrogen effect. It is therefore far from sufficient to know NEC, LDx-, LCy- and ECz-values.

Which uncertainty (safety) factors reflect the amount of uncertainty that must be taken into account when experimental laboratory data or empirical estimation methods are extrapolated to real situations? Usually, safety factors of 10 - 1000 are used. If good knowledge about the chemical is available, then a safety factor of 10 may be applied. If, on the other hand, it is estimated that the available information has a very high uncertainty, then a safety factor of 10,000 may be recommended. Most frequently, safety factors of 50-100 are applied. NEL (non-effect level) times the safety factor is named the predicted non-effect level, PNEL. The complexity of environmental risk assessment is often simplified by deriving the predicted no effect concentration, PNEC, for different environmental components (water, soil, air, biotas and sediment).

What are the sources and quantities of emissions? The answer requires thorough knowledge of the production and use of the chemical compounds considered, including an assessment of how much of the chemical is wasted in the environment by production and use? The chemical may also be a waste product which makes it very difficult to determine the amounts involved. For instance, the very toxic dioxins are waste products from incineration of organic waste.

What is (are) the actual exposure concentration(s)? The answer to this question is named the predicted environmental concentration, PEC. Exposure can be assessed by measuring environmental concentrations. It may also be predicted by a model (Jørgensen and Fath, 2011) when the emissions are known.

What is the ratio PEC/PNEC? This ratio is often called the risk quotient. It should not be considered an absolute assessment of risk but rather a relative ranking of risks. The ratio is usually found for a wide range of ecosystems such as aquatic ecosystems, terrestrial ecosystems, and ground water.

How will you classify the risk? Risk valuation is made to decide on risk reductions (step 9). Two risk levels are defined: 1) the upper limit, i.e., the maximum permissible level (MPL), and 2) the lower limit, i.e., the negligible level, NL. It may also be defined as a percentage of MPL, for instance 1% or 10% of MPL.

The two risk limits create three zones: a black, unacceptable, high risk zone > MPL, a grey, medium risk level and a white, low risk level < NL. The risk of chemicals in the gray and black zones must be reduced. If the risk of the chemicals in the black zone cannot be reduced sufficiently, then it should be considered to phase out the use of these chemicals.

What is the relation between risk and benefit? This analysis involves examination of socioeconomic, political and technical factors, which are beyond the scope of this volume. The cost-benefit analysis is difficult because the costs and benefits are often of a different order and dimension.

How can the risk be reduced to an acceptable level? The answer to this question requires deep technical, economic and legislative investigation. Assessment of alternatives is often an important aspect in risk reduction.

In North-America, Japan and EU medicinal products are considered similarly to other chemical products as there is in principle no difference between a medicinal product and other chemical products. At present, technical directives for human medicinal products do not in the EU include any reference to ecotoxicology and the assessment of their potential risk (Jensen et al., 1998).

However, a detailed technical draft guideline issued in 1994 (Joint Research Centre, 2003) indicates that the approach applicable for veterinary medicine would also apply to human medicinal products. Presumably, ERA will be applied to all medicinal products in the near future when sufficient experience with veterinary medicinal products has been achieved. Veterinary

medicinal products on the other hand are released in larger amounts to the environment as manure. For instance, veterinary medicine is utilized as fertilizer on agricultural fields. It is also possible to perform an environmental risk assessment where the human population is in focus. It uses the nonadverse effect level and non-observed adverse effect level to replace the predicted non-effect concentration and the predicted environmental concentration is replaced by the tolerable daily intake. This type of environmental risk assessment has particular interest for veterinary medicine which may contaminate food products for human consumption. For instance, the use of antibiotics in pig feed has attracted a lot of attention, as they may be found as residue in pig meat or may contaminate the environment through the application of manure as natural fertilizer.

2.1.8 Eco-toxicological model of chemical compounds

Ecosystems are open systems, which implies that all the chemicals that we are using sooner or later may reach the environment. A model attempts to relate the "leakage" to the environment with the corresponding concentrations in various parts of the environment: soil, water, porewater, groundwater, sediment, air, specific species, our food, entire populations and so on. Mankind has always made more or less descriptive models of the environment, which often have been used to solve problems or to obtain knowledge about how nature functions. In eco-toxicological model, mathematical equations are used as a tool to describe nature. Selection of a proper ecotoxicological model is the first initial step in the development of an environmental exposure model (Jørgensen and Fath, 2011).

2.1.8.1 Modelling Elements

In its mathematical formulation, a model in environmental sciences has five components. Forcing functions, or external variables, which are functions or variables of an external nature that influence the state of the ecosystem. In a management context the problem to be solved can often be reformulated as follows: if certain forcing functions are varied, how will this influence the state of the ecosystem? The model is used to predict what will change in the ecosystem when forcing functions are varied with time. The forcing functions under our control are often called control functions. The control functions in eco-toxicological models are, for instance, inputs of toxic substances to the ecosystems. Other forcing functions of interest could be climatic variables, which influence the biotic and abiotic components and the process rates. They are not controllable forcing functions. State variable, as the name indicates, describe the state of the ecosystem. The selection of state variable is crucial to the model structure, but often the choice is obvious. If, for instance, we want to model the bioaccumulation of a toxic substance, the state variable should be the organisms in the most important food chains and concentrations of the toxic substance in the organisms. In eutrophication models the state variables will be the concentrations of nutrients and phytoplankton. When the model is used in a management context, the value of state variables predicted by changing the forcing functions can be considered as the results of the model, because the model will contain relationships between the forcing functions and the state variable.

Mathematical equations are used to represent the biological, chemical and physical processes. They describe the relationship between the forcing functions and state variables. The same type of process may be found in many different environmental contexts, which implies that the same equations can be used in different models. This does not imply, however, that the same process is always formulated using the same equation. First, the considered process may be better described

by another equation because of the influence of other factor. Second, the number of details needed or desired to be included in the model may be different from case to case due to a difference in complexity of mathematical formulation of processes as sub-models.

Parameters are coefficients in the mathematical representation of processes. They may be considered constant for a specific ecosystem or part of an ecosystem. In causal models the parameter will have a scientific definition, for instance, the excretion rate of cadmium from a fish. Many parameters are not indicated in the literature as constants but as ranges, but even that is of great value in the parameter estimation. Our limited knowledge of parameters is one of the weakest points in modeling. Furthermore, the application of parameters as constants in our models is unrealistic due to the many feedbacks in real ecosystems. The flexibility and adaptability of ecosystems is inconsistent with the application of constant parameters in the model. A new generation of models that attempts to use parameters varying according to some ecological principle seems a possible solution to the problem, but a further development in this direction is absolutely necessary before we can achieve an improved modeling procedure reflecting the processes in real ecosystems. Universal constants, such as the gas constant and atomic weights, are also used in most models.

Models are formal expressions of the essential elements of a problem in mathematical terms. The first recognition of the problem is often verbal. This may be recognized as an essential preliminary step in the modeling procedure. However, the verbal model is difficult to visualize and it is, therefore, more conveniently translated into a conceptual diagram, which contains the state variables, the forcing functions and how these components are interrelated by mathematical formulations of processes. Three significant steps in the modelling procedure need to be defined: they are verification, calibration and validation.

Verification is a test of the internal logic of the model. Typical questions in the verification phase are: Does the model react as expected? Is the use of units consistent? Verification is to some extent a subjective assessment of the behaviour of the model. To a large extent, the verification will go on during the use of the model before the calibration phase, which has been mentioned above.

Calibration is an attempt to find the best accordance between computed and observed data by variation of some selected parameters. It may be carried out by trial and error or by use of software developed to find the parameters giving the best fit between observed and computed values. In some static models and in some simple models, which contain only a few welldefined, or directly measured, parameters, calibration may not be required.

Validation must be distinguished from verification. Validation consists of an objective test of how well the model outputs fit the data. We distinguish between a structural (qualitative) validity and a predictive (quantitative) validity. A model is said be structurally valid, if the model structure represents reasonably accurately the cause- effect relationship of the real system. The model exhibits predictive validity if its predictions of the system behaviour are reasonably in accordance with observations of the real system. The selection of possible objective tests will be dependent on the aims of the model, but the standard deviations between model predictions and observations and comparison of observed and predicted minimum or maximum value of particularly important state variable are frequently used. If several state variables are included in the validation, they may be given different weights.

2.1.8.2 The modeling procedure

The initial focus of research is always the definition of the problem. This is the only way in which the limited research resources can be correctly allocated instead of being dispersed into irrelevant

activities. The first modelling step is therefore a definition of the problem and the definition will need to be bound by the constituents of space, time and subsystems. The bounding of the problem in space and time is usually easy, and consequently more explicit, than the identification of the subsystems to be incorporated in the model.

System thinking is important in this phase: you must try to grasp the big picture. The focal system behaviour must be interpreted as a product of dynamic processes, preferably describable by causal relationships. Fig. 2.9 show the procedure proposed, but is important to emphasize that this procedure is unlikely to be correct at the first attempt, so there is no need to aim at perfection in one step. The procedure should be considered as an iterative process and the main requirement is to get started (Jeffers, 1978).

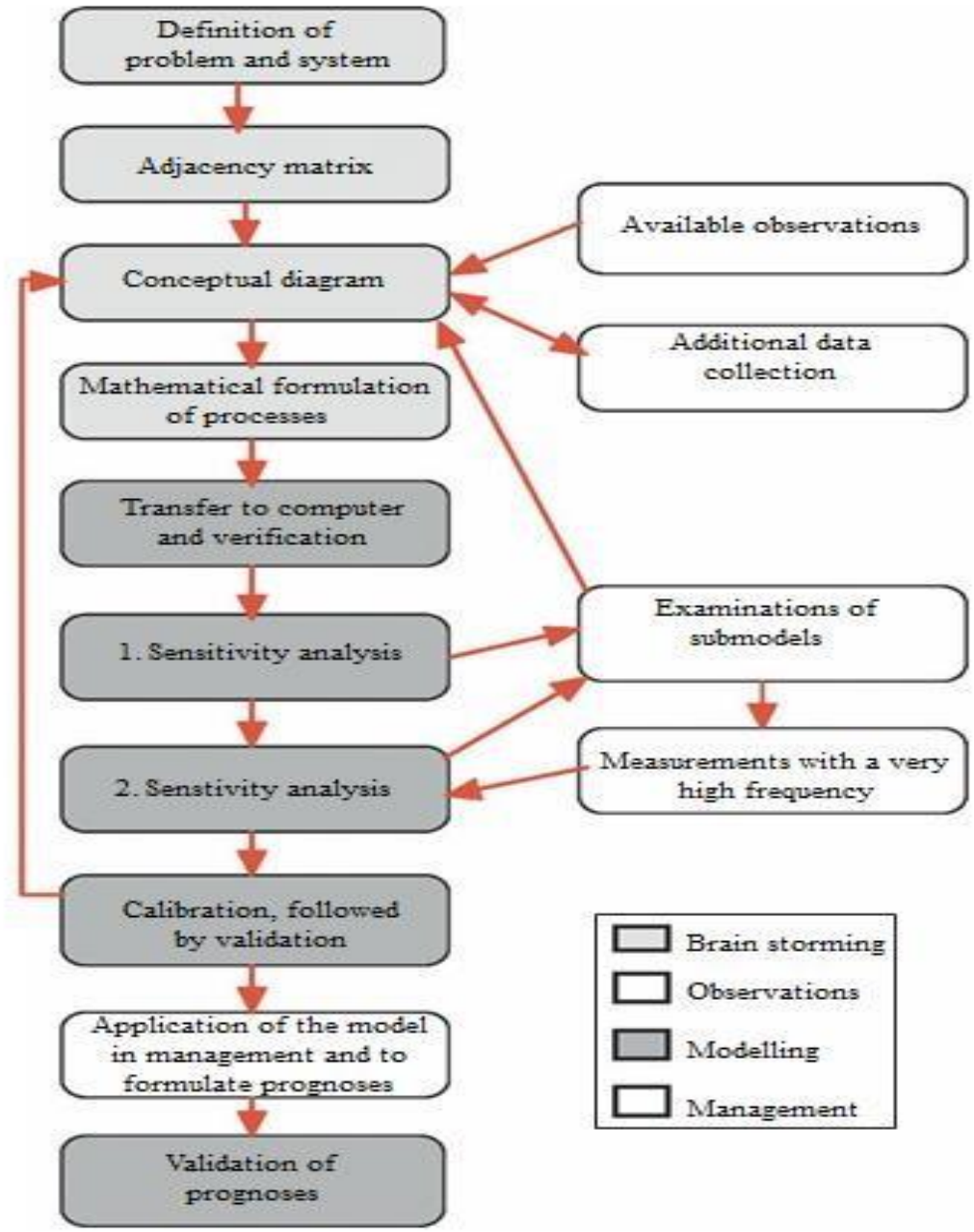


Fig. 2.9: Modeling procedure
Source: (Jørgensen and Fath, 2011)

It is difficult, at least in the first instance, to determine the optimum number of subsystems to be included in the model for an acceptable level of accuracy defined by the scope of the model. Due to lack of data, it will often become necessary at a later stage to accept a lower number than intended at the start or to provide additional data for improvement of the model. It has often been argued that a more complex model should account more accurately for the reactions of a real system, but this is not necessarily true. Additional factors are involved. A more complex model contains more parameters and increases the level of uncertainty, because parameters have to be estimated either by more observations in the field, by laboratory experiments, or by calibrations, which again are based on field measurements. Parameter estimations are never completely without errors, and the errors are carried through into the model, thereby contributing to its uncertainty.

A first approach to the data requirement can be made at this stage, but it is most likely to be changed at a later stage, once experience with the verification, calibration, sensitivity analysis and validation has been gained. In principle, data for all the selected state variable should be available; in only a few cases would it be acceptable to omit measurements of selected state variable, as the success of the calibration and validation is closely linked to the quality and quantity of the data.

Several model formulations are always available and the ability to choose among them requires that sound scientific constraints are imposed on the model. Possible constraints are introduced and discussed. A mathematical model will usually require the use of a computer and therefore a computer language. Although the selection of a computer language is not discussed, because there are many possibilities and new language emerge from time to time The well-established computer languages include STELLA, PowerSim, Berkeley Madonna, Vensim, Simile, etc.

2.1.8.3 Eco-toxicological software: STELLA

A computer model was developed using the Structural Thinking and Experiential Learning Laboratory with Animation (STELLA) software, a user-friendly and commercial software package for building a dynamic modeling system. STELLA is a popular system dynamics modeling language, which helps to put together conceptual diagrams and converts them into numeric computer models. STELLA was one of the first dynamic modeling systems to achieve broad recognition and use, due to its user-friendly graphic interface. One of the reasons that system dynamics software became so popular in modeling is that they provide handy tools to put together conceptual diagrams, and provide tools to convert them into numeric computer models. It uses an iconographic interface to facilitate construction of dynamic system models. The key features of STELLA consist of the following four tools (Fig. 2.10): (1) Stocks, which are the state variables for accumulations. They collect whatever flows into and out of them; (2) Flows, which are the exchange variables and control the arrival or the exchanges of information between the state variables; (3) Converters, which are the auxiliary variables. These variables can be represented by constant values or by values depending on other variables, curves or functions of various categories; and (4) Connectors, which are to connect among modeling features, variables, and elements.

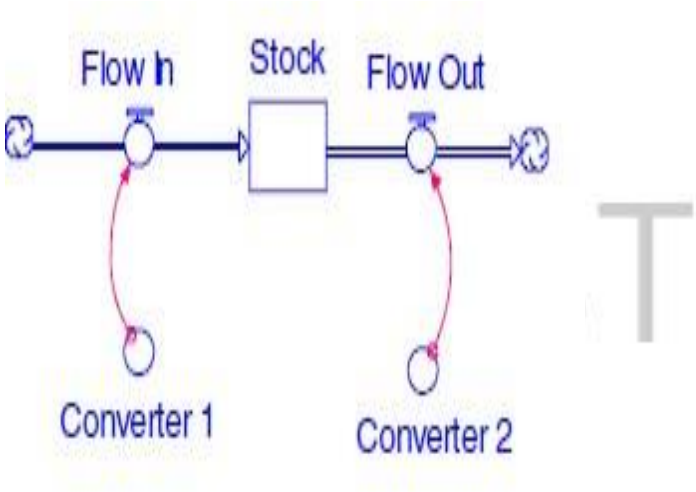


Fig. 2.10 Key features of STELLA

STELLA offers a practical way to dynamically visualize and communicate how complex systems and ideas really work (Naimi and Voinov, 2012). STELLA has been widely used in biological, ecological, and environmental sciences (Aassine and El Jai, 2002; Azanu et al., 2016a; Costanza et al., 2002; Hannon and Ruth, 1994; Peterson and Richmond, 1996; Spaepen et al., 1997).

2.1.8.4 Applications of modelling

Fugacity models have been developed for pharmaceutical in the environment both for ecosystems and for regions, both with and without effect (Jørgensen and Bendoricchio, 2001; Mackay, 1991). The following pharmaceuticals have been modeled by fugacity models: cyclophosphamide, diazepam, ivermectin (Kümmerer, 2001). Khan and Ongerth, (2005) has developed a fugacity model describing the distribution of pharmaceuticals in a sewage treatment plant: how much is decomposed? which concentration is found in the treated sewage water? and which concentration has the sludge? All the here mentioned fugacity models are no effect models, but could easily be

expanded to include also an effect by translation of the found concentration into a relevant effect. Montforts, (1999) and Spaepen et al., (1997) have developed an exposure model for oxytetracycline on grassland and how it contaminate the groundwater. The model is biogeochemical. Another bio-geo-chemical model was developed for olaquindox and tylosine (Jørgensen and Fath, 2011).

2.2 WASTEWATER MANAGEMENT AND VEGETABLE PRODUCTION IN KUMASI METROPOLIS: REVIEW

Ghana's population is 24.7 million with 2.5% growth rate annually (Ghana Statistical Service, 2012). Out of total land area of 23 million hectares, 13 million hectares is suitable for agricultural production and 5.3 million hectares is under cultivation. The city of Kumasi is the second largest in Ghana and lies about 150 km north-west of Accra, the country's capital at the south coast of western Africa. Kumasi is the capital of the Ashanti Region and commercial capital of central Ghana. The Kumasi Metropolitan Area is one of the 18 administrative districts of the Ashanti region. Its area was extended in 1996 and now comprises 254 km^2 out of which around 80% are developed. In 2005, 75 km^2, or 30%, were either referred to as open space or undeveloped land, such as river valleys or other unpopulated areas (Drechsel and Keraita, 2014).

Kumasi's population per year 2010 census is 2 million with growth rate of 5.2% (Ghana Statistical Service, 2012). Kumasi is best described as a lively African city still containing its rich cultural heritage while being slowly modernized in the process of rapid city growth and development. Kumasi plays an important role as a commercial trade center, the level of industrialization has remained rather low though. Elevation above sea level is between 230-290 m, the terrain is dominated by undulating hills with gentle slopes generally less than 5%. The ecological zone is moist semi-deciduous forest and the climate of the area is wet, semi-equatorial with a mean annual rainfall of 1350 mm (1967-2006). Minimum and maximum temperatures are around 21 °C and 30 °C, respectively, with only little variability throughout the year. Mean minimum and maximum annual humidity are 59% and 94%, respectively. It is generally less humid in the dry season between November and February. Rainfall is slightly bimodal with a short dry period in August. Nearly 90% of rainfall is recorded in the 7 months of the two wet seasons.

Kumasi is located on a drainage divide. 28% of the developed area drain to the west eventually joining the Offin river. 72% of the developed area drain to the Oda river in the south of the city. Most streams originate within the administrative boundaries of Kumasi. The only considerable inflow from outside is noted from Sisa and Wiwi rivers to the north of Kumasi. Their watershed size upstream of the city is around 42 km^2 (Cornish et al., 1999; Maoulidi, 2010).

2.2.1 Wastewater Generation, Pollution Sources and Pathways

Analysis in 2003 showed that out of the 60,000 m^3 of daily produced wastewater in Kumasi, over 9,000 m^3 is blackwater (including sewage) (Drechsel et al., 2004) while Agodzo et al., (2003) probably overestimated the amount to over 100,000 m^3 in 2000. Most of the blackwater is generated by the 0.5 million inhabitants who use water toilets. Fifty-five percent of the total household waste is organic (Drechsel et al., 2004). In a year the total amount of organic waste produced in Kumasi is 4,000 tons from households and 60,000 tons from markets (Drechsel et al., 2004). When including other sources of waste available for composting such as: sawdust, industry and livestock manure, Kumasi has a total of 230,000 – 250,000 tons available for composting every year. Based on these values it is clear that there is no shortage of fresh supply for composting in Kumasi (Drechsel et al., 2004).

2.2.1.1 Human Waste

In many less developed countries like Ghana, water toilets are not connected to sewage systems as predominantly found in high income countries, because their installation is expensive (Salifu, 2013). Furthermore, experience has shown that installed systems such as treatment plants suffer frequent breakdowns because of bad operational conditions, lack of maintenance and spare parts.

Common sanitation facilities in developing countries are dry toilets such as bucket latrines or pit latrines. Bucket latrines are toilets where it is defecated into a bucket that needs to be emptied on a regular basis. They have been phased out in Kumasi because of their unhygienic nature (Mensah, 2006).

In Kumasi, a more sophisticated pit latrine has been developed and installed: the KVIP (Kumasi Ventilated Improved Pit Latrine) (Mensah, 2006). It consists of two alternately used tanks and a improved ventilation system. Due to poor maintenance they are usually malfunctioning and not as popular as initially expected. Flushing toilets are also popular and are predominantly found in high-income areas. They are either connected to a sewage system or to a septic tank. A septic tank collects the solid wastes and has an outlet for the overflowing urine and water (Drechsel et al., 2004).

A survey by Owuso-Addo, (2006) of 50 septic tanks in Kumasi showed that the majority of tanks are not properly designed and do not have a soil absorption system.

Most faecal sludge from public toilets, septic tanks and latrines is collected by desludging trucks and brought to the faecal sludge treatment plant at Dompoase-Kaase. This practice started in 2004 after closing down of the treatment plant in Buobai because of conflicts with the local population (Abuenyi, 2010; Obuobie et al., 2006).

According to Mensah, (2006), KMA increased faecal sludge collection up to 90-95%. It is assumed that the remaining part of faecal sludge is dumped elsewhere, most probably into streams. Another 5% do not have toilet facilities and defecate into surface water or onto land (Drechsel et al., 2004). In Kumasi, only a small fraction of the population is connected to a sewage system. The two largest sewage systems at KNUST and Asafo serve about 40000 people. In total, less than 60000 people

are connected to a sewage system. The treatment plant at KNUST, used by about 20000 persons, has been out of order for over 20 years.

2.2.1.2 Industrial waste

Relatively little industrial activity can be found in Kumasi. Except for the many timber processing companies, there are one brewery, the Guinness Ghana bottling company, an abattoir and some other manufacturers such as yarn dyeing, rubber and foam works and a soap factory (Simon et al., 2001). Furthermore, an "industrial complex" called Suame Magazine employs about 30,000 people in small enterprises such machining, sheet metal fabrication, wood working, vehicle salvage and sale of parts, and automobile repairs and manufacture. The Suame Magazine is worth mentioning as it is located at the head of the Owabi watershed that drains into the Owabi reservoir which provides about a sixth of the piped water to the Kumasi metropolis.

2.2.1.3 Hospital Waste

There are 14 private and public hospitals in Kumasi, of which Komfo Anokye Teaching hospital (KATH) is the largest and also a teaching hospital. It serves the people in the Northern sector of Ghana. Waste generated in each hospital are managed by the individual hospital. Hospital waste consist of solid waste and liquid waste. Solid wastes are mostly sent to landfills except KATH and Tafo hospital where there are simple incinerators. These incinerators are made of bricks and concrete with local firewood as the source of energy. According to (Mensah and Larbi, 2005), incinerators at hospitals in Kumasi, have no environmental controls and often comprise nothing more than combustion of medical and chemical waste in an oven or open pit. All hospitals have on site liquid waste treatment facility (septic tank).

2.2.2 Waste Treatment Facilities in Kumasi

Sewage treatment plants (STPs) are designed to remove heavy metals from domestic and municipal wastewater through adsorption of suspended matter and precipitation of hydroxides and carbonates (Mara, 2000, 1987). Waste stabilization ponds (WSPs) are the most common type of low-cost natural STPs and comprise of a series of engineered basins through which the wastewater flows by gravity, usually over a period of several weeks. In a properly functioning system, the water in the tertiary (or maturation) ponds and the final effluent should meet applicable standards for in situ aquaculture, and for discharge to the environment or cultivated land (Von Sperling and Chernicharo, 2005; WHO, 2006).

Waste Stabilization Ponds (WSPs) are large, shallow basins in which raw sewage is treated entirely by natural processes involving both algae and bacteria. They are used for sewage treatment in temperate and tropical climates, and represent one of the most cost-effective, reliable and easilyoperated methods for treating domestic and industrial wastewater. Waste stabilization ponds are very effective in the removal of faecal coliform bacteria. Sunlight energy is the only requirement for its operation. WSP systems comprise a single string of anaerobic, facultative and maturation ponds in series, or several such series in parallel. In essence, anaerobic and facultative ponds are designed for removal of Biochemical Oxygen Demand (BOD), and maturation ponds for pathogen removal, although some BOD removal also occurs in maturation ponds and some pathogen removal in anaerobic and facultative ponds (Mara, 1987).

Most of Kumasi's grey water is released to drains that discharge untreated into one of the streams flowing through or originating from Kumasi. Unlike most developed countries, where all wastewater including storm runoff is brought together, a large fraction of Kumasi's blackwater is separated from other wastewater and brought to a central treatment plant. In-house treatment plants (septic tank

system) are mostly found in major industries and institutions in Kumasi. A significant fraction of mostly organic waste from the large markets in Kumasi are thrown or swept to drains and washed down by storm water. Five small-scale sewerage systems with target coverage of about 40,000 people currently exist in Kumasi. They include:

- The Conventional Sewerage System at KNUST
- Asafo Simplified Sewerage System built in 1994
- Ahinsan Satellite Sewerage System rehabilitated under UESP in 2001
- Chirapatre Satellite Sewerage System rehabilitated under UESP in 2001
- Dompoase feacal desludge treatment system built in 2004

Komfo Anokye Teaching Hospital (KATH), City Hotel and the central parts of the 4BN Army barracks Conventional Sewerage System. The treatment facilities to the University (KNUST) and KATH systems are not functioning (Abuenyi, 2010; Kumasi Metropolitan Assembly, 1995).

2.2.2.1 Asafo waste stabilization ponds

The Asafo waste stabilization ponds (WSP) were built in the high-density area in 1994 (Fig. 2.11) as part of UNDP/World Bank, Kumasi Sanitation Project (GHA/87/0160) (Salifu, 2013). The WSP was constructed to cover an area of approximately 45 ha and connect 4,000 households making up approximately 20,000 people (Salifu, 2013). The WSP has 2 anaerobic ponds, 1 facultative pond, 2 maturation ponds. Effluent from Asafo WSPs joins the Subin River, which runs through the commercial centre of Kumasi and merges with the River Oda downstream at Asago (6° 8' 450" N 18° 360" W), which is the site of a rural farming community.

The Komfo Anokye Teaching Hospital (KATH) sampling site is the largest teaching hospital in

Kumasi and the second largest teaching hospital in Ghana with 1200 beds. University hospital (USTH) is a referral hospital for the University community and patronized by the surrounding submetro population. These were selected based on their potential to have high levels of antibiotic residues, in wastewater effluent. It is significant of note that these institutions sewage line are directly entering into nearby streams.

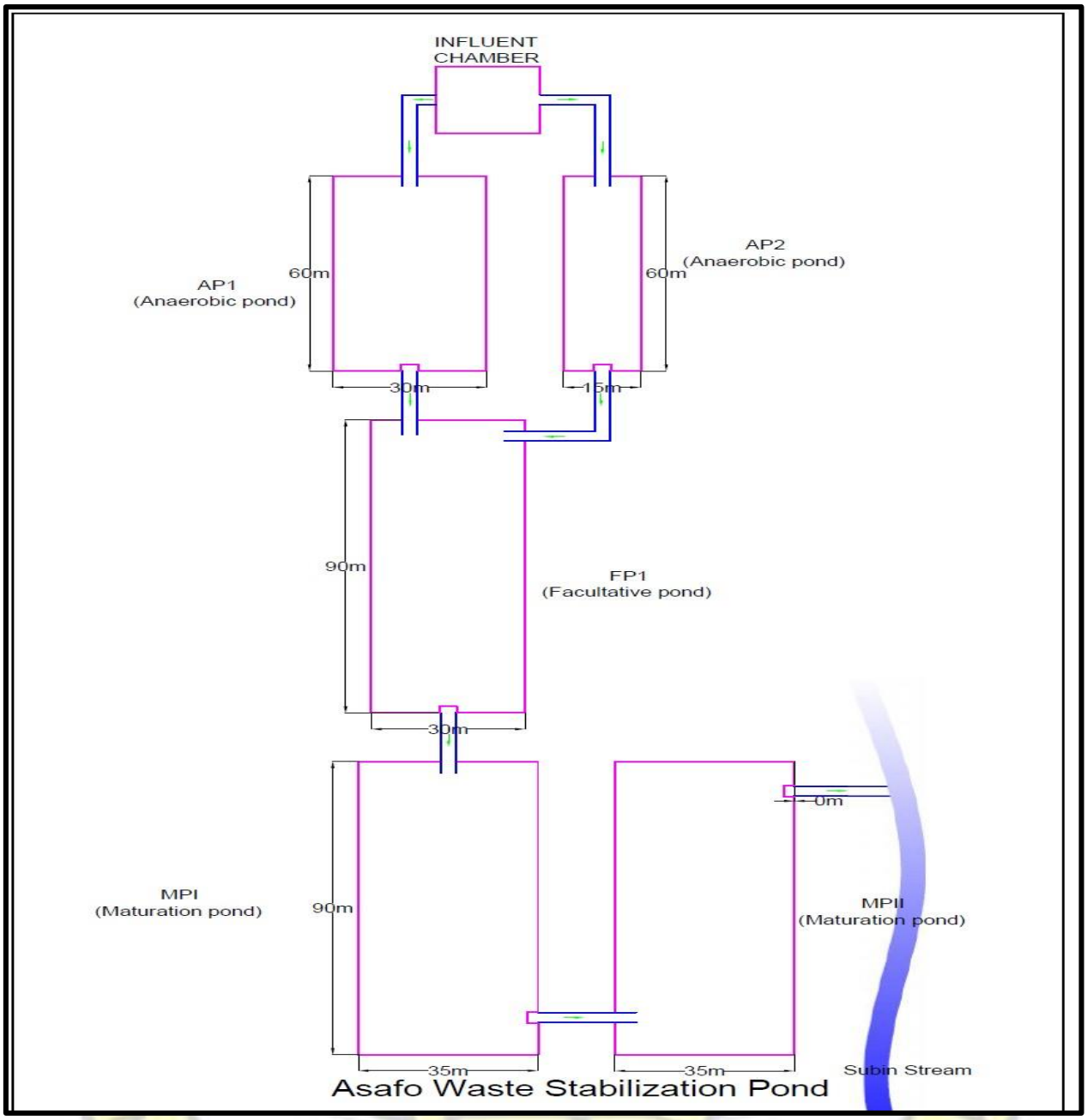


Fig. 2.11 Map of Asafo waste stabilization ponds

2.2.2.2 Kumasi Sanitary Landfill and Septage treatment facility

The Kumasi Sanitary Landfill and Septage treatment facility is located at Dompoase a suburb of

Kumasi. The facility was built under the World Bank financed Urban Environmental Sanitation Project (Urban IV), and the Government of Ghana, under the Ministry of Local Government's Urban Environmental Sanitation Project. It commenced in January 2004 and was designed to handle both solid waste and seepage produced in the city of Kumasi. The site has an overall area of 40 hectares and is bordered on the south by the Oda River.

A 1.5 km long access road to the landfill links the Dompoase road through the industrial township to the north-western corner of the site. There is a security fence around the perimeter of the site. The landfill area is a valley, with a mound located in the north - eastern half forming a smaller separate catchment area.

The septage ponds (Fig. 2.12) are located at the lowest elevation of the site and are situated in the valley that is present on the site. The septage ponds are designed to treat both municipal septage and leachate from the landfill. These effluents after treatment are discharged into the Oda river through a nearby stream.

The facility has a 15-year design life to cater for the current daily generations rates of 860 tons solid waste and 500 cubic metres faecal sludge including the projected future increases after which new facilities would be required (Abuenyi, 2010; Olufunke and Koné, 2009).

Fig. 2.12 Aeral view Dompoase site

2.2.2.3 Ahensan waste stabilization ponds

Ahensan waste stabilization ponds (Fig 2.13) was built in the late seventies with the intention to handle sewage from Ahensan suburb. Currently about 200 houses, with an estimated population of 1500 have been connected to the facility (Murray and Yeboah-Agyepong, 2012). The effluents from the WSP, are discharged into the Wiwi River through a nearby stream which is used for vegetable irrigation downstream. The WSP has 1 anaerobic pond, 1 facultative pond, 2 maturation ponds. The maturation ponds are used for aquaculture. The effluent after treatment is discharged into the Wiwi River through a nearby stream.

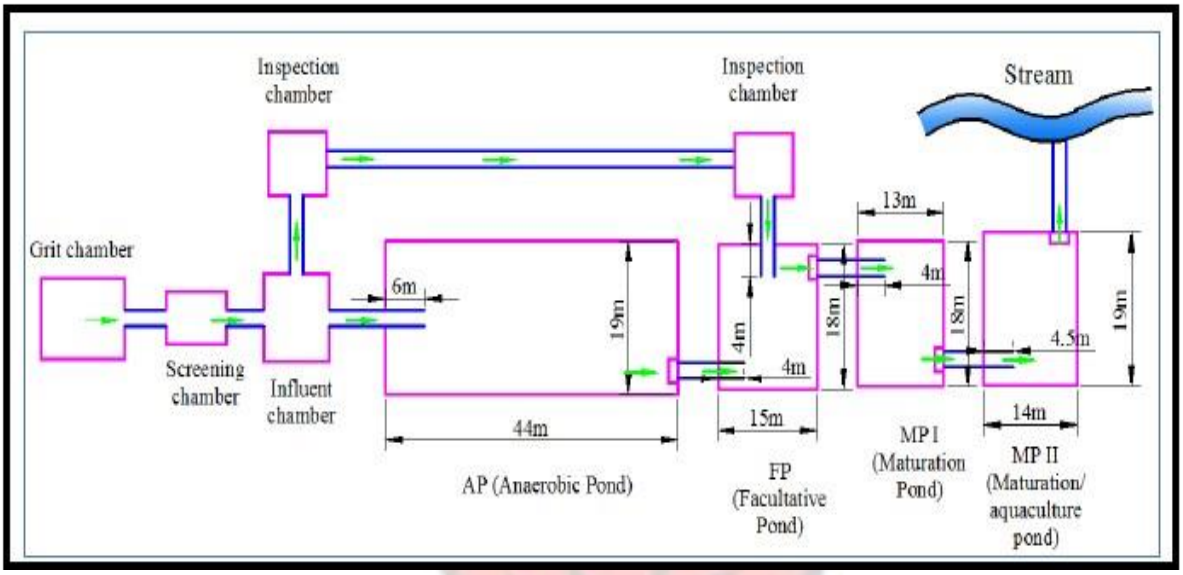


Fig. 2.13 Schematic diagram of Ahensan waste stabilization ponds

2.2.2.4 Chirapatre waste stabilization ponds

Chirapatre waste stabilization ponds (Fig 2.14) also built in the late seventies has a network of sewer lines connected to the Chirapatre Estate surburb WSP. The WSP was designed to connect to 300 household with an estimated population of 1800 people (Murray and Yeboah-Agyepong, 2012). The WSP has 1 anaerobic pond, 2 facultative ponds, 2 maturation ponds. The maturation ponds are used for aquaculture. The effluent after treatment is discharged into the Oda River through a nearby Oti stream. There are several farms within a distance of 2 km from the WSP, utilizing the stream water in which the effluent from the waste stabilization is discharged for vegetable irrigation.

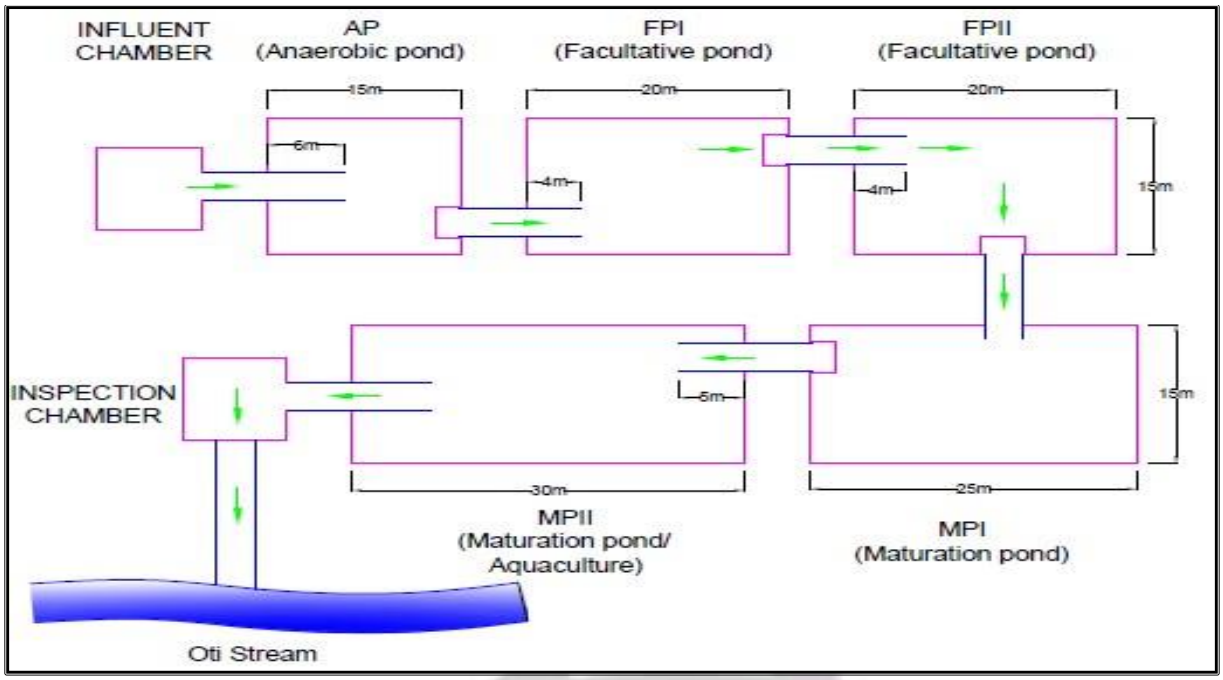


Fig. 2.14 Schematic diagram of Chirapatre waste stabilization ponds

2.2.3 Wastewater reuse in urban vegetable farming

The agricultural reuse of untreated, partially treated and diluted wastewater is a common reality in and around three out of four cities in low-income countries. It is practiced in both dry and wet climates, mostly as an informal activity. In several instances, raw wastewater is used in irrigation; in the majority of the cases, however, wastewater is discharged into water bodies and thus used in diluted form. The main driving forces identified for the growth of this practice include general global scarcity of freshwater resources and contamination of water bodies traditionally used for irrigation, especially around urban centres. Estimates on the extent of this practice range widely, but figures point at about 20 million ha of land irrigated in this way, most of it in Asia, Latin America, and sub-Saharan Africa (Drechsel et al., 2010).

Farmers located along the river Subin (one of the four main rivers) use the river for irrigation. The rivers in the Kumasi metropolis are heavily polluted and exhibit high faecal colifiorm concentrations (Mensah et al., 2001).

2.2.4 Major Irrigated Vegetable Farming Sites in Kumasi

In urban Kumasi, most land where farming occurs belongs to government institutions and private developers and not by individual. The total area used for open space farming in the city was about 70 ha in 2005 with 41 hectares under irrigated vegetable farming (Obuobie et al., 2006). However, Drechsel and Keraita, (2014) estimated 59 hectares (145 acres) of vegetables in the dry season and 48 hectares (118 acres) in the rainy season, cultivated by 300 men and 23 women farmers on about 20 farming sites (Table 2.2). This shows increase in the total area used for vegetable farming. The largest agglomeration of farms (Fig. 2.15) remains in the lowlands around the KNUST University (Drechsel and Keraita, 2014).

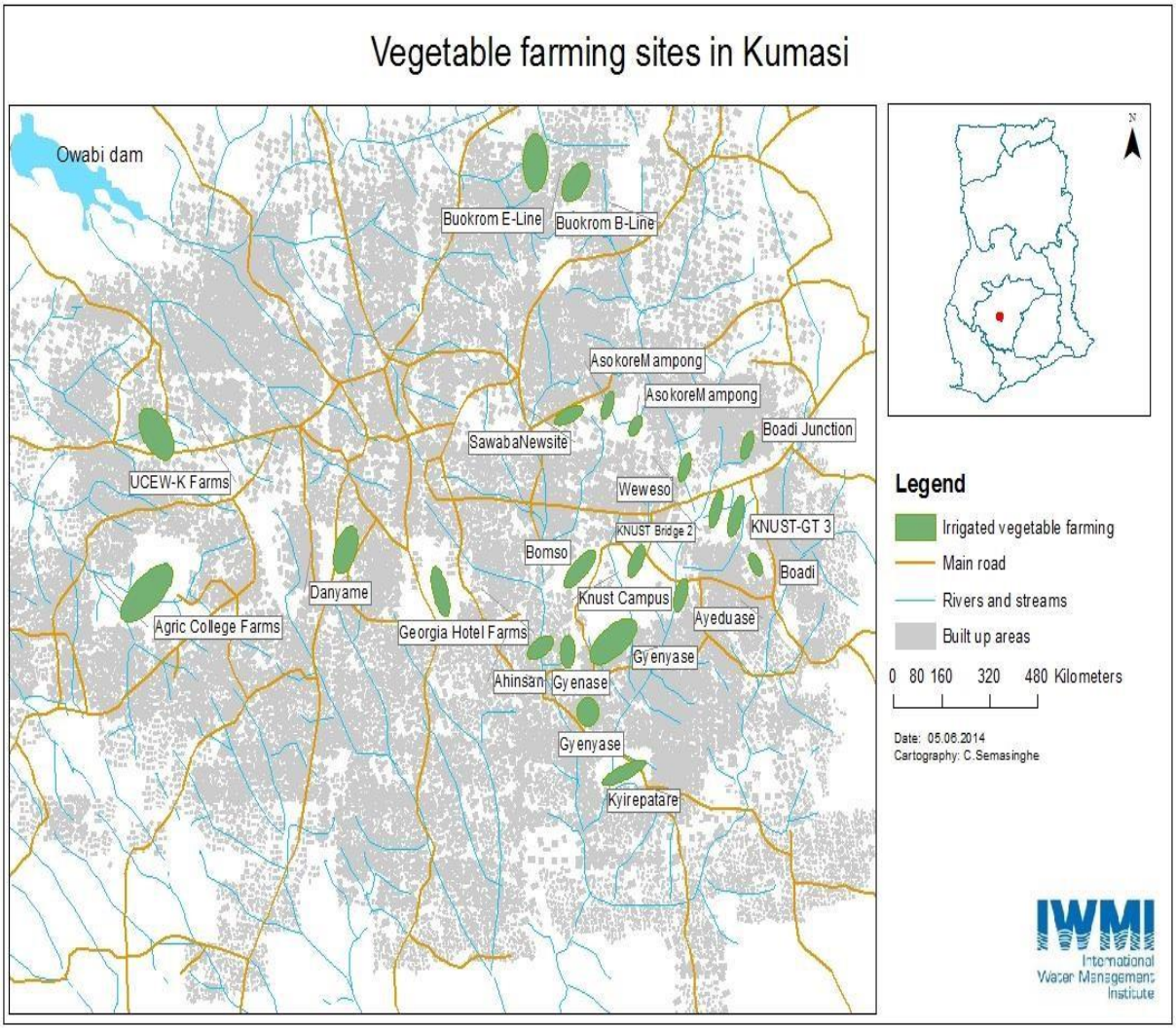


Fig. 2.15 Vegetable farming site in Kumasi in 2014
Source: (Drechsel and Keraita, 2014)

Table 2.2 Features of urban vegetable irrigation sites in Kumasi.

Adapted from (Drechsel and Keraita, 2014)

Kumasi Sub metro	Farming site names	Acres dry/wet season	No. of farmers (male/female)	Commonly grown vegetables	Common water sources	Irrigation methods

KWADASO	Agric. College Farms and UCEW-K Farms	21/10	20 (19/1)	Cabbage, Green pepper, Carrot, Lettuce	Stream, pipe	Watering can, pumps
NHYIASO	Danyame and Georgia Hotel	3/3	6 (6/0)	Spring onions, Lettuce, Cabbage, Carrot	Shallow well, Stream	Watering can, pumps
MANHYIA	Buokrom B- and E- Line	3/2	7 (5/2)	Spring onions, Lettuce	Shallow well, Stream	Watering can
ASAWASI	Asokore Mampong, Sawaba New site	19/19	41 (41/0)	Cabbage, Spring onions, Cucumber	Stream, shallow well	Watering can, pumps
OFORIKROM	All sites at KNUST north of Gyenase	46/37	152 (134/18)	Spring onions, Cabbage, Lettuce, Pepper, Spinach, Garden eggs	Shallow well, Stream	Pump, watering can
ASOKWA	Chirapatatre, Ahensan, Gyenase, Quarters	53/47	97 (95/2)	Cabbage, Lettuce, Spring onions, Cauliflower	Stream, Shallow well, pipe	Pump, watering can
Total		145/118	323 (300/23)			

2.2.5 Vegetable Market channels

2.2.5.1 Traditional Marketing Structures

Wholesale marketing of exotic vegetables, which are produced in peri-urban areas, takes place at certain distribution points on specific days during the week. The presence of traders from outside Kumasi can influence market prices as they usually make better offers (Cornish and Aidoo, 2000). Urban and growing numbers of peri-urban farmers who are not selling to traders on farm, send their produce to various distribution points relatively early in the morning (normally by 5.30 am), where wholesalers, retailers and hawkers converge to purchase the vegetables. In addition to the

main markets there are other small sale points located at strategic places within the city. Once the local market has been satisfied and the nonlocal traders are gone, the market for exotic vegetables on any other day is small and prices are highly erratic (Cornish et al., 2001).

However a significant proportion of the exotic vegetables produced in the city is sold at the farm gate, i.e. directly to wholesalers or retailers who harvest the (best) crops themselves. While many agriculture sectors in Ghana are being financed either by the government or external aid, urban farmers specialized in market production can only rely on self-financing (usually to start the business) or market women who can provide credit for the purchase of inputs (especially seeds and chemicals). These women can be intermediaries, wholesalers or actual market sellers. They visit the urban farms and reserve beds of vegetables in advance, thus financing the venture. The contract is oral. The price per bed depends on the season, crop and the size of the bed (approximately USD1.4 to 3.6 per bed) and farmers are not allowed to sell the vegetable to any other person. The total amount of money eventually received may differ from the agreed values as demand and supply might have changed during the growing period, but seldom to the advantage of the (male) farmers (Henseler et al., 2005).

2.2.5.2 Alternative Marketing Strategies

There are only a few examples of (male) urban farmers who escaped from their dependency on market women. Danso and Drechsel, (2003) reported the case of a small group of about three to five farmers within the La area in Accra who farm around a wastewater treatment pond. In 2003 two senior farmers managed the group: one supervised crop production, while the other tried to market the vegetables. The junior members of the group were a mix of laborers or apprentices in charge of vegetable bed preparation, cultivation, watering of crops, spraying of pesticides and harvesting of produce. The ‘marketing manager’, who is still today (2014) at the site, was

responsible for input supply and marketing of the produce and all the necessary farming information concerning production techniques and marketing strategies. He had a long history in trading of nonagricultural commodities from Nigeria to Ghana, but had never worked as a farmer before he started cultivation in the La area. At peak production, each of the junior farmers was supposed to have up to 100 beds under cultivation. According to the marketing manager, their cropping pattern depends entirely on the demand (price) for a particular product at a particular time. He studied the market in order to know when to produce what to meet demand peaks and used the following commodity chart at the time of interview (Drechsel and Keraita, 2014).

As many vegetables are grown in short rotation (e.g. lettuce can be cultivated nine to 11 times a year) flexibility is crucial, and the main crop is accompanied by others. Marketing of the vegetables was carried-out in two ways: a greater portion of the produce will be sent to certain vegetable markets in Accra while the remainder is sold on the farm. During high demand periods, the marketing manager purchases from other producers at different sites in order to improve his profits but also to provide sufficient produce to meet demand. In this way he is partially taking over the function of a wholesaler. The leaders paid themselves at the time of the interview a monthly wage of USD57 each and USD29 each to the other five members leaving a quarterly net profit of USD286. This amount was used to buy the necessary input for the next crop. In addition, the farmers managed to have a special budget, which was used only when there was loss in production or a member of the group had a problem (family, health, funeral, etc.) (Danso and Drechsel, 2003).

2.2.6 Quantifying Lettuce Flows

According to Henseler et al., (2005), 95% of the lettuce consumed in Kumasi come from urban farms. In Accra 35% came from urban sources within Accra, whereas a reasonable amount

(approximately 20% of total lettuce on the market) also came from peri-urban farms. Rural farms obviously contribute very little to lettuce supply in the two cities, probably due to its easily perishable nature. As the lettuce brought from other cities has its origin mostly in urban farms, it is assumed that this source ('irrigated urban agriculture') accounts for about 95% (in Kumasi) of the total lettuce sold in the cities' markets (Fig. 2.16).

The importance of the different sources of lettuce certainly varies during different seasons. During the major dry season in Accra (November to April), more lettuce is brought from other cities. The shares of the different external sources (i.e. Kumasi and Lomé) again depend on the climatic conditions in those areas.

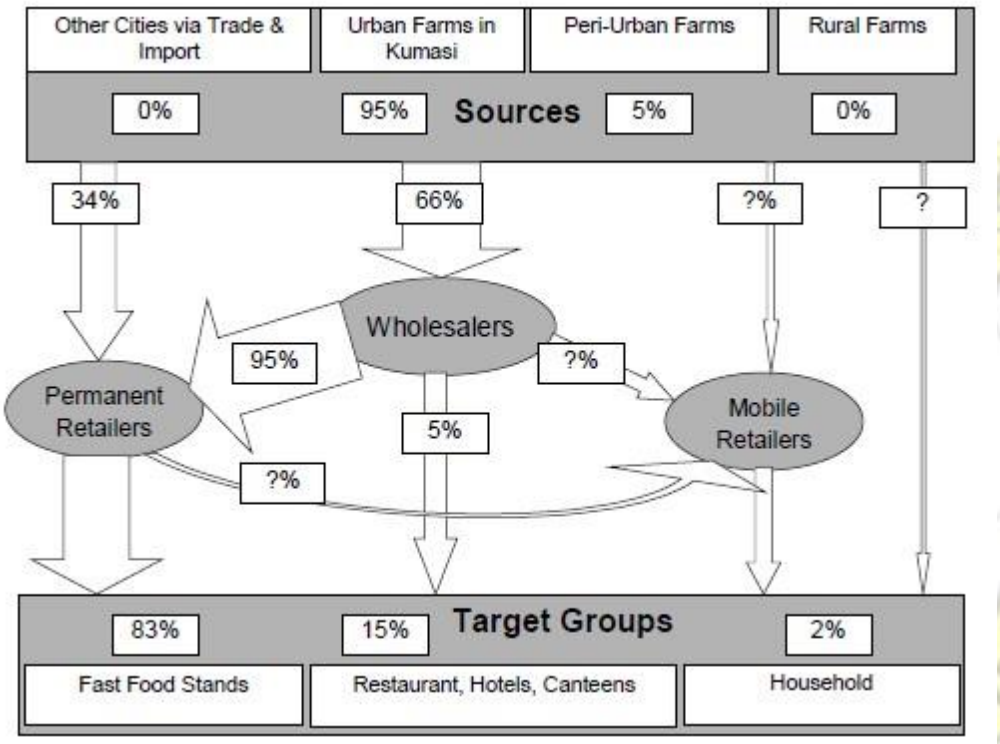


Fig. 2.16 Flow chart of lettuce distribution in Kumasi
Source: (Henseler et al., 2005)

The major sources of lettuce in Accra are urban farms in Accra and other cities. Trade and Import means in most cases lettuce harvested at farms from urban Kumasi. In a few cases Aburi and Koforidua in Ghana, and Lomé in Togo were mentioned as sources. A large fraction of the lettuce coming from these cities is organized by a small group of seven to 10 female wholesalers. They bring their produce (five to 10 sacks each) in public buses (USD1.1 fee per sack in 2005) or lorries to Accra's Agbogbloshie market and sell it there for USD16 to 39 (depending on the season) to other wholesalers and retailers. The Agbogbloshie market therefore plays a crucial role in lettuce distribution in Accra. One of such sacks weighs on average 50 kg (wet weight). Sacks used for sales within Kumasi are smaller (average of 30 kg lettuce per sack). These sacks are sold in Kumasi for USD3 to 13, depending on the season. Some wholesalers in Kumasi bring their produce to local bus stations where they sell them to other wholesalers who transport the lettuce in lorries or buses to Accra, Takoradi/Tarkwa, Cape Coast and other cities in Ghana (Fig. 2.17). Lettuce from Togo is transported with lorries that also carry other vegetables, including carrots and spring onions. allowed to dominate the market and hardly any lettuce is brought to Agbogbloshie from other cities. Based on these numbers, it was estimated that in 2005 a total of over 300 t of lettuce per year is brought to Agbogbloshie market from these sources. There are probably other smaller markets in Accra for lettuce wholesale.

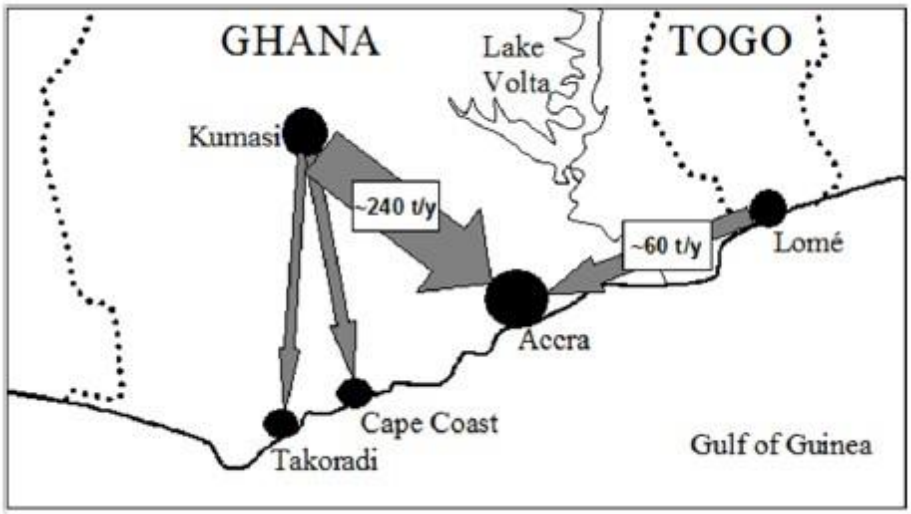


Fig. 2.17 Trade of lettuce in Ghana

Source: (Henseler et al., 2005)

2.2.7 Lettuce Distribution Pathways

Henseler et al., (2005), investigated the major distribution pathway of lettuce in Ghana (Fig. 2.17) from farm gate to wholesalers, from wholesalers to permanent retailers and from retailers to the final target groups. For Kumasi, three larger and 18 neighborhood markets, about 20 wholesalers and 160 permanent retailers have been estimated (Henseler et al., 2005). In Kumasi, hardly any independent vegetable sellers be found (Henseler et al., 2005). The total amount of lettuce handled and purchased in Kumasi in 2005 was calculated on the basis of cultivation area and information on productivity that about 1,000 t of lettuce are produced per year (Henseler et al., 2005). Henseler et al., (2005), investigation revealed that large quantities of lettuce produced on Kumasi's urban farms are transported to Accra and other cities without ever going to Kumasi markets. Further, it agrees with the values calculated by (Leitzinger, 2000), who estimated total lettuce consumption of 615 t year-1 in Kumasi. His survey was based on interviews at the household level. It can be confidently expressed that nearly all lettuce actually consumed in Kumasi is also produced there.

CHAPTER THREE 3 ANTIBIOTICS OCCURRENCE IN WASTE STABILIZATION PONDS AND HOSPITAL WASTEWATER IN KUMASI, GHANA

3.1 SUMMARY

Occurrence of 12 antibiotics in wastewater from hospitals and waste stabilization ponds in Kumasi were investigated. Hospital wastewater and effluents from WSP in Kumasi are directly discharged into streams in the city. They are eventually used as low quality water for vegetable irrigation. Antibiotics in the water samples were extracted using SPE and then analyzed on HPLC-MS/MS. The mean concentrations of the 12 antibiotics in two hospital wastewaters and 3 waste stabilization ponds ranged from 2.0 to 15,733.3 and from <LOD to 7,743.5 ng/L, respectively. Antibiotic removal efficiency of the 3 WSP studied ranged from 89% to 96%. Thus, the WSP are efficient systems in removing the residual antibiotics entering the streams. The total load discharged through the WSP effluents and hospital wastewater was in the range of 9.5 – 3066 mg/day. This translates to 4.2 g of 12 antibiotics being discharged into surface waters in Kumasi every day. Risk quotient (RQ) calculated for ciprofloxacin and amoxicillin antibiotics to sewage-bacteria, were 3.9 x 10^{-1} and 1.6 x 10^{-1} respectively. All antibiotics would cause minimal risk except for ciprofloxacin which would cause medium risk in algae. Due to the lack of municipal wastewater treatment plants in Kumasi, the viability of on-site treatment systems should be encouraged and implemented.

3.2 OBJECTIVES

This work aims at determining occurrence of antibiotics in wastewater, as a potential source on introducing antibiotics in to the aquatic environment in Kumasi, Ghana.

The specific objectives are:

i. To determine the concentrations of 12 antibiotics in wastewater from hospitals (KATH and University hospital (USTH)) and waste stabilization ponds (Ahensan WSP, Chirapatre
WSP and Asafo WSP).

ii. To calculate the antibiotics removal efficiency for the 3 waste stabilization ponds iii. To estimate the risk quotient of antibiotics to aquatic organisms.

3.3 MATERIAL AND METHODS

3.3.1 Selection of antibiotics

A total of 12 antibiotics, all on the Ghana Essential Medicines List and the 2011 National Health Insurance Drug List (Ministry of Health, 2010; NHIA, 2011) were studied. These were ciprofloxacin (quinolone), erythromycin (macrolide), trimethoprim and sulfamethoxazole (sulphonamides), amoxicillin, ampicillin and cefuroxime (β-lactams), metroimidazole (nitroimidazole) as well as doxcycline, tetracycline, chlorotetracycline and othrotetracycline (tetracyclines).

Selection of the antibiotics was based on four factors: 1) frequency of prescribed usage for human use in Ghana (Ministry of Health, 2004), 2) known or suspected environmental and species impact (Ash et al., 1999), 3) persistence in aqueous environments or previous detections in wastewater and surface waters (Kolpin et al., 2002), and 4) inclusion in previous studies of antibiotics in urine samples from outpatients in Ghana (Lerbech et al., 2014).

The relevant physicochemical properties of the studied antibiotics are presented in Table 2.1.

Amoxicillin (CAS #: 267-87-780, 98% pure) was obtained from Duchefa (Haarlem, Holland). Ampicillin (CAS #: 69-53-4, 97% pure), metronidazole (CAS #: 443-48-1, 98% pure), cefuroxime

(CAS #: 55268-75-2, 97% pure), ciprofloxacin (CAS #: 85721-33-1, 96% pure), erythromycin (CAS #: 114-07-8, 97% pure), trimethoprim (CAS #: 738-70-5, 98% pure), and sulfamethoxazole (CAS #: 723-46-6, 97% pure) were purchased from Fluka (Brøndby, Denmark). Tetracycline hydrochloride (CAS #: 60-54-8, >96% pure), oxytetracyline (CAS #: 79-57-2, 97% pure) and chlorotetracycline (CAS #: 64-72-2, 97% pure) were purchased from Sigma- Aldrich (Steinheim, Germany), and doxycycline hydrochloride (CAS #: 564-25-0, 98% pure) was obtained from Takeda Pharma (Roskilde, Denmark). The internal standard (IS) d_4-sulfamethoxazole was purchased from Toronto Research Chemicals (Toronto, Canada), and the ISs d_3-trimethoprim and d_8-ciprofloxacin from Qmx Laboratories (Thaxted, UK). Methanol (HPLC grade) was purchased from Lab-Scan (Gliwice, Poland) and formic acid (98-100% pure, Ph Eur) was purchased from Merck KGaA (Darmstadt, Germany). Pure water was produced in - house with a Milli-Q water gradient system (Millipore, Bedford, MA, USA).

Stock solutions were prepared with methanol and stored in freezer at -18 °C. Internal standard solution mixture with a concentration of 2.5 mg/mL for each IS and standard antibiotic-mix with a concentration of 5 mg/L of all 12 analytes were prepared from the stock solutions. These were prepared by mixing a known amount of each stock solution with methanol in a 5 mL volumetric flask. The standard antibiotic-mix and internal standard solution -mix were kept in brown bottles to protect them from light and stored at -18˚C.

3.3.2 Study area

Kumasi was divided into three sampling zones. These are Ahensan sampling zones, Chirapatre sampling area and Asafo sampling area (Fig. 3.1).

The Komfo Anokye Teaching hospital (KATH) sampling site is the only teaching hospital in

Kumasi and the second largest teaching hospital in Ghana with 1200 beds. University hospital (USTH) is a referral hospital for the University community and patronized by the surrounding submetro population. These were selected based on their potential to have high levels of antibiotic residues, in wastewater effluent.

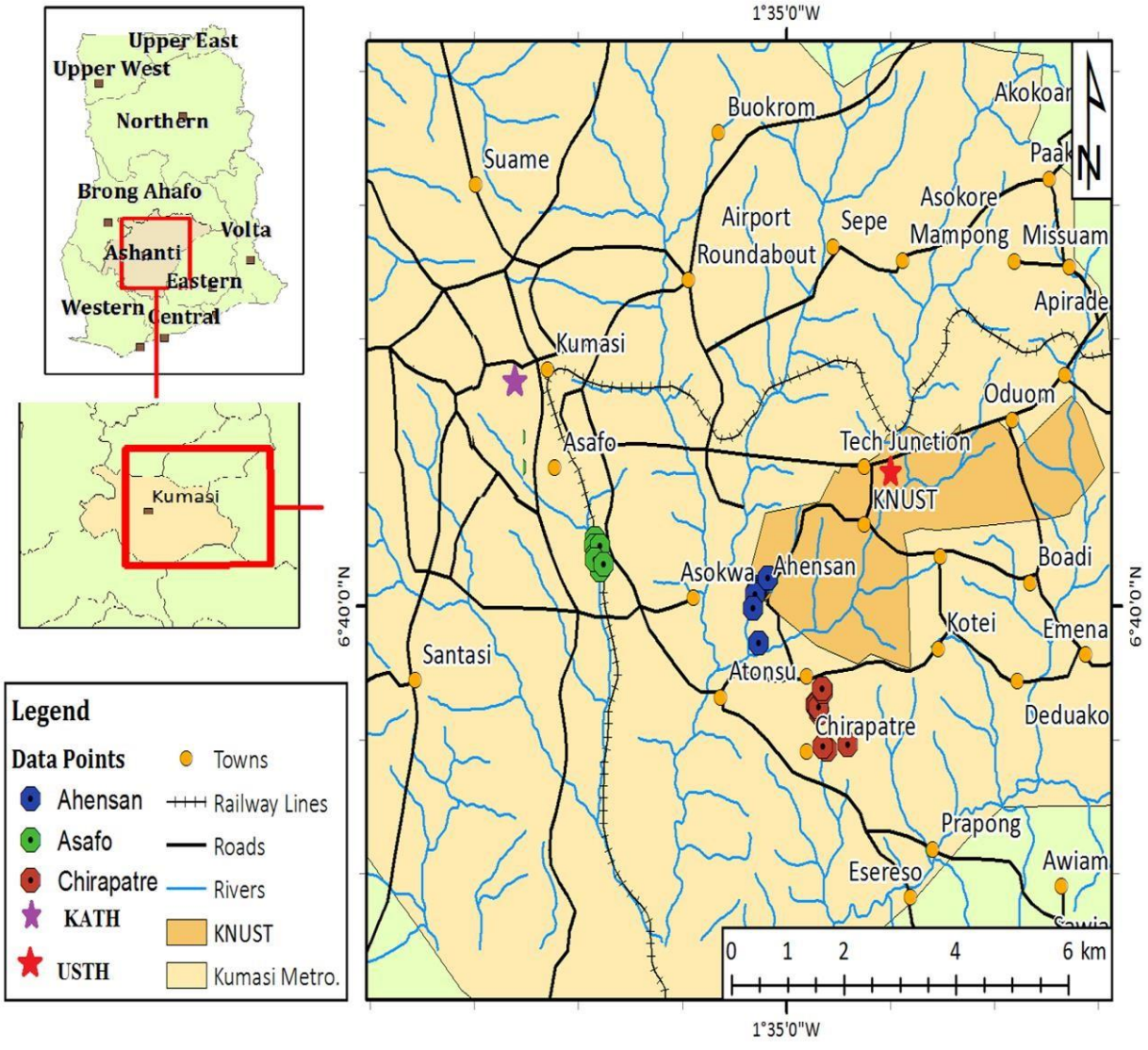


Fig. 3.1: Map of Ghana showing Kumasi and the study areas

3.3.2.2 Sampling

Nineteen sampling points were identified in the sampling areas. Sampling was conducted in three sampling period; 12-14th March 2014 (first), 2- 4th April 2014 (second) and 23 - 25th April 2014 (third). At each sampling point, two replicates composite water samples were collected. A total volume of 1 L (pooling 200 mL aliquots for 5 times) was collected from the same site within 30 minutes interval into 1.5 L brown HDPE bottles.

In all, 120 water samples were collected and transported to the laboratory where they were filtrated twice. The first filtration was through a grade 5 filter paper (Munktell Filter AB, Falun, Sweden) with particle retention of 20 µm. The second filtration was through a grade 120H filter paper (Munktell Filter AB, Falun, Sweden) with particle retention of 1-2 µm. After filtration, the pH was measured using universal pH indicator strips and was adjusted to 7 ± 0.3 with 2 M H_2SO_4 (SigmaAldrich) or 2 M NaOH (Merck). The filtered samples were divided to 2 x 100 mL into brown HDPE bottles and spiked with 100 µL aliquot of 2.5 mg/L internal standard mix (IS mix). The IS mix contained ciprofloxacin-d_8 (d-Cip), sulfamethoxazole-d_4 (d-Sul) and trimethoprim-d_3 (dTrim).

3.3.3 Solid phase extraction

The water samples were extracted on a reversed phase solid-phase extraction (SPE) using Oasis HLB cartridges (hydrophilic-lipophilic balance, 200 mg sorbent, 30 µm, 6 cm^3) purchased from Waters Oasis (Massachusetts, USA). The SPE cartridges were conditioned with 2 mL of MeOH followed by 2 mL of 0.01 M citrate buffer and lastly with 2 mL of distilled water. A 100 mL portion of water samples were loaded onto SPE columns at a flow rate of 1.5 mL/min. The dried SPE columns were then bulked and kept in a refrigerator at -4 °C before shipping to Denmark where they were stored at -18°C until use.

In Denmark, the dried SPE columns were washed with 3 mL of 5% MeOH in water. The sorbents were allowed to dry for a couple of minutes under vacuum before the antibiotics were eluted with 3 mL MeOH acidified with 0.1% formic acid at a flow of approximately 1 mL min^{-1}. Eluates were evaporated to dryness under a gentle flow of nitrogen at 30 °C and then reconstituted in 1 mL 1% MeOH into brown flat-cap HPLC-vials for analysis.

3.3.4 LC-MS Analysis

The liquid chromatographic (LC) system consisted of an Agilent 1290 Infinity Binary System (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a degasser, a cooled autosampler (4 °C), and a column oven (30 °C). The chromatographic separations were achieved by use of a reversed-phase column (Kinetex® Biphenyl 100 Å column, 2.1 mm x 50 mm x 2.6 µm), coupled to a guard column (Ultra cartridges BP, 2.1 mm x 2.6 µm), both from Phenomenex ApS, (Milford, MA, USA) and application of binary gradient flow rate of 400 µL/min at 20 °C. The injection volume was set at 10 µL. The mobile phase A consisted of water acidified with 0.1% formic acid whereas mobile phase B consisted of MeOH acidified with 0.1% formic acid. The initial proportion between the mobile phases was 99% A and 1% B. This gradient was held for 1 minute followed by a 5 min linear gradient ending with 50% A and 50% B. This proportion was soon changed in a 1 min linear gradient from 6 to 7 min to 1% A and 99% B. This gradient was maintained for 2 min followed by a 1 min linear gradient ending with the initial conditions. This gradient was then maintained for 2 min resulting in a total analysis time of 12 min.

Mass spectrometry was performed with an AB SCIEX QTRAP® 4500 System with CEM detector (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source (Turbo Ionspray). For MS detection, electrospray ionization was performed in positive

mode (ESI+) for metronidazole, ciprofloxacin, erythromycin, trimethoprim, tetracycline, oxytetracycline, chlorotetracycline, doxycycline, amoxicillin, ciprofloxacin-d_8 and trimethoprimd_3. The negative mode (ESI-) was performed for ampicillin, cefuroxime, sulfamethoxazole and sulfamethoxazole-d_4. The temperature was set to 300 ˚C with a nebulizer gas flow of 8 L/min, curtain gas flow of 12 L/min and collision gas flow of 6 L/min. The ionspray voltage was set to 5000 V. The MS system was set to operate in the multiple reaction monitoring (MRM) mode with the parameters listed in Table 3.1. Collection and treatment of data were performed using Analyst v. 1.4.2 software (Applied Biosystems) and a Savitzky-Golay smoothing factor at 3 on a Windows XP platform-based data-processing system.

Table 3.1: MS instrument parameters

Compound	Precursor ion (Q1) (m/z)	Product ion (Q3) quantifier/qualifier (m/z)	Retention Time	DP(volts)	EP (Volts)	CE (Vollts)	CXP(Volts)
Metronidazole (MET)	172.1	128/82	4.13	60/60	11/11	19/31	10/6
Ciprofloxacin (CIP)	332.5	313.7/288.2	6.71	75/75	10/10	30/25	36/5
Erthromycin (ERY)	735	158/83	7.31	10/10	4/4	35/80	11/5
Trimethoprim (TRIM)	291.1	230.3/261.2	5.58	85/85	10/10	35/35	11/11
Tetracycline (TC)	445	410/427.2	6.33	25/25	10/10	30/20	11/16
Oxytetracycline (OTC)	461.2	426.2/443	6.03	60/60	10/10	30/25	11/16
Cyclotetracycline (CTC)	479.1	444.1/461	7.14	25/25	10/10	30/30	16/21
Doxycycline (DC)	444.9	428.1/154.1	7.27	20/20	10/10	20/40	21/11
Amoxicillin (AMX)	398.2	159.9/348.8	5.15	45/45	10/10	27/22	12/14
Ampicillin (AMP)	348	206.7/74	5.65	-63/-63	-9/-9	-16/-35	-8/-5
Cefuroxime (CEF)	423.4	206.7/317.7	6.6	-12/-12	-4/-4	-17/-11	-9/-12
Sulfamethoxazole (SUL)	252.3	63.8/91.7	6.53	-60/-60	-7/-10	-52/-34	-4/-6
d4- Sulfamethoxazole	256	159.6/63.8	6.51	-64/-64	-2/-2	-20/-56	-5/-4
d8- Ciprofloxacin	340.1	322/296	6.63	15/15	14/14	30/25	25/20
d3-Trimethoprim	294.1	123/230.3	5.54	75/75	10/10	35/35	11/11

3.3.5 Validation of analytical Procedure

The method was validated in compliance with the requirements in standard guidelines (European Medical Agency 2012; ICH Harmonised Tripartite Guideline 2005; U.S. Food and Drug Adminstration 2001). The linear calibration curves were constructed by analyzing standard solutions ranged from 0.001 to 1000 ng/mL followed by calculating the ratios of analyte peak area to that of the internal standards. Precision was determined by injecting a 1 ng/mL standard antibiotics-mix solution 8 times. The limit of detection (LOD) and limit of quantification (LOQ) of the HPLC-MS/MS system was determined from the standard deviation (σ) of the response from the lowest calibration standard (0.001 ng/mL) injected 6 times and by the slope (S) of the calibration curve. Matrix effect (recovery) was studied with water from Søstein Lake (55.701316N, 12.565211E), Copenhagen. A 4 L water sample was collected into 5 L brown HDPE bottles and transported to the laboratory. Samples were then filtered and pH adjusted as described above. The filtered water was divided to 12 x 100 mL into brown bottles. The first four bottles containing the water were pre-spiked (prior to SPE) with 20 µL of 5 mg/L antibiotics mixture and post-spiked with 100 µL of 2.5 mg/L IS - mixture before LC determination. The subsequent four samples were post-spiked (after SPE) with 20 µL of 5 mg/L antibiotics mixture and 100 µL of 2.5 mg/L IS -mixture. The last four samples were pre-spiked with 100 µL of 2.5 mg/L IS -mixture and post-spiked with 20 µL of 5 mg/L antibiotics mixture before the LC determination.

The concentration of antibiotics in the samples were determined on calibration curves constructed for each individual analyte. Quality control (QC) samples were run in parallel during the quantification process. Positive controls consisting of matrix spiked (fortified) with antibiotic-mix and IS were used whereas negative controls consisting of matrix and internal standards were used to exclude possible procedural contaminations.

3.3.6 Statistical analysis

The results obtained were subjected to statistical evaluation. Mean, standard deviation and coefficient of variation were evaluated with Microsoft Office Excel 2013 (Version 15, Microsoft, USA). Pearson's correlation coefficient was performed as a measure of dependence between antibiotics studied. The closer the coefficient is to either -1 (decreasing linear relationship) or 1 (direct linear relationship), the stronger the correlation between the variables. In general, interpretation of correlation analysis was done using correlation coefficients values higher than 0.5.

Discriminant analysis was performed for evidence on sampling period variation using SPSS version 22. The classical Wilks' Lambda statistics was used as a significance test for the equality of the group means (Nath and Pavur, 1985). This is to test whether or not there is a statistically significant relationship between the independent variables (antibiotics concentration) and the dependent variables (sampling periods). The key statistic indicating whether or not there is a relationship between the independent and dependent variables is the significance test for Wilks' lambda. Wilks' lambda is the proportion of the total variance in the discriminant scores not explained by differences among the groups. Smaller values of Wilks' lambda are desirable (Nath and Pavur, 1985). One-way ANOVA was performed to test the differences in various sources of water, with a Tukey's Honesty Significant Differences (HSD) as a post test. The p-value was considered statistically significant at $p<0.05$. The one-way ANOVA was performed by using GraphPad Prism version 5.01 for Windows (GraphPad Software Inc., USA). Principal components analysis (PCA), a multivariate statistical technique capable of discerning patterns in large environmental datasets and where complex inter-relationships between variables are difficult to identify and visualize (Shaw, 2003) was performed. In simple terms PCA is a data reduction

technique whereby new variables (principal components or factors) are calculated from linear combinations of the original variables. The first principal component, or factor, accounts for the greatest variability in the data, and there can be an infinite number of new factors with each accounting for less data variability than the previous (Webster, 2001). Detailed descriptions of PCA and its applications can be found in a number of texts (Field, 2000; Shaw, 2003) Principal component analysis (PCA) based on antibiotics concentrations in samples was done, to determine the distribution pattern of antibiotics in sampling area and water sources, using JMP statistical software v. 10 (SAS Institute). The principal components were extracted with eigenvalues >1.

3.3.7 Environmental Risk Assessment

The potential risk posed by each antibiotics was assessed by calculating its risk quotient (RQ) as the ratio between its maximum measured environmental concentration (MEC) and its predicted no-effect concentration (PNEC), as suggested by EMEA, (2006). To cover all food chain in the water, RQ was calculated at three different trophic levels of the ecosystem, algae, daphnids and fishes. PNEC values assumed for this risk analysis correspond to the lowest ecotoxicological PNEC values found in the literature. For 2 out of the 12 investigated compounds, ecotoxicological data were available in literature. The toxicity data for the antibiotics which could not be found in literature were calculated from the ecological structure activity relationships (ECOSAR) model (US-EPA, 2012). The aquatic organisms from three different trophic levels, i.e. green algae, daphnid and fish were chosen in the ECOSAR model. PNEC was calculated by dividing the lowest short-term L(E)C50 or long-term NOEC (no-observed-effect-concentration) value available in the literature, by an assessment factor (AF). The AF is an arbitrary factor to consider the inherent uncertainty in the obtained laboratory toxicity data. In this study, an assessment factor of 100 was

used. Table 3.2 provides all the toxicity data with the corresponding assayed species, endpoint and references. A commonly used risk ranking criterion; RQ < 0.1, minimal risk to aquatic organisms, 0.1 ≤ RQ ≥ 1, median risk; RQ ≥1, high risk was applied (Hernando et al., 2006).

Table 3.2: Toxicity data collected from literature and ECOSAR

Antibiotics	EC50 sewage sludge bacteria (µg/L)	Reference	EC50 algae (µg/L)	Reference	EC50 daphnid (µg/L)	Reference	EC50 fishes (µg/L)	Reference
MET	100000	Halling-Sørensen et al, 2001	2.93E+06	ECOSAR	3.20E+06	ECOSAR	8.85E+06	ECOSAR
CIP	610	Halling-Sørensen et al, 2000	6.10E+02	Halling-Sørensen et al, 2000	9.91E+05	Sanderson et al., 2003	2.46E+08	Sanderson et al., 2003
ERY			1.21E+05	ECOSAR	1.30E+05	ECOSAR	2.24E+05	ECOSAR
TRIM	17800	Halling-Sørensen et al, 2000	1.10E+04	Ando et al., 2007	5.48E+04	Park and Choi, 2008	1.00E+05	Kim et al., 2007
TC	2200	Halling-Sørensen et al, 2001	9.12E+07	ECOSAR	1.00E+08	ECOSAR	3.56E+08	ECOSAR
OTC	1200	Halling-Sørensen et al, 2001	1.68E+09	ECOSAR	1.87E+09	ECOSAR	8.89E+09	ECOSAR
CTC	400	Halling-Sørensen et al, 2001	6.46E+09	ECOSAR	7.20E+09	ECOSAR	3.93E+10	ECOSAR
DC			9.76E+07	ECOSAR	1.07E+08	ECOSAR	3.84E+08	ECOSAR
AMX	3.7	Halling-Sørensen et al, 2000	1.01E+06	ECOSAR	1.09E+06	ECOSAR	2.51E+06	ECOSAR
AMP			3.92E+05	ECOSAR	4.24E+05	ECOSAR	8.88E+05	ECOSAR
CEF			4.21E+06	ECOSAR	4.59E+06	ECOSAR	1.20E+07	ECOSAR
SUL			1.75E+06	ECOSAR	1.90E+06	ECOSAR	4.78E+09	ECOSAR

3.4 RESULT

3.4.1 Validation of analytical Procedure

The regression coefficient (r^2) expressed as percentage for all the 12 antibiotics ranged from 98.7 to 99.9%. The precision expressed as coefficient of variation ranged from 3 to 6%. The LOD determined for all antibiotics (Table 3.3) using the slope and the standard deviation ranged from 0.80 to 2.75 ng/L, while the LOQ ranged from 2.42 to 8.33 ng/L (Table 3.3).

Table 3.3: Result for validation of water analytical procedure

Analyte	Linearity r^2 (%)	LOD (ng/L)	LOQ (ng/L)	Accuracy (%CV)	Precision (%CV)	Matrix Effect	
						AR ± RSD (%)	RR ±RSD (%)
MET	98.7	3	8	3	3	71.4±22.0	83.1±12.2
CIP	99.9	1	3	4	3	73.1±21.2	97.4±11.5
ERY	99.9	2	5	4	3	62.6±14.6	82.4±24.1
TRIM	99.9	2	5	3	4	101.0±9.0	91±17.9
TC	99.5	1	3	5	4	64.7±23.8	84.3±14.5
OTC	99.9	1	3	3	3	66.8±20.9	91.4±23.1
CTC	99.9	2	6	4	3	72.6±12.0	89.2±22.4
DC	99.8	2	5	5	6	78.7±15.8	88.5±11.5
AMX	99.9	1	2	4	4	67.3±10.1	97.5±20.6
AMP	99.9	3	8	4	3	68.0±11.6	89.7±23.5
CEF	99.9	2	6	3	4	87.6±20.2	98.7±24.2
SUL	99.9	2	7	4	5	98.9±26.4	105.2±16.4

The matrix absolute recovery (%) ranged from 62.6 to 101.0 (Table 3.3) and the relative recovery ranged from 82.4 to 105.2 (Table 3.3). They were all above the minimum acceptable target value of 60% (Venn, 2008).

3.4.2 Occurrence of antibiotics in WSPs

Sulfamethoxazole recorded the highest concentration (7743.5 ng/L) in all the WSP samples followed by ciprofloxacin (Table 3.4). Amoxicillin concentrations in all the samples analysed were generally low ranging from <LOD to 8.5 ng/L.

Table 3.4: Antibiotics concentrations (ng/L) in hospital and waste stabilization ponds investigated.

Antibiotics	KATH and USTH, Kumasi	3 WSPs, Kumasi	3 WSPs Effluent, Kumasi
MET	246.8 – 419.8	3.0[a]-26.9	3.0[a]-19
CIP	11352.0 – 15733.3	27.0-2875.1	27-262
ERY	7943.9 – 10613.4	37.9-1982.3	47-882
TRIM	93.5 – 4826.3	30.6-1854.4	31-255
TC	57.7 – 116.3	11.1-242.5	11-24
OTC	75.1 – 252.3	2.4[a]-278.9	2.4[a]-24
CTC	16.3- 23.9	5.6-98.1	6.0-19
DC	23.6 – 120.0	14.4-196.9	14-49
AMX	2.0 - 6.0	<LOD -8.5	<LOD-1.3[a]
AMP	106.8 – 324.0	50.5-572.5	51-97
CEF	1052.4 – 1556.7	58.0-1327.9	58-345
SUL	2314.9-3590.3	103.0-7743.5	103- 320

Tetracyclines found in the WSP samples were up to few hundreds of ng/L. The maximum concentration of tetracyclines was 278.9 ng/L (OTC). The concentrations in all WSPs samples (Table 3.4) following increasing order as: AMX > MET > CTC >DC > TC > OTC > AMP>CEF>TRIM> ERY > CIP > SUL

3.4.3 Correlation analysis

Correlation analysis of the concentration of antibiotics in the WSPs showed moderate to strong correlations. With exception of metronidazole, the 11 antibiotics correlated positively with each other and was statistically significant for most of them (Table 3.5). The correlation between ERY and CIP, and OTC and AMX showed positive correlation but was not statistically significant ($p>0.005$). The correlation of AMX with ERY and AMX with AMP was weak and not statistically significant ($p>0.005$).

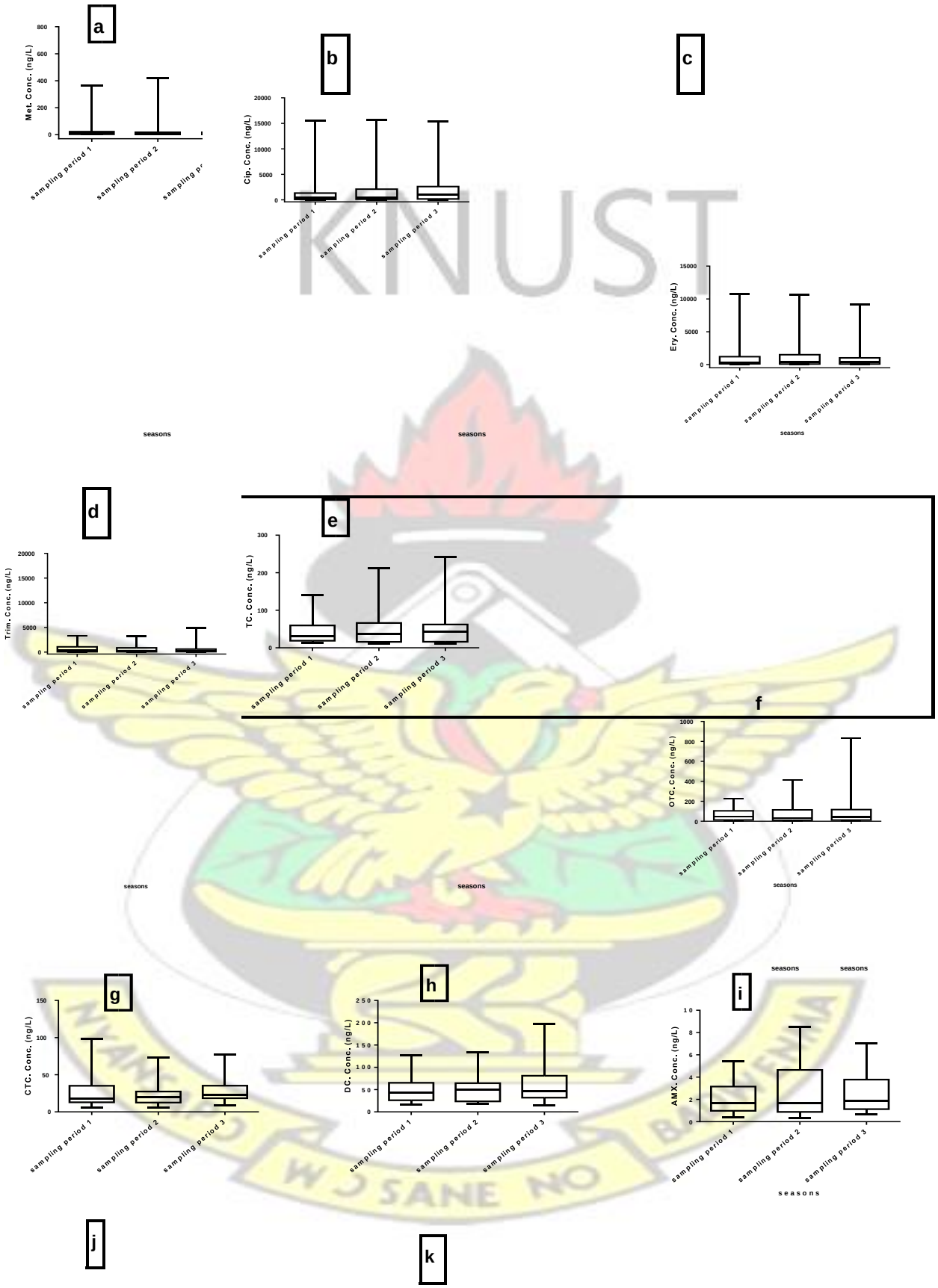
Table 3.5: Correlation matrix for the antibiotics concentrations in waste stabilization ponds wastewater

	MET	CIP	ERY	TRIM	TC	OTC	CTC	DC	AMX	AMP	CEF	SUL
MET												
CIP	-0.03											
ERY	-0.58	0.65										
TRIM	-0.31	0.83*	0.91*									
TC	-0.09	0.92*	0.68*	0.83*								
OTC	-0.35	0.85*	0.89*	0.92*	0.92*							
CTC	0.05	0.93*	0.66*	0.87*	0.94*	0.87*						
DC	0.10	0.87*	0.63*	0.82*	0.95*	0.85*	0.97*					
AMX	0.34	0.82*	0.43	0.68*	0.80*	0.64	0.89*	0.90*				
AMP	0.27	0.93*	0.47	0.71*	0.86*	0.73*	0.92*	0.89*	0.92*			
CEF	-0.12	0.87*	0.84*	0.95*	0.86*	0.90*	0.92*	0.90*	0.81*	0.85*		
SUL	-0.03	0.75*	0.60*	0.76*	0.93*	0.85*	0.89*	0.92*	0.72*	0.73*	0.78*	

* $p<0.005$

3.4.4 Sampling period variation

The mean concentrations of antibiotics measured in various sampling period were quite similar (Fig. 3.2). Wilks' lambda was 0.76, and the p value was 0.63. The chi-square statistics corresponding to Wilks' lambda was 20.56. The results indicate that the concentrations at various sampling period are not significantly different.


a
Met. Conc. (ng/L)
800
600
400
200
0
sampling period 1
sampling period 2
b
Cip. Conc. (ng/L)
20000
15000
10000
5000
0
sampling period 1
sampling period 2
sampling period 3
c
Ery. Conc. (ng/L)
15000
10000
5000
0
sampling period 1
sampling period 2
sampling period 3
seasons
seasons
seasons
d
Trim. Conc. (ng/L)
20000
15000
10000
5000
0
sampling period 1
sampling period 2
sampling period 3
e
TC. Conc. (ng/L)
300
200
100
0
sampling period 1
sampling period 2
sampling period 3
f
OTC. Conc. (ng/L)
1000
800
600
400
200
0
sampling period 1
sampling period 2
sampling period 3
seasons
seasons
seasons
g
CTC. Conc. (ng/L)
150
100
50
0
sampling period 1
sampling period 2
sampling period 3
h
DC. Conc. (ng/L)
250
200
150
100
50
0
sampling period 1
sampling period 2
sampling period 3
seasons
seasons
i
AMX. Conc. (ng/L)
10
8
6
4
2
0
sampling period 1
sampling period 2
sampling period 3
seasons
j
k

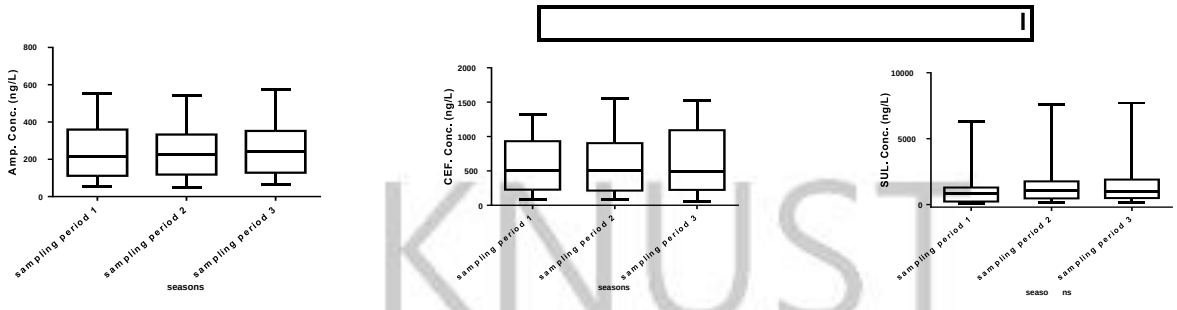


Fig. 3.2: Box-and-whisker plots of antibiotics organized by sampling period
(a) MET, (b) CIP, (c) ERY, (d) TRIM, (e) TC, (f) CTC, (g) OTC, (h) DC, (i) AMX, (j) AMP, (k) CEF and (l) SUL

3.4.5 Load and removal of antibiotics in influent and effluent of WSPs

Concentrations of the antibiotics in WSPs influent and effluent were multiplied by the flow rates, to obtain loads expressed in mg/day. The total load (sum of the loads of the 12 antibiotics substances) for the influent and effluents of the 3 WSPs was in the range of 93.1 to 1353.7 mg/day and 9.5 to 71.0 mg/day respectively (Fig. 3.3). The removal efficiency, however, of the 3 WSP studied ranged from 89% to 96 %. The removal efficiency was highest for Asafo WSP with percentage being 96%. Total loads of Asafo WSP was 1700.9 for the influent, this is about 100 times higher followed by Chirapatre (160.4) and Ahensan (93.1).

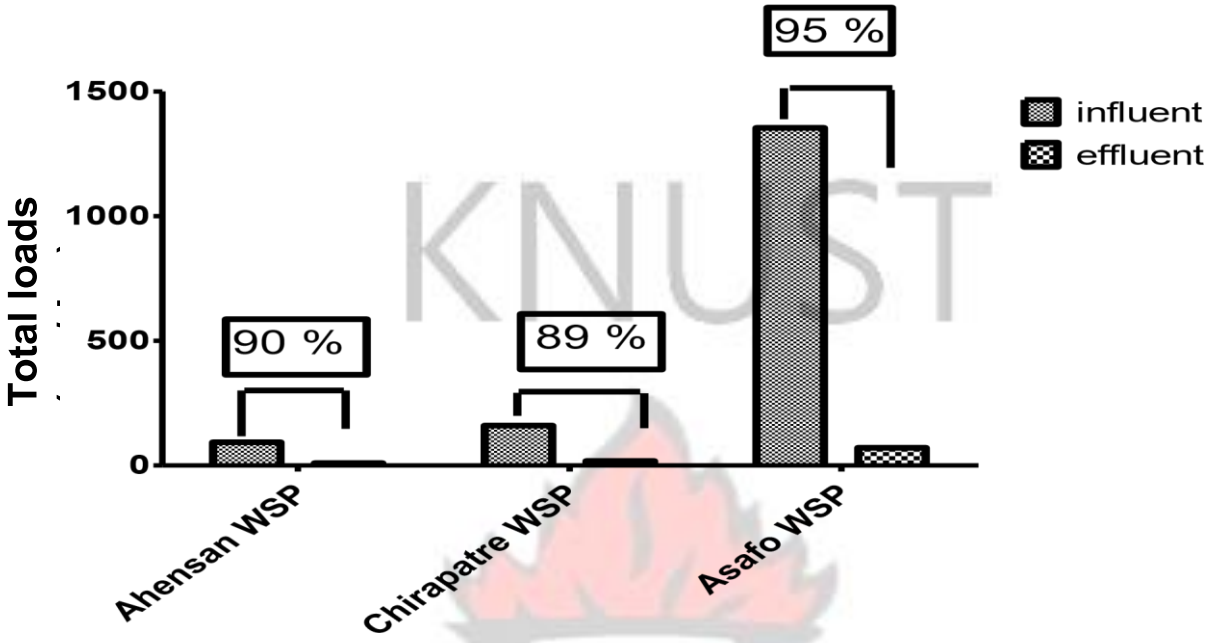


Fig. 3.3. Removal rates in WSPs obtained from total loads of antibiotics in influent and effluent. For comparison, loads for each of the three WSP were normalized for the population equivalent of the plants and expressed as mg/day/inhabitant. Daily mass load per capita, in µg/day/person, of individual antibiotics in the current study were obtained by multiplying the concentrations in sewage and the average daily flow rate during the sampling period and normalizing this value to the catchment population. The total load discharged through the WSP effluents and hospital wastewater was in the range of 9.5 – 3066 mg/day (Fig. 3.4).

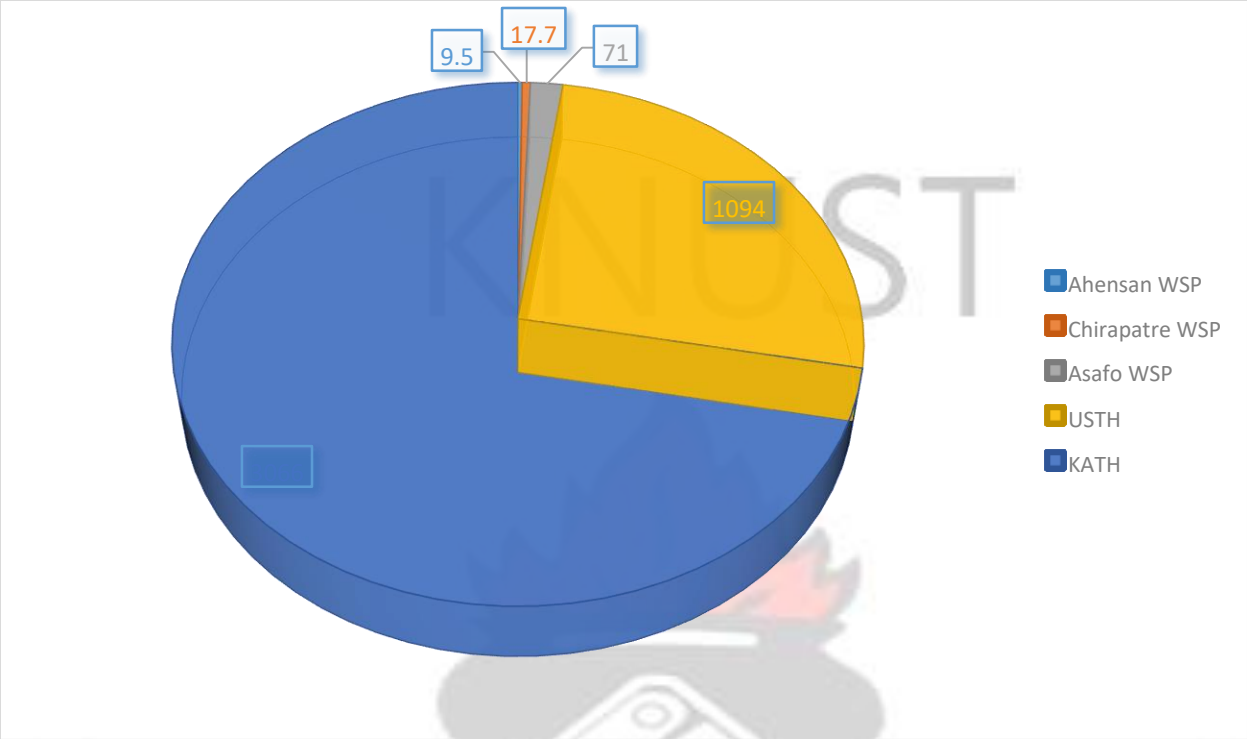


Fig. 3.4. Total amount of antibiotics discharged into the environment through the WSPs effluent and hospitals wastewater studied

3.4.6 Removal of antibiotics in WSPs Based on various processes occurring in WSP.

The removal efficiency of various processes occurring in the 3 WSP was determined. The mean percent removal (37.3%) for facultative ponds gave the highest removal of total load followed by anaerobic pond (32.2%) and lastly the maturation pond (16.0%) (Fig. 3.5). For the case of Asafo WSPs, percentage removal load was higher in anaerobic pond (57.2 %) than facultative pond (30.0%). In all, the highest removal of antibiotic load occurred in anaerobic pond (66.6 %). In general removal in anaerobic pond was higher followed by facultative pond and maturation pond. However, the differences are not statistically significant ($p = 0.4$, $F = 0.94$).

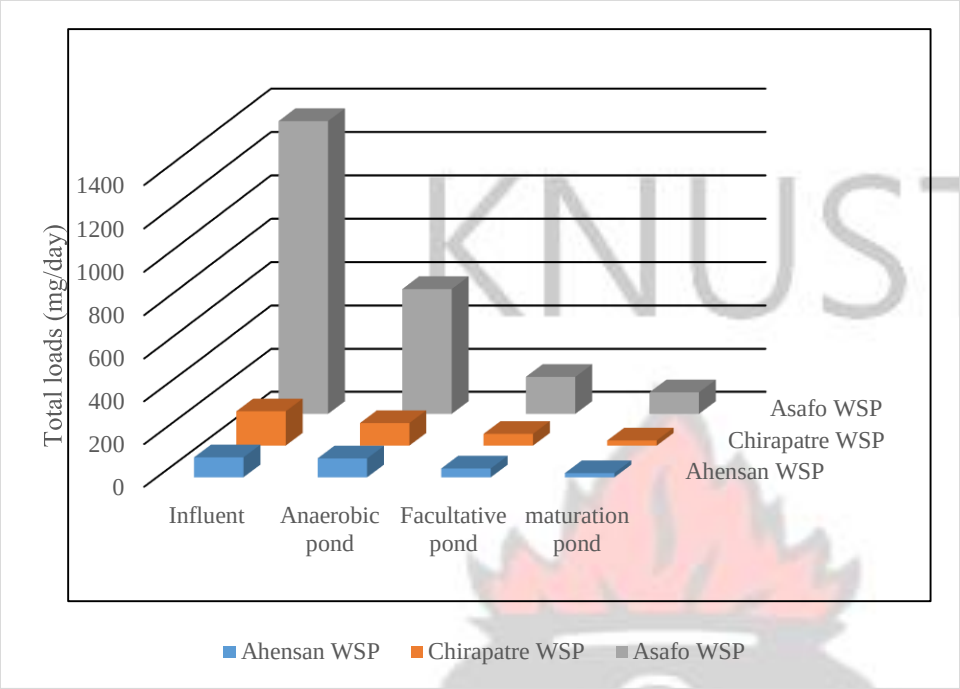


Fig. 3.5. Overall removal efficiencies of antibiotics by different waste stabilization levels

3.4.7 Antibiotics in Hospitals

The concentrations in all hospital samples (Table 3.4) ranged from 246.8 to 419.8 ng/L for MET; 11352.0 to 15733.3 ng/L (CIP), 7943.9 to 10613.4 ng/L (ERY), 93.5 to 4826.3 ng/L (TRIM), 57.7 to 116.3 ng/L (TC), 75.1 to 252.3 ng/L (OTC), 16.3 to 23.9 ng/L (CTC), 23.6 to 120.0 ng/L (DC), 2.0 to 6.0 ng/L (AMX), 106.8 to 324.0 ng/L (AMP), 1052.4 to 1556.7 ng/L (CEF) and 2314.9 to 3590.3 ng/L (SUL). The hospitals wastewater studied revealed higher concentrations of antibiotics in Komfo Anokye Teaching Hospital compared to University (KNUST) Hospital (Fig. 3.6).

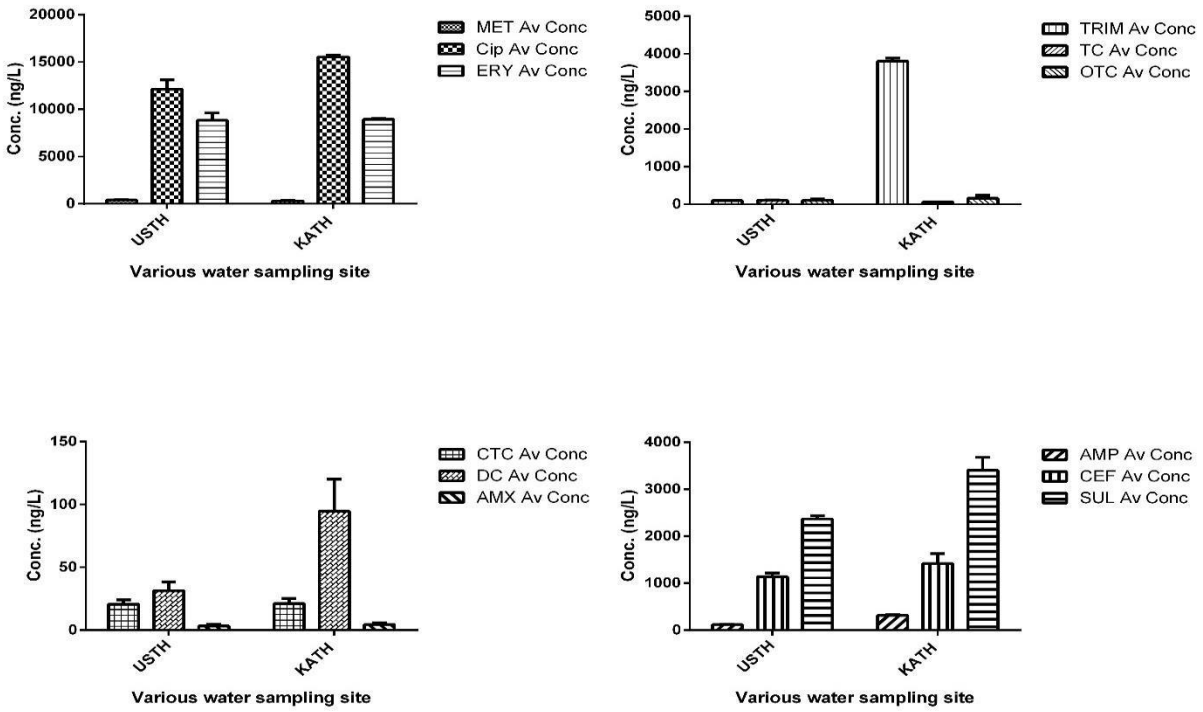


Fig. 3.6. Bar chart of antibiotics in wastewater
University Hospital (USTH) and Komfo Anokye Teaching Hospital (KATH)

3.4.8 Principal Component analysis

The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.815, above the recommended value of 0.6, and Bartlett's test of sphericity was significant ($\square^2$ (245) = 845.1, $p < 0.05$). Bartlett's test of sphericity, tests the null hypothesis, this indicate that the correlation matrix is an identity matrix. Considering these tests results, the data passed the minimum standard which should be passed before a principal components analysis could be conducted.

Distribution of antibiotics in water sources and sampling area were analyzed using PCA. PCA results showed a significant separation between Ahensan effluent water (H), grouped in the bottom left

corner, Chirapatre effluent water (G), clustered on the top left quadrant, and finally Asafo effluent water (D), separated into right top and bottom quadrant (Fig. 3.7).

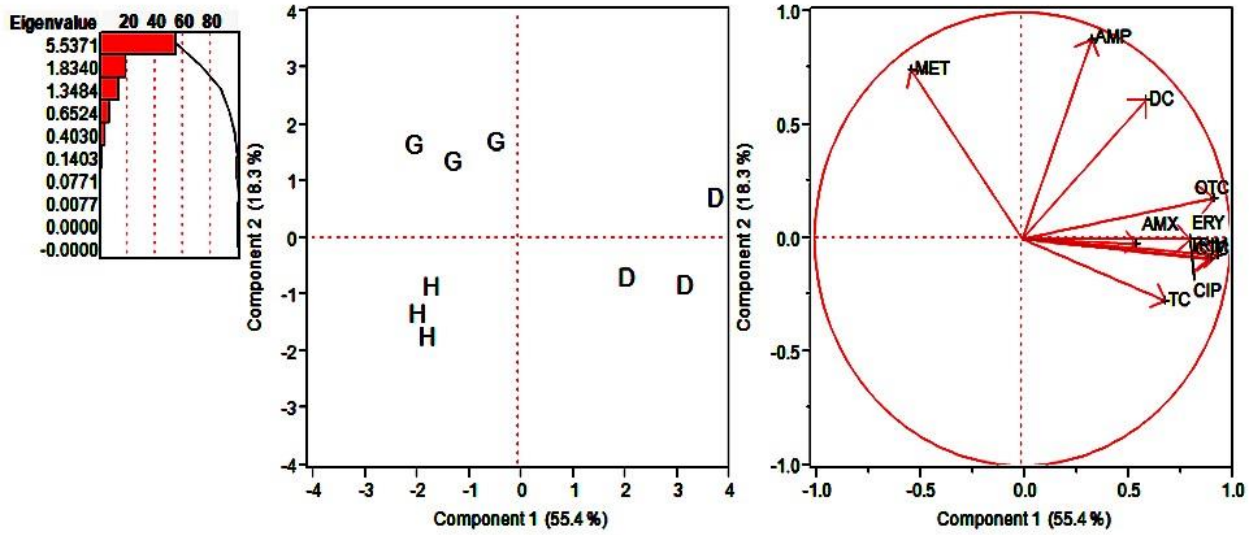


Fig. 3.7. Distribution pattern of antibiotics in WSP effluent characterised by PCA (D: Asafo WSP effluent, G: Chirapatre WSP effluent, H: Ahensan WSP effluent)

Interestingly, Chirapatre effluent water correlated with metronidazole and Asafo effluent samples strongly correlated with erythromycin, tetracycline, sulfamethoxazole, ciprofloxacin and trimethoprim (Fig. 3.7). Ahensan effluent water, however did not have any antibiotics correlating with it (Fig. 3.7).

The distribution pattern of antibiotics in WSP and hospital wastewater showed hospital wastewater separated to the top right corner quadrant while WSP wastewater are distributed within the rest of the quadrant. Hospital wastewater were correlated with metronidazole, erythromycin, ciprofloxacin and cefuroxime (Fig. 3.8).

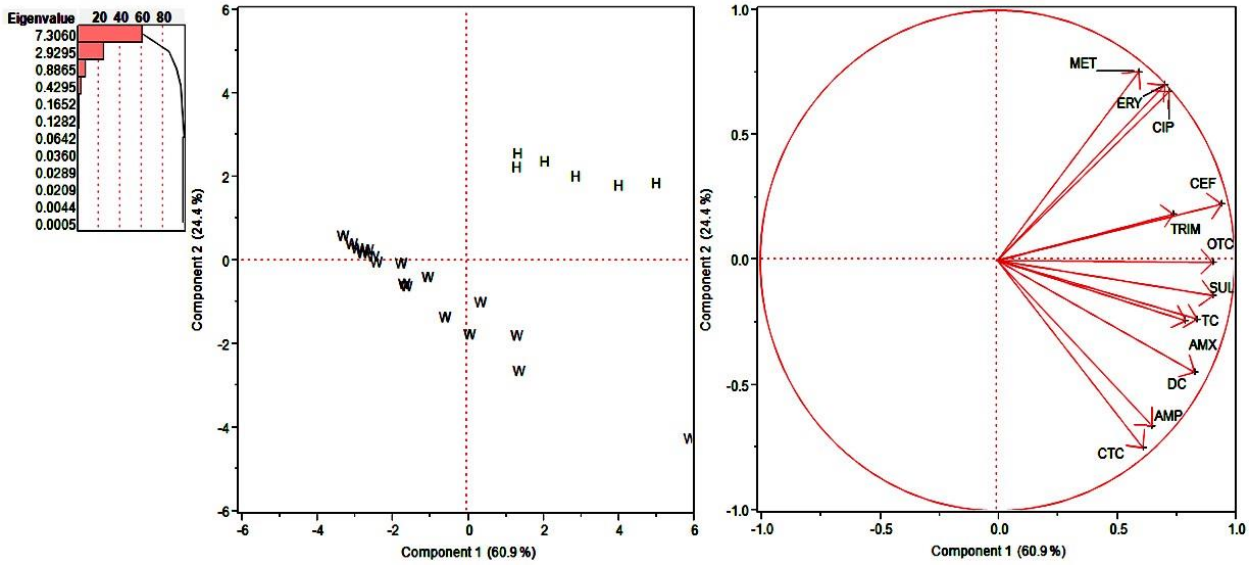


Fig. 3.8: Distribution pattern of antibiotics in wastewater characterised by PCA (W: WSPs wastewater and H: Hospital wastewater)

3.4.9 Environmental risk assessment

PNEC values and risk quotients deemed for each analyte are shown in Table 3.6. The risk quotients for sewage-bacteria ranged from 2.4 x 10^{-5} to 3.9 x 10^{-1}, for algae for all antibiotics from 1.4 x 10^{-8} to 3.9 x 10^{-1}, for daphnid from 1.2 x 10^{-9} to 1.5 x 10^{-3} and for fishes from 2.1 x 10^{-10} to 1.7 x 10^{-3}.

Table 3.6: Maximum environmental concentrations (MEC) of antibiotics in wastewater from waste stabilization ponds. PNEC and RQ for fish, daphnids (all species belonging to their trophic level) and algae (or bacteria) for the studied antibiotics.

Antibiotics	MEC (µg/L)	PNEC sewage sludge bacteria (µg/L)	RQ sewage bacteria	PNEC algae(µg/L)	RQ algae	PNEC daphnid (µg/L)	RQ daphnid	PNEC fishes (µg/L)	RQ fishes
MET	0.024	1.0E+03	2.4E-05	2.9E+04	8.0E-07	3.2E+04	7.4E-07	8.8E+04	2.7E-07
CIP	2.371	6.1E+00	3.9E-01	6.1E+00	3.9E-01	9.9E+03	2.4E-04	2.5E+06	9.6E-07
ERY	1.931	n.a	n.a	1.2E+03	1.6E-03	1.3E+03	1.5E-03	2.2E+03	8.6E-04
TRIM	1.668	1.8E+02	9.4E-03	1.1E+02	1.5E-02	5.5E+02	3.0E-03	1.0E+03	1.7E-03
TC	0.199	2.2E+01	9.0E-03	9.1E+05	2.2E-07	1.0E+06	2.0E-07	3.6E+06	5.6E-08
OTC	0.233	1.2E+01	1.9E-02	1.7E+07	1.4E-08	1.9E+07	1.2E-08	8.9E+07	2.6E-09
CTC	0.083	4.0E+00	2.1E-02	6.5E+07	1.3E-09	7.2E+07	1.2E-09	3.9E+08	2.1E-10
DC	0.153	n.a	n.a	9.8E+05	1.6E-07	1.1E+06	1.4E-07	3.8E+06	4.0E-08
AMX	0.006	3.7E-02	1.6E-01	1.0E+04	5.9E-07	1.1E+04	5.4E-07	2.5E+04	2.4E-07
AMP	0.556	n.a	n.a	3.9E+03	1.4E-04	4.2E+03	1.3E-04	8.9E+03	6.3E-05
CEF	1.277	n.a	n.a	4.2E+04	3.0E-05	4.6E+04	2.8E-05	1.2E+05	1.1E-05
SUL	7.194	n.a	n.a	1.7E+04	4.1E-04	1.9E+04	3.8E-04	4.8E+04	1.5E-04

n.a, indicate no toxicity data was available in literature

3.5 DISCUSSION

3.5.1 Occurrence of antibiotics in point sources

3.5.1.1 Antibiotics in Hospitals

The concentrations in the two hospital effluent samples are presented in Table 3.4. Ciprofloxacin was the most abundant of the 12 antibiotics studied in hospital wastewater from Kumasi, Ghana. Ciprofloxacin concentrations were between 11.4 µg/L and 15 µg/L. The CIP levels were comparable to those measured in studies in European hospitals such as in Switzerland 0.3–29 µg /L (Alder et al., 2004), in Sweden 3.6–101 µg/L (Lindberg et al., 2004) and in Germany 0.7–125 µg/L (Hartmann et al., 1999; Ohlsen et al., 2003). In a study performed in the USA, CIP occurred in hospital effluents at concentrations up to 2l µg/L (Brown et al., 2006). The average CIP concentration measured in Huu Nghi hospital raw wastewater was 25.8 ± 8.1l µg/L. It was at least two times higher than those found in this study (13.8 ± 2.0 µg/L). The lower CIP concentrations can be explained by the different consumption pattern in various hospital. The antibiotics concentration in the hospitals wastewater depend on the specialization of a particular hospital, the number of patients and on the prescription practice of the doctors. However, it is difficult to quantify the consumption of antibiotics in the hospitals because they can be provided by the hospital or can be bought in pharmacies outside the hospital and consumed by the patients in the hospital. Under this constraint, a comparison of mass fluxes and the number of patients in the different hospitals can still be attempted.

Previous study showed that quinolone antibiotics like ciprofloxacin, have been shown to exert genotoxic effects for the genetically modified bacterial strain, Salmonella typhimurium, at concentrations as low as 25.0 µg/L (Hartmann et al., 1998). Therefore, the concentration of CIP in

this study may not cause any genotoxic effect, but due to the pseudo-persistent and bio magnification nature of antibiotics, there might be some effect in the future.

3.5.1.2 Antibiotics in WSPs

The WSPs influent was collector for most of the residential facilities and some institutions and also contained high concentrations of the 12 antibiotics detected in this study. Metronidazole concentrations in all WSPs samples (Table 3.4) were up to 26.9 ng/L. K'oreje et al. (2012), analysed samples from Dandora sewage plant in Kenya and detected metronidazole up to 700 ng/L. Additionally, metronidazole found in effluent samples in Europe ranged from 55-561 ng/L (Verlicchi et al., 2012). The maximum concentration found in this study is below the maximum concentration recorded by these reported studies. The low concentration of metronidazole found in current study could be due to consumption rate of drug and amount of wastewater input into waste treatment ponds in various countries. Accordingly, Halling-Sorensen determined that no observed effect concentration (NOEC) of metronidazole on sewage sludge bacteria to be 100 mg/L. The predicted no - effect concentration (PNEC) for metronidazole was determined to be 2500 ng/L (Kümmerer and Henninger, 2003).

Ciprofloxacin, a fluoroquinolones class of antibiotics, measured in this study ranged from 26.5 to 2,875.1 ng/L in WSPs samples. Ciprofloxacin, found in WSPs samples worldwide ranged from 75,700 ng/L (Verlicchi et al., 2012), indicating that the maximum concentration found in this study is lower than the maximum reported worldwide. As an important group of antibiotics, fluoroquinolones are widely used to treat a great deal of human and animal diseases. Ciprofloxacin have been proven to have genotoxic effects on sewage sludge bacteria at EC50 of 0.61 mg/L (Bent Halling-Sørensen et al., 2000). Moreover, a PNEC of total fluoroquinolones for organisms in STPs

is estimated to be 8,000 ng/L (Golet et al., 2002). Sanderson et al. (2003) also estimated PNEC for ciprofloxacin to be 938,000 ng/L. In the present study, the maximum concentrations of ciprofloxacin in all WSPs wastewater (2,875.1 ng/L) was lower than the value of PNEC reported in literature. Therefore, it is unlikely for ciprofloxacin to have adverse effects on microorganisms involved in sewage treatment processes.

Erythromycin, a macrolide, was detected in all of the WSP effluents examined ranging from 37.9 to 1,982.3 ng/L. Concentrations of erythromycin reported in literature are; up to 6,000 ng/L in Germany waste treatment ponds (Hirsch et al., 1999); and up to 287 ng/L in Switzerland by Giger et al., (2003). The maximum concentration of erythromycin found in this study was higher than the maximum of concentration found in STP in Germany, but lower than 6000 ng/L found by Hirsch et al., (1999) in STP effluent in Germany.

The β-lactam class of antibiotics are the most frequently prescribed class of antimicrobials in Ghana, particularly amoxicillin, followed by ampicillin and cefuroxime (Tagoe and Attah, 2010). In this study, amoxicillin was detected in low concentrations in all of the 3 WSPs examined were up to 8.5 ng/L. Amoxicillin is chemically unstable due to the β-lactam ring which readily undergo hydrolysis (Gáspár et al., 2002). This could account for the low concentration found in the samples. Amoxicillin was not detected in STP sample in Germany (Hirsch et al., 1999). However, Andreozzi et al., (2004), found amoxicillin concentrations ranging from <1.8 ng/L to 120 ng/L in Italian STPs. The lower concentrations detected in STPs effluents in Ghana as compared with those in Italy probably reflect differences in prescription patterns for macrolide antibiotics in the two countries. Unfortunately, information on prescription rates of antibiotics in Ghana are not available, so it is not possible to confirm this hypothesis.

The PNEC concentration has been estimated for amoxicillin to be 3.7 ng/L (Kümmerer and Henninger, 2003). The maximum concentration found in this study was higher than the PNEC, which indicate that there could be an adverse effect of bacteria in the sewage treatment plant. There is a need for further investigation to assess their effect. The second most used β- lactam antibiotics, ampicillin and cefuroxime were measured in WSPs ranging from 50.5 to 572.5 ng/L and 58.0 to 1,327.9 ng/L respectively. These antibiotics have not been well reported in literature.

Among the sulphonamides, sulfamethoxazole and trimethoprim are the two most abundant antibiotics in the samples analysed, with concentrations ranging from 103.0 to 7,743.5 ng/L and 30.6 to 1,854.4 ng/L respectively. K'oreje et al. (2012), analysed samples from Dandora sewage plant in Kenya and detected sulphamethoxazole up to 6,000 ng/L and trimethoprim up to 1,000 ng/L.

The highest concentration of sulfamethoxazole detected in this study was lower than the 6,000 ng/L reported in Dandora STP in Kenya (K'oreje et al., 2012). Trimethoprim in this study, however was higher than what was reported in Kenya and 180 ng/L reported in STP in New Mexico (Brown et al., 2006). Although sulfamethoxazole/trimethoprim is administered as one drug (cotrimoxazole) at a mass ratio of 1:5, the measured aqueous concentration of sulfamethoxazole exceeds that of trimethoprim by a factor of 3. This may be explained by their different fates both in the human body and in the environment (Madureira et al., 2010). Although the fraction that is excreted in unchanged form by humans is lower for sulfamethoxazole (15%) than for trimethoprim (60%), the latter is more readily removed in STPs (Madureira et al., 2010) and via photolytic degradation up to 99% (Abellán et al., 2009).

The tetracyclines (TCs) are broad-spectrum antibacterial including tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycline (DC) are widely used as

veterinary and human antibiotics. The tetracyclines detected in all the WSPs wastewater samples, had concentrations ranging from 11.1 to 242.5 ng/L for TC, 2.4 to 278.9 ng/L for OTC, 5.6 to 98.1 ng/L for CTC, and 14.4 to 196.9 ng/L for DC respectively. The concentrations of TC detected in this study were much lower than 1,200 ng/L detected in several wastewater treatment facilities in Wisconsin, USA. The estimated EC50 toxicity for sewage sludge bacteria are 0.4 mg/L, for CTC; 1.2 for OTC and 2.2 for TC. In the present study, the highest concentrations of the TCs are far below the EC50 values hence may not cause any adverse effect.

Applying the risk ranking scale (Hernando et al., 2006), on RQ of antibiotics calculated for sewage-bacteria, ciprofloxacin and amoxicillin antibiotics had RQ of 3.9×10^{-1} and 1.6×10^{-1} respectively which would cause medium risk. For algae, except ciprofloxacin which could cause median risk, the rest of the antibiotics could cause minimal risk. Comparatively a study focussed on the occurrence of pharmaceuticals in water from Pego-Oliva Marsh, Spain, found ciprofloxacin RQ to be 6.9 for algae (Vazquez-Roig et al., 2012). Previously, other studies have reported high RQs in surface water due to the presence of pharmaceuticals in high concentrations, such as analgesics, psychiatric drugs and antibiotics: ibuprofen, naproxen, ketoprofen and carbamazepine (Hernando et al., 2006), diclofenac (Hernando et al., 2006; Zhao et al., 2010), mefenamic acid (Jones et al., 2002; Tauxe-Wuersch et al., 2005), sulfamethoxazole (García-Galán et al., 2011), paracetamol, amoxicillin and oxytetracycline (Jones et al., 2002).

These data all indicate that the potential toxicological effects posed by antibiotics for the aquatic environment could be reduced when the hydraulic characteristics of the receiving water bodies are taken into consideration in their management (Gros et al., 2010).

This risk evaluation has its limitations, such as lack of long-term toxicological studies and the nonfeasibility to carry out chronic studies during the lifespan of the organisms (especially in fishes),

but on the other hand since mixture of compounds with the same pharmacological mechanism present in waters, synergistic effects could be expected (Gros et al., 2010), making the real hazard greater than that calculated. Nevertheless, the presence of antibiotics in the environment is not limited only to ecological problem but most importantly to antibiotics resistance. Antibiotics resistance due to bacteria exposure to sub-MIC could occur in the study area, as Gullberg et al., (2011), found minimum selective concentration (MSC) for antibiotic resistant mutant with ciprofloxacin to be 100 pg/mL and 15 ng/mL for tetracycline. The fact that antibiotic levels of several hundred-fold below the MIC of the susceptible strains can select resistant bacteria means that the sub-MIC selective window is much larger than the traditional selective window.

3.5.2 Removal of antibiotics in WSPs

3.5.2.1 Load and removal of antibiotics in influent and effluent of WSPs

The WSP effluents still contained antibiotics that were therefore discharged in to the receiving water. The removal efficiency, however, of the 3 WSP studied ranged from 89% to 96 %. The removal efficiency was highest for Asafo WSP with percentage of 96 %. Asafo WSP is well managed with frequent desludging and maintenance, and this could be reason why it showed high removal efficiency. Obviously, Chirapatre showed less removal efficiency because there is little maintenance of the WSP. Total loads of Asafo WSP was 1700.9 for the influent, which is about 100 times higher followed by Chirapatre (160.4) and Ahensan (93.1). The difference agrees perfectly with the population connected to the various ponds.

For comparison, loads for each of the three WSP were normalized for the population equivalent of the plants and expressed as expressed in mg/day/inhabitant. Daily mass load per capita, in µg/day/person, of individual antibiotics in the current study were obtained by multiplying the

concentrations in sewage and the average daily flow rate during the sampling period and normalizing this value to the catchment population. Effluent loads have been previously used for estimating the total loadings of local water bodies (Minh et al., 2009).

Generally, Asafo WSPs influent contained higher (100 fold) influent loads than other residential WSPs. These elevated normalized mass flows reflected the presence of certain antibiotic sources, probably higher proportions of influent volume originating from hospitals in the catchment regions. However, detailed information on effluent outputs from the corresponding public hospitals is not available.

Mass loads of CEF in this study, ranged from 5.5–18.8 μg/day/person in influents and 0.5–1.1 μg/day/person in effluents and were up to fifty times lower than those detected in Brisbane, Australia (influent: 920 μg/day/person), where CEF is one of the most prescribed antibiotics (Watkinson et al., 2007). AMX levels (0.07±0.04 μg/day/person) in all influent samples were also far lower than those reported for the mean of influent STPs in six towns in Italy (13 μg/day/ person) and Brisbane (38 μg/day/person) by a factor of at least 190 (Castiglioni et al., 2006; Watkinson et al., 2007). Due to the unstable β-lactam ring, amoxicillin antibiotic is less persistent in the aquatic environment (Cha et al., 2006; Hirsch et al., 1999). In this context, the occurrence as well as "pseudo-persistence" of β-lactam antibiotics could be predominantly due to the extensive and continuous usage of these antibiotics. The average mass loads of ERY-H_2O (4.5±3.3 μg/day/person) in influent samples and (1.4±0.1 μg/day/person) in effluent samples of the 3 WSPs were almost 200 times lower than those measured in China (influent: 753; effluent: 782 μg/day/person) (Xu et al., 2007) but were almost 100 times as lower as those detected in North America (influent: 497; effluent: 164 μg/day/person) (Karthikeyan and Meyer, 2006).

Average loads of SUL (26.5±4 µg/day/person) and TRIM (6.5±1.0 µg/day/person) in influent were lower than those encountered in North America (Karthikeyan and Meyer, 2006) and Europe (Lindberg et al., 2005). The highest loads of SUL in influents samples in this study of 31.1 µg/d/person was lower than 1410 µg/d/person measured in Guangzhou, China (Peng et al., 2006). These results reflect variations in antibiotic prescription habits among different countries. The degree of antibiotic contamination in Kumasi, Ghana can thus be seen to lie far below those of western countries, China and developing cities in the PRD region. Nevertheless, daily antibiotic loads were greatest quite significant and comparing the unregulated use of antibiotics in Ghana, there is a need for more comprehensive monitoring work in receiving waters in the city.

3.5.2.2 Removal of antibiotics in WSPs Based on various processes occurring in WSP

The mean percent removal (37.3%) for facultative ponds was the highest of the total load removal efficiency followed by anaerobic pond (32.2%) and lastly the maturation pond (16.0%). This suggest that processes occurring in the facultative ponds cause most of the attenuation of antibiotics load in WSPs. The low removal of antibiotics in anaerobic ponds, could be due to sedimentation, which is the principal removal mechanism occurring in these ponds. Since anaerobic ponds in most WSPs are design to hold wastewater for 1-1.5 days, settling of antibiotics would be low. The principal mechanisms for removal in facultative and maturation ponds are now known to be: (a) time and temperature; (b) high pH (> 9); and (c) high light intensity, combined with high dissolved oxygen concentration. Time and temperature are the two principal parameters used in designing maturation ponds. Additionally, the sun plays a role in direct antibiotic removal in WSP through photo degradation. There is also the hydroxyl ions accumulation from algal photosynthesis, often

raising the pH to values above 10 and resulting in high dissolved oxygen concentrations, which are necessary for promoting photo-oxidative damage of antibiotics.

There were some exceptions which also brings to bear some parameters that enhance the removal of antibiotics in WSPs. For the case of Asafo WSPs, percentage removal load was higher in anaerobic pond (57.2 %) than facultative pond (30.0%). The reason being that, Asafo WSPs has two anaerobic ponds in parallel which are relatively larger than the other ponds. The synergistic effect of the two ponds makes its removal efficiency higher. Hence pond size and number of ponds in each process could affect the percentage removal of antibiotics. In the case of Chirapatre, the anaerobic pond although small in size holds the waste matter for a long time. The retention time in this pond is relatively higher than what occurs in the rest of the ponds and could account for the slightly higher percentage removal than the facultative pond.

In all, the highest removal of antibiotic load occurred in anaerobic pond (66.6 %). In general removal in anaerobic ponds are higher followed by facultative pond and maturation ponds. However, the differences are not statistically significant ($p = 0.4$, $F = 0.94$).

3.5.3 Loads of antibiotics discharged into environment

The results showed that antibiotics are partially eliminated in WSP. The rest pass through the effluent and may end up in the environment, mainly in the water compartment. Residual amounts can reach surface waters, groundwater or sediments (Kümmerer, 2009). Again wastewater from hospitals are discharged directly into surface water. The total load (sum of the loads of the 12 antibiotics substances) discharged through the WSP effluents and hospital wastewater was in the

range of 9.5 – 3,066 mg/day (Fig. 3.4). Therefore, about 4.2 g of the 12 antibiotics analysed reach surface waters in this way every day in Kumasi.

PCA between 3 WSPs effluents (Fig. 3.7), revealed correlation of metronidazole with Chirapatre effluent water. This could result in possible contamination of nearby stream which is used for vegetable farming. Vegetables exposure to metronidazole at Chirapatre warrant further study. To reduce the concentrations of antibiotics in river water, pharmaceutical waste disposal in developing countries should be monitored.

3.6 Conclusion

This study provided an overview on the occurrence of antibiotics in hospital and waste stabilization ponds for wastewater in Kumasi. As expected, hospitals are important point sources contributing to the release of all the antibiotics studied. The total load discharged through the WSP effluents and hospital wastewater were in the range of 9.5 – 3066 mg/day. Therefore, about 4.2 g of 12 antibiotics analysed reach surface waters every day in Kumasi, Ghana. Considering sewagebacteria, ciprofloxacin and amoxicillin antibiotics with RQ determined to be 3.9×10^{-1} and 1.6×10^{-1} respectively would cause medium risk. For algae, except ciprofloxacin which would cause median risk, the rest of the antibiotics would cause minimal risk. WSPs help reduce antibiotics entering the environment, hence treatment of hospital wastewater before discharging into rivers should be considered as a viable option and consequently implemented. Further work would be required to determine the extent of exposure and the risk associated. Since these point sources of antibiotics in Ghana are discharged into streams and nearby rivers used for farming, there is a need to investigate the possibility of contamination of foods.

CHAPTER FOUR

4 OCCURRENCE AND RISK ASSESSMENT OF ANTIBIOTICS IN LOW QUALITY WATER USED FOR VEGETABLE IRRIGATION

4.1 SUMMARY

Occurrence of 12 antibiotics in low quality water (untreated wastewater, effluents of waste stabilization ponds, rivers and surface water) used for vegetable irrigation was determined in different water bodies in Ghana. Antibiotics in water samples were extracted using SPE and analyzed by HPLC-MS/MS. All studied compounds were detected and their concentrations were significantly higher ($p > 0.05$) in untreated wastewater than in other water sources. Interestingly, the concentrations in irrigation water were not significantly different from that of the river ($p = 0.03$) but there is a statistical difference between irrigation water and effluents ($p = 0.004$). This could indicate similar pollution signature. The antibiotics found with high concentrations in all the samples were sulfamethoxazole, erythromycin, ciprofloxacin, cefuroxime and trimethoprim. Hence they could also be considered as or added to the list of potential critical compounds from an environmental risk point of view in Ghana. Irrigation water samples analysed had concentrations of antibiotics up to 0.2 ppb. When environmental risk assessment based on the available long-term data was performed, the results showed actual risk for the lowest trophic level, due to the presence of ciprofloxacin. However, the presence of antibiotics in the environment is not limited only to ecological problems since contamination also affects drinking water and vegetables being a potential risk to human health. The outcomes of this study suggest there could be indirect exposure of humans to antibiotics through vegetable consumption and drinking water in Ghana.

4.2 OBJECTIVES

The purposes of this work was to:

- study the occurrence and distribution of 12 antibiotics in wastewater, river, effluent of waste stabilization ponds, and low quality water used for vegetable irrigation.
- evaluate whether or not there is a relationship between antibiotic concentrations in different water sources.
- perform risk assessment on the antibiotics found in low quality water.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals

A total of 12 antibiotics were studied. These were ciprofloxacin (quinolone), erythromycin (macrolide), trimethoprim and sulfamethoxazole (sulphonamides), amoxicillin, ampicillin and cefuroxime (β-lactams), metroimidazole (nitroimidazole) as well as doxcycline, tetracycline, chlorotetracycline and othrotetracycline (tetracyclines). Basis of selection are presented in chapter 3 section 3.3.1 and relevant physicochemical properties of the studied antibiotics are presented in Table 2.1.

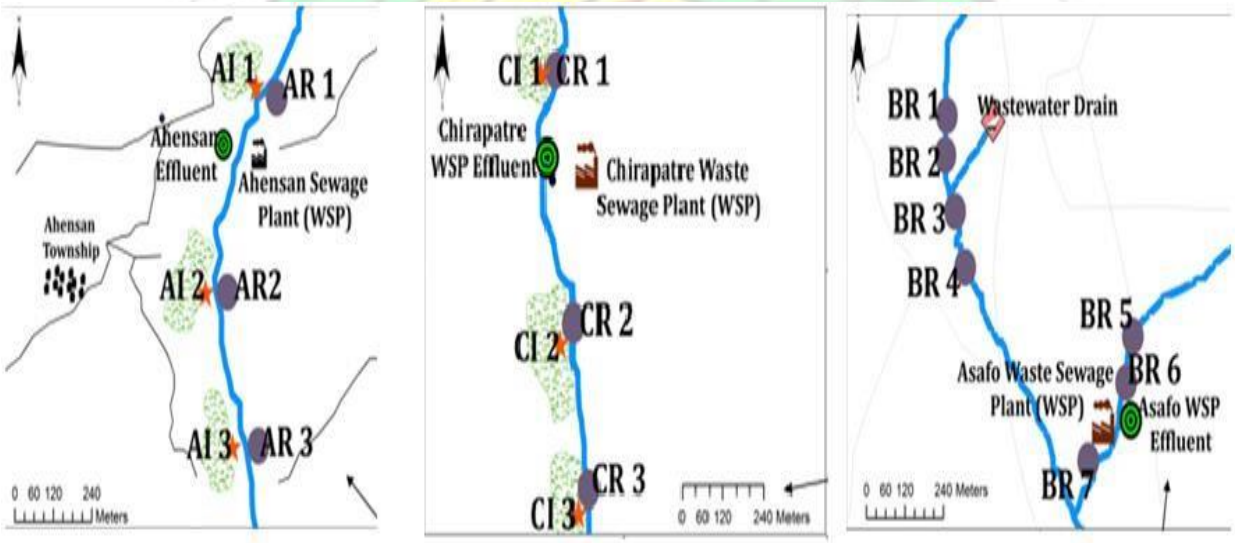
4.3.2 Study Area

For easy comparison of data, the study city, Kumasi, was group into three zones. These are Ahensan sampling zones (Fig. 4.1a), Chirapatre sampling area (Fig. 4.1b) and Asafo sampling area (Fig. 4.1c). Ahensan sampling zone is located in Asokwa sub-metro of Kumasi. The area is drained by Wiwi and Sisa Rivers. There is waste stabilization ponds (WSP) located at Ahensan Estate of

this sub-metro in Kumasi. The effluents from the WSP, is discharged into the Wiwi River through a nearby stream which is used for vegetable irrigation downstream.

Chirapatre sampling zone is in the Asokwa sub-metro and is located on a slope towards river Oda. Chirapatre area has a network of sewer lines connected to the Chirapatre WSP. There are several farms within a distance of 2 km from the WSP, utilizing the stream water in which the effluent from the waste stablisation is discharged for vegetable irrigation.

The Asafo sampling zone is in Bantama sub-metro. Effluent from Asafo WSPs joins the Subin River, which runs through the commercial centre of Kumasi and merges with the River Oda downstream at Asago (6° 8' 450" N 18° 360" W), which is the site of a rural farming community. At Asafo sampling zone, a broken wastewater drain believed to be the sewer line linking Komfo Anokye Teaching Hospital (KATH) and the 4BN soldier Barracks was selected as untreated wastewater contributing a point source pollution.



a b c

Fig. 4.1: Map of sampling area
(a) Ahensan sampling area, AIs are Ahensan irrigation water and ARs are Ahensan river samples (b) Chirapatre sampling area; CIs are Chirapatre irrigation water, CRs are Chirapatre river samples and (c) Asafo sampling area; BRs are Asafo river samples

4.3.3 Sampling

At Ahensan sampling area, effluent from Ahensan WSP was sampled. The irrigation water used by three vegetable farms in Ahensan sampling zone was also sampled. The first farm is about 100 m upstream from where the Ahensan WSP effluent enters the Wiwi River. The second farm is 50 m after the effluent of Ahensan WSP enters the Wiwi River. The third farm is about 1 km downstream. At each farm, the main surface water used by farmers was sampled. The river that passes along the farm which is either diverted to the farm for irrigation or fetched directly for watering vegetable was also sampled.

At Chirapatre sampling zone, the effluent from the WSP was sampled. The irrigation water used at three vegetable farms at Chirapatre being Karikari farms (about 200 m upstream to where the Chirapatre WSP effluent enters the Oti stream), Chirapatre farms (1 km downstream from the entry of the effluent into the Oti stream) and Ramseyer farms (about 1.5 km downstream) was sampled

At Asafo sampling zone, the effluent from Asafo WSP was sampled. The river that receives the effluent, which also runs through the city center, was sampled at; upstream (200 m before the entry of the effluent), midstream (50 m after the entry of effluent) and downstream (300 m from the entry point of the effluent). A tributary of Subin River passing behind 4BN soldiers barrack receives untreated wastewater from Komfo Anokye Teaching Hospital sewage drain which is broken before joining the Subin River. The untreated wastewater which flows through the lowlands before joining the river was sampled 20 m before joining the river. The Subin River was sampled 1 km upstream

from point of entry of the untreated wastewater, 50 m before the entry of the untreated wastewater, 20 m after the entry of untreated wastewater and 1km downstream from the entry of the untreated wastewater.

In total, 23 sampling points (Fig. 4.1a-c) were sampled in the three sampling zones. Sampling was conducted by sampling one sampling zone on each date for the three sampling period; 12-14th March 2014 (first), 2- 4th April 2014 (second) and 23 - 25th April 2014 (third). At each sampling point, two replicates composite water samples were collected. A total volume of 1 L (pooling 200 ml aliquots for 5 times) was collected from the same site within 30 minutes interval into 1.5 L brown high density polyethene (HDPE) bottles. In all, 138 water samples were collected and transported to the organic laboratory at Department of Chemistry, KNUST. The samples were filtrated twice. The first filtration was through a grade 5 filter paper (Munktell Filter AB, Falun, Sweden) with particle retention of 20 µm. The second filtration was through a grade 120H filter paper (Munktell Filter AB, Falun, Sweden) with particle retention of 1-2 µm. After filtration, the pH was measured using universal pH indicator strips and was adjusted to 7 ± 0.3 with 2 M H_2SO_4 (Sigma-Aldrich) or 2 M NaOH (Merck). The filtered samples were divided to 2 x 100 mL into brown HDPE bottles and spiked with 100 µL aliquot of 2.5 mg/L internal standard mix (IS mix). The IS mix contained ciprofloxacin-d_8 (d-Cip), sulfamethoxazole-d_4 (d-Sul) and trimethoprim-d_3 (d-Trim).

4.3.4 Solid Phase Extraction

The water samples were cleaned up on a reversed phase solid-phase extraction (SPE) using Oasis

HLB cartridges (hydrophilic-lipophilic balance, 200 mg sorbent, 30 μm, 6 cm^3) purchased from Waters Oasis (Massachusetts, USA). The SPE cartridges were conditioned with 2 mL of MeOH followed by 2 mL of 0.01 M citrate buffer and lastly with 2 mL of distilled water. A 100 mL portion of water samples were loaded onto SPE columns at a flow rate of 1.5 mL/min. The dried SPE columns were then bulked and kept in a refrigerator at -4 °C before shipping to Denmark where they were stored at -18°C until use.

In Denmark, the dried SPE columns were washed with 3 mL of 5% MeOH in water. The sorbents were allowed to dry for a couple of minutes under vacuum before the antibiotics were eluted with 3 mL MeOH acidified with 0.1% formic acid at a flow of approximately 1 mL min^{-1}. Eluates were evaporated to dryness under a gentle flow of nitrogen at 30 °C and then reconstituted in 1 mL 1% MeOH into brown flat-cap HPLC-vials for analysis.

4.3.5 LC-MS Analysis

The liquid chromatographic system consisted of an Agilent 1290 Infinity Binary System (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a degasser, a cooled autosampler (4 °C), and a column oven (30 °C). The chromatographic separations were achieved by use of a reversedphase column (Kinetex® Biphenyl 100 Å column, 2.1 mm x 50 mm x 2.6 μm), coupled to a guard column (Ultra cartridges BP, 2.1 mm x 2.6 μm), both from Phenomenex ApS, (Milford, MA, USA) and application of binary gradient flow rate of 400 μL/min at 20 °C. The injection volume was set at 10 μL. The mobile phase A consisted of water acidified with 0.1% formic acid whereas mobile phase B consisted of MeOH acidified with 0.1% formic acid. The initial proportion between the mobile phases was 99% A and 1% B. This gradient was held for 1 minute followed by a 5 min linear gradient ending with 50% A and 50% B. This proportion was soon changed in a 1 min linear gradient from 6 to 7 min to 1% A and 99% B. This gradient was maintained for 2 min

followed by a 1 min linear gradient ending with the initial conditions. This gradient was then maintained for 2 min resulting in a total analysis time of 12 min.

Mass spectrometry was performed with an AB SCIEX QTRAP® 4500 System with channel electron multiplier detector (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source (Turbo Ionspray). Electrospray ionization was performed in positive mode (ESI+) for metronidazole, ciprofloxacin, erythromycin, trimethoprim, tetracycline, oxytetracycline, chlorotetracycline, doxycycline, amoxicillin, ciprofloxacin-d_8 and trimethoprimd_3. The negative mode (ESI-) was performed for ampicillin, cefuroxime, sulfamethoxazole and sulfamethoxazole-d_4. The temperature was set to 300 ˚C with a nebulizer gas flow of 8 L/min, curtain gas flow of 12 L/min and collision gas flow of 6 L/min. The ionspray voltage was set to 5000 V. The MS system was set to operate in the multiple reaction monitoring mode with the parameters listed in Table 3.1. Collection and treatment of data were performed using Analyst v. 1.4.2 software (Applied Biosystems) and a Savitzky-Golay smoothing factor at 3 on a Windows XP platform-based data-processing system.

4.3.6 Validation of analytical Procedure

The method was validated in compliance with the requirements in standard guidelines (European Medical Agency 2012; ICH Harmonised Tripartite Guideline 2005; U.S. Food and Drug Adminstration 2001). The linear calibration curves were constructed by analyzing standard solutions ranging from 0.001 to 1000 ng/mL followed by calculating the ratios of analyte peak area to that of the internal standards. Precision was determined by injecting a 1 ng/mL standard antibiotics-mix solution for 8 times. The limit of detection (LOD) and limit of quantification

(LOQ) of the HPLC-MS/MS system was determined from the standard deviation (σ) of the response from the lowest calibration standard (0.001 ng/mL) injected 6 times and by the slope (S) of the calibration curve. Matrix effect (recovery) was studied with water from Søstein Lake (55.701316N, 12.565211E), Copenhagen. A 4 L water sample was collected into 5 L brown HDPE bottles and transported to the laboratory. Samples were then filtered and pH adjusted as described above. The filtered water was divided to 12 x 100 mL into brown bottles. The first four bottles containing the water were pre-spiked (prior to SPE) with 20 µL of 5 mg/L antibiotics mixture and post-spiked with 100 µl of 2.5 mg/L IS - mixture before LC determination. The subsequent four samples were post-spiked (after SPE) with 20 µL of 5 mg/L antibiotics mixture and 100 µl of 2.5 mg/L IS -mixture. The last four samples were pre-spiked with 100 µl of 2.5 mg/L IS -mixture and post-spiked with 20 µL of 5 mg/L antibiotics mixture before the LC determination.

The concentration of antibiotics in the samples were determined from calibration curves constructed for each individual analyte. Quality control (QC) samples were run in parallel during the quantification process. Positive controls consisting of matrix spiked (fortified) with antibioticmix and IS were used whereas negative controls consisting of matrix and internal standards were used to exclude possible procedural contaminations.

4.3.7 Statistical Analysis

The results obtained were subjected to statistical evaluation. Mean, standard deviation (SD) and coefficient of variation (CV %) were evaluated with Microsoft Office Excel 2013 (Version 15, Microsoft, USA). Pearson's correlation coefficient was performed as a measure of dependence between antibiotics studied. The closer the coefficient is to either −1 (decreasing linear relationship) or 1 (direct linear relationship), the stronger the correlation between the variables. In

general, interpretation of correlation analysis was done using correlation coefficients values higher than 0.5. However, some values close to 0.5 were also included (round-up to 0.5) to produce grouping such as 0.47 for tetracycline and ciprofloxacin; 0.46 for chlorotetracycline and erythromycin and 0.48 for chlorotetracycline and trimethoprim.

Discriminant analysis was performed for evidence on sampling period variation using SPSS version 22. The classical Wilks' Lambda statistics was used as a significance test for the equality of the group means (Nath and Pavur, 1985). This is to test whether or not there is a statistically significant relationship between the independent variables (antibiotics concentration) and the dependent variables (sampling periods). The key statistic indicating whether or not there is a relationship between the independent and dependent variables is the significance test for Wilks' lambda. Wilks' lambda is the proportion of the total variance in the discriminant scores not explained by differences among the groups. Smaller values of Wilks' lambda are desirable (Nath and Pavur, 1985). One-way ANOVA was performed to test the differences in various sources of water, with a Turkey's Honesty Significant Differences (HSD) as a post test. The P-value was considered statistically significant at $P<0.05$. The one-way ANOVA was performed by using GraphPad Prism version 5.01 for Windows (GraphPad Software Inc., USA). Principal components analysis (PCA), a multivariate statistical technique capable of discerning patterns in large environmental datasets and where complex inter-relationships between variables are difficult to identify and visualize (Shaw, 2003) was performed. In simple terms PCA is a data reduction technique whereby new variables (principal components or factors) are calculated from linear combinations of the original variables. The first principal component, or factor, accounts for the greatest variability in the data, and there can be an infinite number of new factors with each accounting for less data variability than the previous (Webster, 2001). Detailed descriptions of PCA and its applications can be found in a number of texts (Field, 2000; Shaw, 2003) Principal component analysis (PCA) based on

antibiotics concentrations in samples was done, to determine the distribution pattern of antibiotics in sampling area and water sources, using JMP statistical software v. 10 (SAS Institute). The principal components were extracted with eigenvalues >1.

4.3.8 Environmental Risk Assessment

The potential risk posed by each antibiotics, was assessed by calculating its risk quotient (RQ) as the ratio between its maximum measured environmental concentration (MEC) and its predicted no-effect concentration (PNEC), as suggested by EMEA, (2006). To cover all parts of the food chain in the water, RQ was calculated at three different trophic levels of the ecosystem, algae, daphnids and fishes. PNEC values assumed for this risk analysis correspond to the lowest ecotoxicological PNEC values found in literature. For 2 out of the 12 investigated compounds, ecotoxicological data were available in literature. The toxicity data for the antibiotics which could not be found in literature were calculated from the ecological structure activity relationships (ECOSAR) model (US-EPA, 2012). Aquatic organisms from three different trophic levels, i.e. green algae, daphnid and fish were chosen in the ECOSAR model. PNEC is calculated by dividing the lowest short-term L(E)C50 or long-term NOEC (no-observed-effect-concentration) value, by an assessment factor (AF). The AF is an arbitrary factor to consider the inherent uncertainty in the obtained laboratory toxicity data. In this study, an assessment factor of 100 was used. Table 4.1 provides all the toxicity data with the corresponding assayed species, endpoint and references. A commonly used risk ranking criterion; RQ < 0.1, minimal risk to aquatic organisms, 0.1 ≤ RQ ≥ 1, median risk; RQ ≥1, high risk was applied (Hernando et al., 2006).

Table 4.1: Toxicity data collected from literature and ECOSAR

Antibiotics	EC50 algae (µg/L)	Reference	EC50 daphnid (µg/L)	Reference	EC50 fishes (µg/L)	Reference
MET	2.93E+06	ECOSAR	3.20E+06	ECOSAR	8.85E+06	ECOSAR
CIP	6.10E+02	Halling-Sørensen et al, 2000	9.91E+05	Sanderson et al., 2003	2.46E+08	Sanderson et al., 2003
ERY	1.21E+05	ECOSAR	1.30E+05	ECOSAR	2.24E+05	ECOSAR
TRIM	1.10E+04	Ando et al., 2007	5.48E+04	Park and Choi, 2008	1.00E+05	Kim et al., 2007
TC	9.12E+07	ECOSAR	1.00E+08	ECOSAR	3.56E+08	ECOSAR
OTC	1.68E+09	ECOSAR	1.87E+09	ECOSAR	8.89E+09	ECOSAR
CTC	6.46E+09	ECOSAR	7.20E+09	ECOSAR	3.93E+10	ECOSAR
DC	9.76E+07	ECOSAR	1.07E+08	ECOSAR	3.84E+08	ECOSAR
AMX	1.01E+06	ECOSAR	1.09E+06	ECOSAR	2.51E+06	ECOSAR
AMP	3.92E+05	ECOSAR	4.24E+05	ECOSAR	8.88E+05	ECOSAR
CEF	4.21E+06	ECOSAR	4.59E+06	ECOSAR	1.20E+07	ECOSAR
SUL	1.75E+06	ECOSAR	1.90E+06	ECOSAR	4.78E+09	ECOSAR

4.4 RESULTS

4.4.1 Result of validation of analytical Procedure

The regression coefficient (r^2), precision, LOD, LOQ and matrix recovery determined for all antibiotics were within the recommended limit (Table 3.3).

4.4.2 Occurrence of antibiotics in water sources

All 12 antibiotics were detected in at least one sample. Amoxicillin was the least detected and concentrations were up to 3.1 ng/L, while tetracyclines concentrations were below 50 ng/L. In

general, the antibiotics with the highest concentrations were; erythromycin; cefuroxime; sulfamethoxazole trimethoprim and ciprofloxacin.

4.4.2.1 Occurrence of antibiotics in untreated wastewater

All 12 antibiotics studied were detected and their concentrations were above its respective LOQ in untreated wastewater sampled. Ciprofloxacin, erythromycin, trimethoprim, cefuroxime and sulfamethoxazole concentrations in the untreated wastewater were above thousands of ng/L with ciprofloxacin recording the highest concentration of 8,532 ng/L (Table 4.2). All antibiotics indicate a distinct difference between untreated wastewater and other water sources sampled and these were statistically significant ($p<0.05$) (Fig. 4.2a-l).

Table 4.2: Antibiotic concentrations in water types (effluent, rivers irrigation water and untreated wastewater) in ng/L

Antibiotics	Untreated wastewater	3 WSP Effluent, Kumasi	Rivers, Kumasi	Irrigation water, Kumasi
MET	524-625	3[a]-19	<LOD-363	3[a] – 33
CIP	8181-8532	27-262	25-1168	47-146
ERY	1857-5858	47-882	7.0-1149	6.7-136
TRIM	2148-3659	31-255	17-820	19-98
TC	21-23	11-24	11-30	11-16
OTC	46-74	2.4[a]-24	3-26	2.2[a]-9.2
CTC	16-23	6.0-19	5.3[a]-44	4.3[a]-14
DC	36-57	14-49	8.3-68	9.4-25
AMX	2.3-3.1	<LOD-1.3[a]	<LOD – 2.7	<LOD– 1.3[a]

AMP	172-176	51-97	21-184	30-74
CEF	1028-1141	58-345	32-868	21-65
SUL	2262- 2653	103- 320	13-2861	11-56

LOQ<a>LOD

4.4.2.2 Occurrence of antibiotics in WSPs effluent

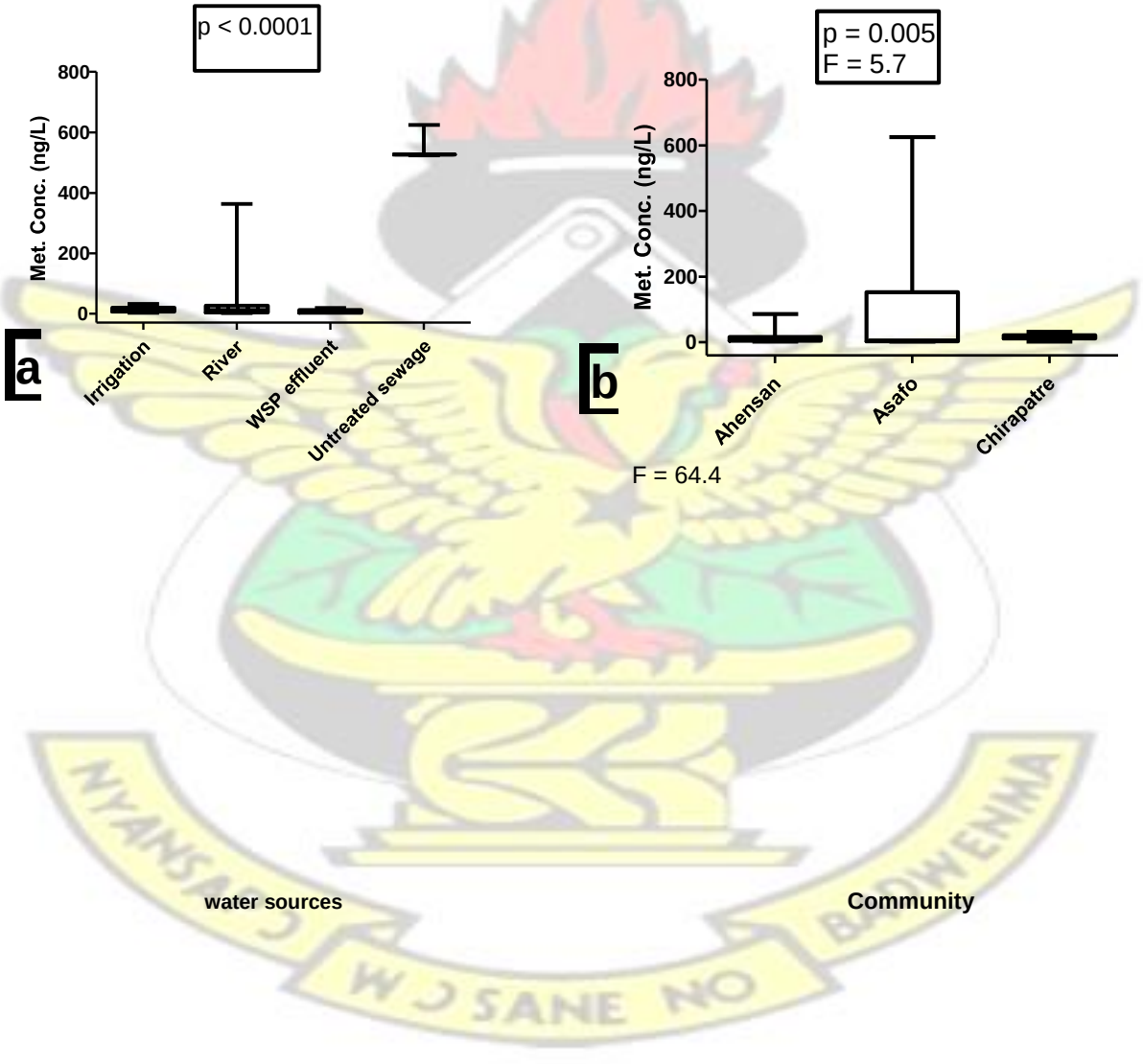
The concentrations of all antibiotics in WSP effluents analysed (Table 4.2) ranged from <LOD to 882 ng/L (erythromycin). The antibiotics with highest concentration was erythromycin followed by cefuroxime and sulfamethoxazole. Ciprofloxacin was the fourth highest antibiotic. Amoxicillin recorded the lowest concentration and tetracyclines were below 50 ng/L. The mean concentrations of all antibiotics was 137 ng/L for Asafo, 46 ng/L for Ahensan, and 57 ng/L for Chirapatre WSP effluent samples studied, however one- way ANOVA showed no statistically differences (p=0.13).

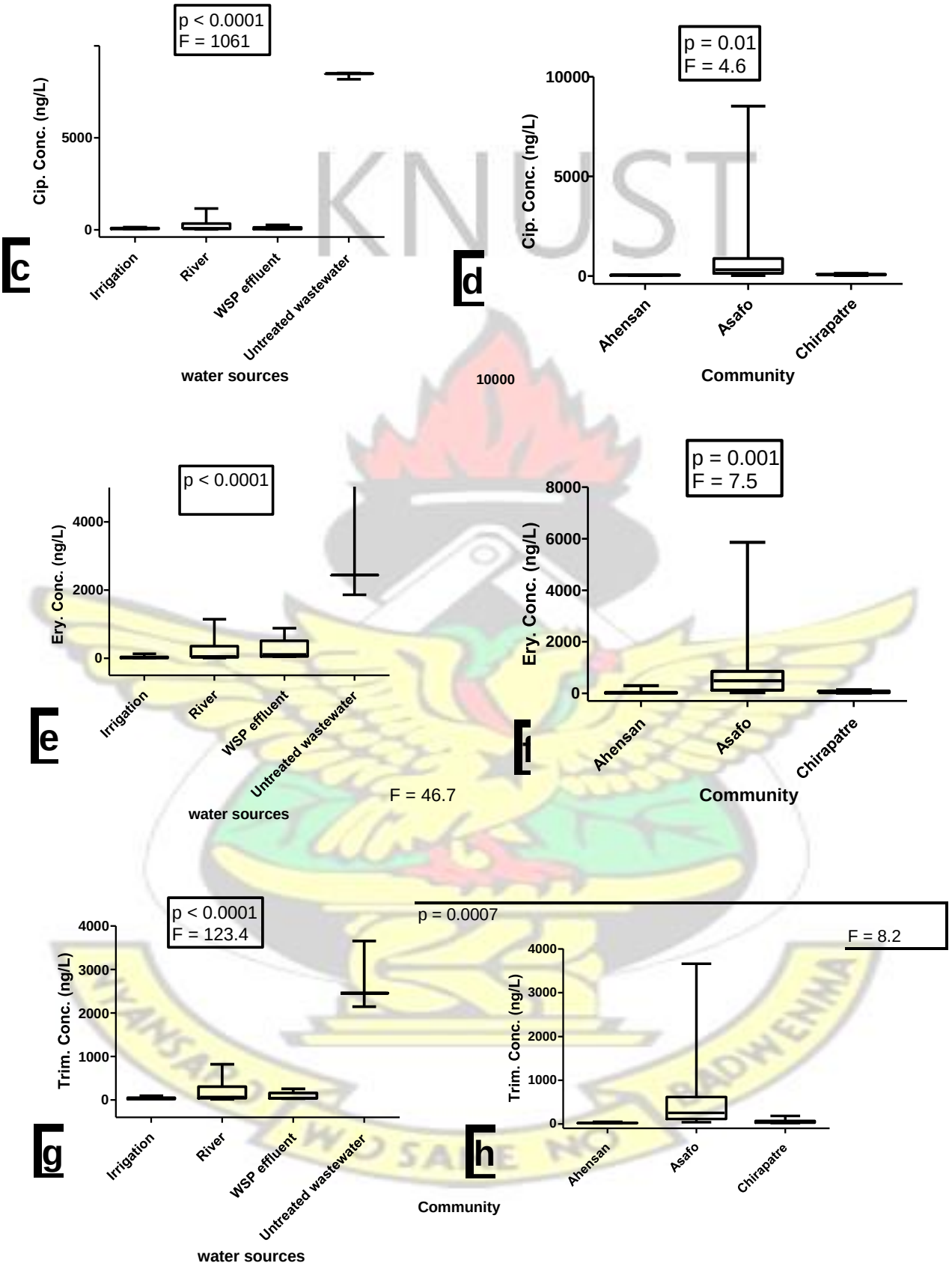
4.4.2.3 Occurrence of antibiotics in rivers

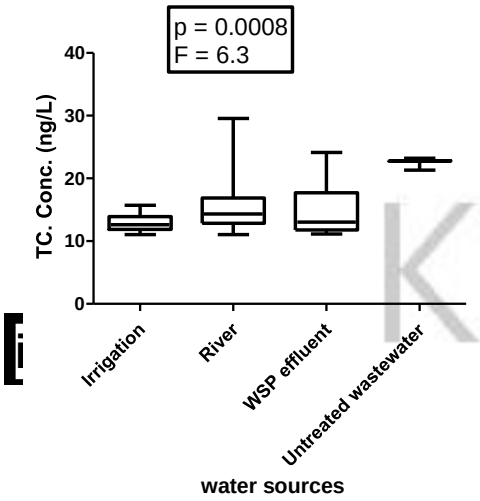
Sulfamethoxazole recorded the highest concentration in the river samples analysed (Table 4.2), followed by CIP and ERY. The concentration of antibiotics in river samples ranged from <LOD to 2861 ng/L (sulfamethoxazole). The concentration of antibiotics in the river samples ranged from 2.4 to 363 ng/L (metronidazole), 25 to 1168 ng/L (ciprofloxacin), 7.0 to 1149 ng/L (erythromycin), 17 to 820 ng/L (trimethoprim), 11 to 30 ng/L (tetracycline), 2.9 to 26 ng/L (oxytetracycline), 5.3 to 44 ng/L (chlortetracycline), 8.3 to 68 ng/L (doxycycline), 0.2 to 2.7 ng/L (amoxicillin), 21 to 184 ng/L (ampicillin), 32 to 868 ng/L (cefuroxime) and 13 to 2861 ng/L (sulfamethoxazole).

4.4.2.4 Occurrence of antibiotics in irrigation water

In general, the concentrations of antibiotics in water used for irrigation of vegetables were lower, ranging from <LOD to 146 ng/L (ciprofloxacin) (Table 4.2). It is worth noting, that the differences between irrigation water and other water sources are not statistically significant for ciprofloxacin (q = 2.5, p >0.05). This trend is also seen in erythromycin (Fig. 4.2c), trimethoprim (Fig. 4.2g), cefuroxime (Fig. 4.2u), metronidazole (Fig. 4.2a), tetracycline (Fig. 4.2l), oxytetracycline (Fig. 4.2k), doxycycline (Fig.2o), amoxicillin (Fig. 4.2q), chlortetracycline (Fig. 4.2m), ampicillin (Fig. 4.2s) and sulfamethoxazole (Fig. 4.2w).




p < 0.0001
F = 1061
Cip. Conc. (ng/L)
5000
0
Irrigation
River
WSP effluent
Untreated wastewater
water sources
c
p = 0.01
F = 4.6
10000
5000
0
Ahensan
Asafo
Chirapatre
Community
d
10000
p < 0.0001
Ery. Conc. (ng/L)
4000
2000
0
Irrigation
River
WSP effluent
Untreated wastewater
water sources
e
F = 46.7
p = 0.001
F = 7.5
8000
6000
4000
2000
0
Ahensan
Asafo
Chirapatre
Community
f
p < 0.0001
F = 123.4
Trim. Conc. (ng/L)
4000
3000
2000
1000
0
Irrigation
River
WSP effluent
Untreated wastewater
water sources
g
p = 0.0007
F = 8.2
Trim. Conc. (ng/L)
4000
3000
2000
1000
0
Ahensan
Asafo
Chirapatre
Community
h


p = 0.0008
F = 6.3
TC. Conc. (ng/L)
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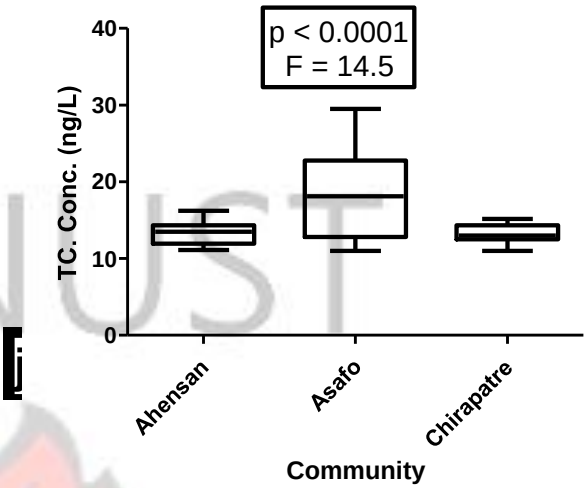
10
20
30
40
Irrigation
River
WSP effluent
Untreated wastewater
water sources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TC. Conc. (ng/L)
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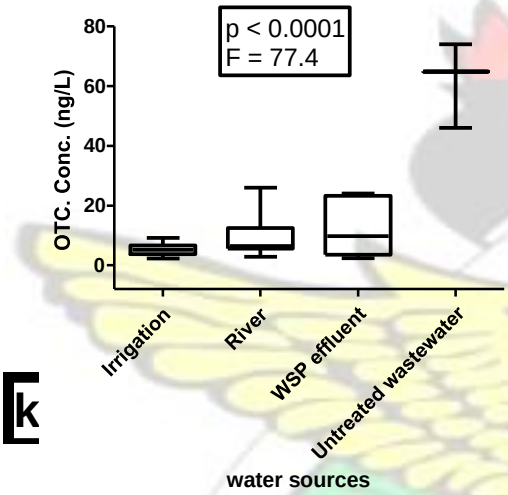
10
20
30
40
Ahensan
Asafo
Chirapatre
Community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OTC. Conc. (ng/L)
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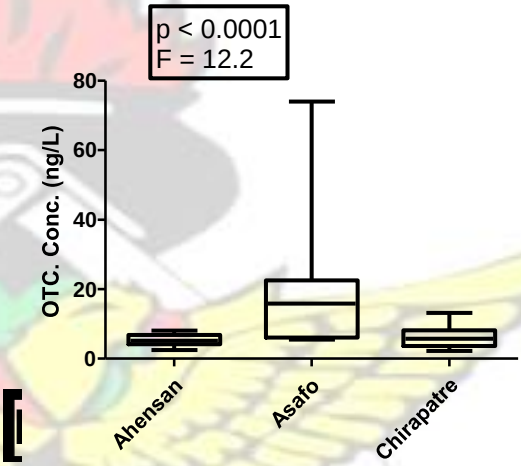
20
40
60
80
Irrigation
River
WSP effluent
Untreated wastewater
water sources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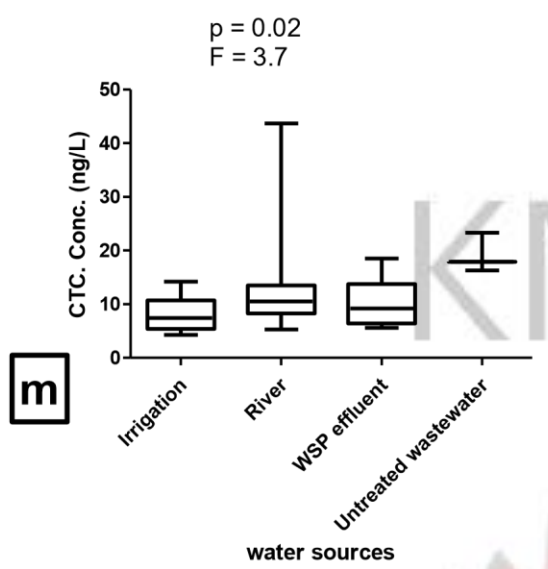
OTC. Conc. (ng/L)
0
20
40
60
80
Ahensan
Asafo
Chirapatre
Community



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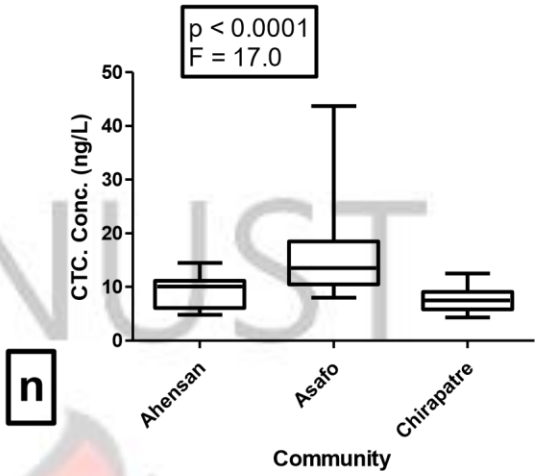

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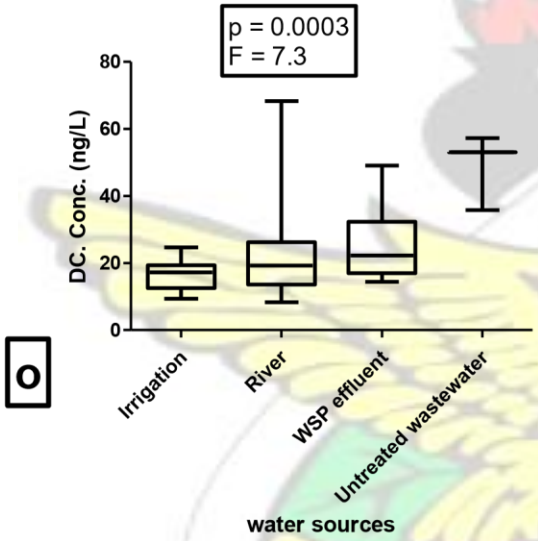

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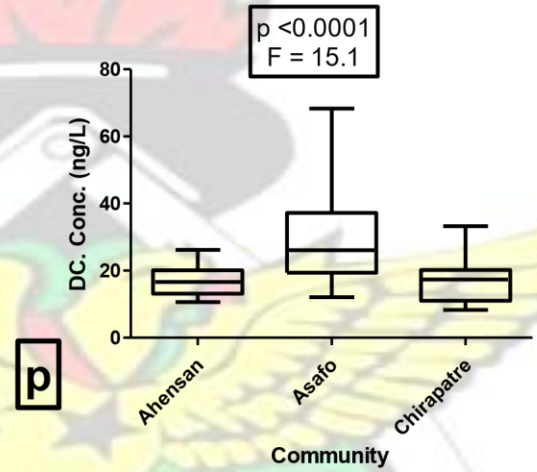
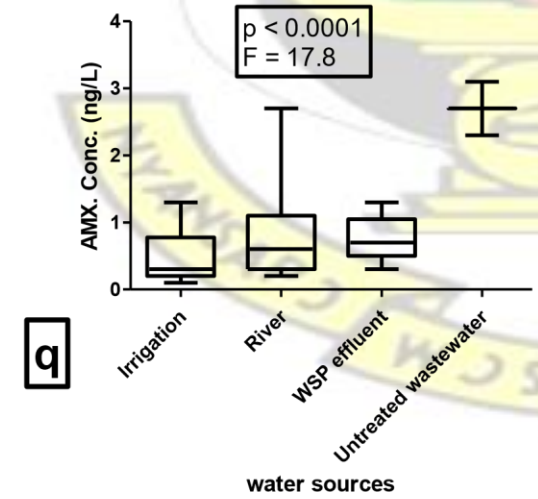


p



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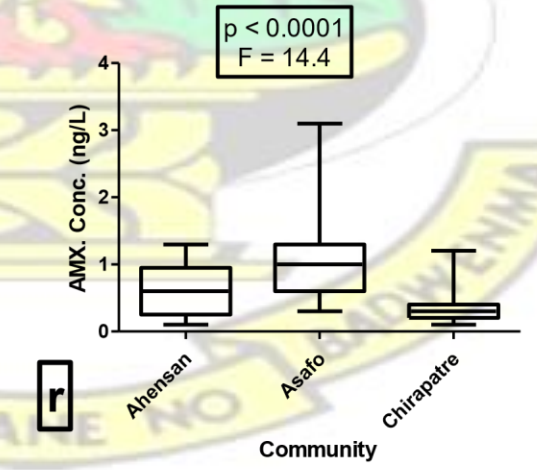

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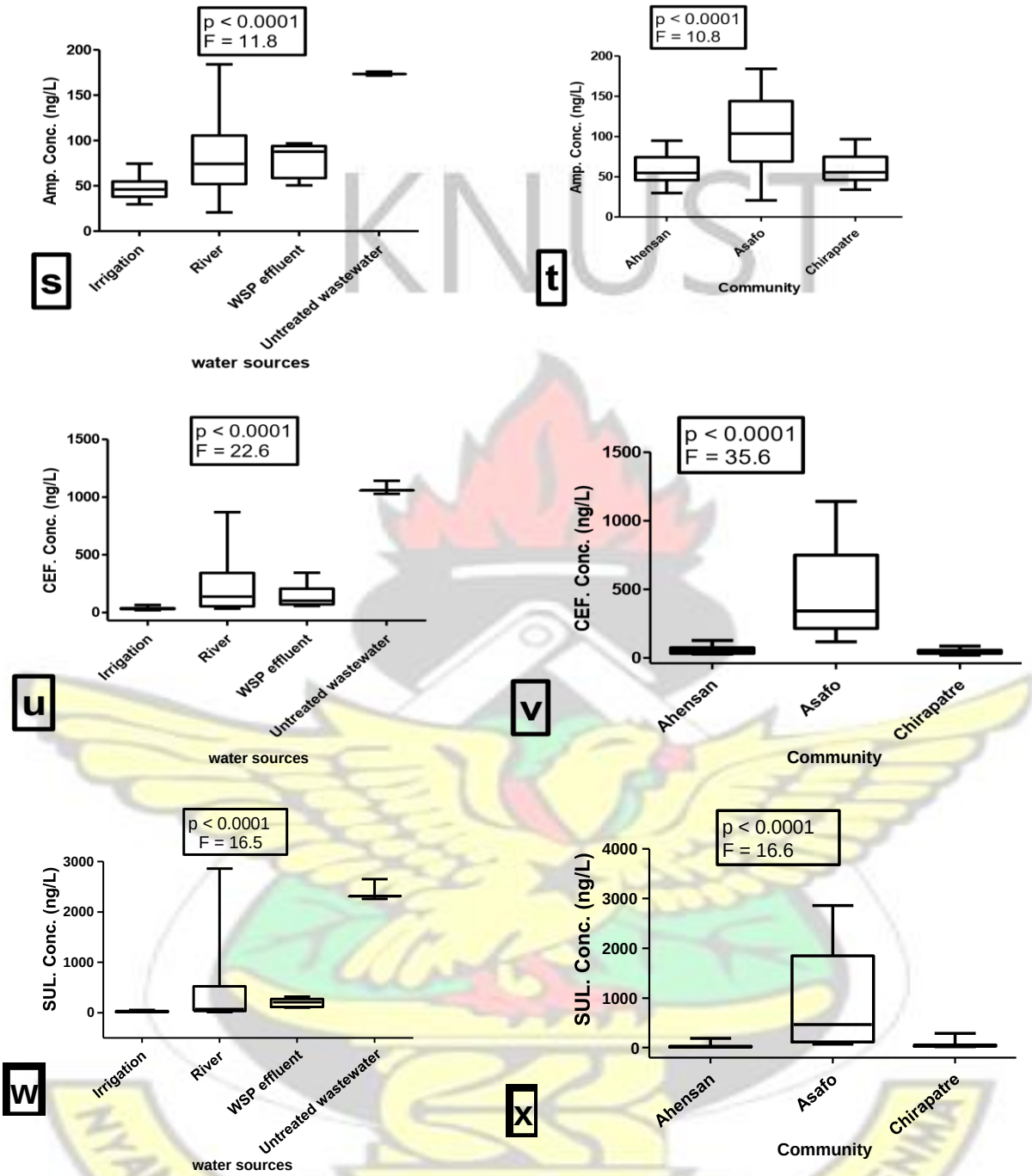
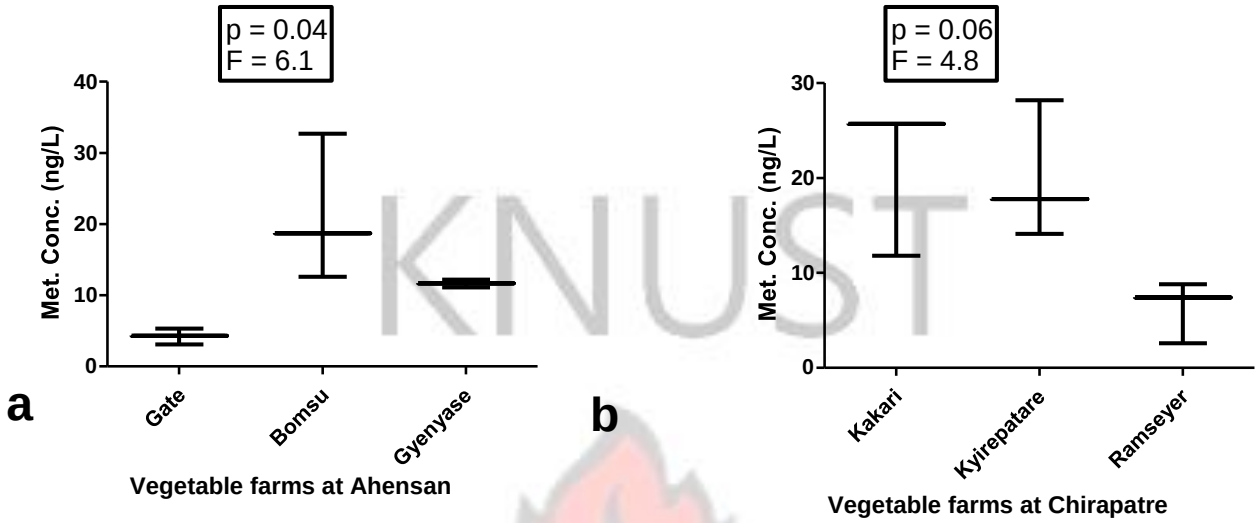


Fig. 4.2: Box-and-whisker plots of low quality water
(a) MET organized by water body type and (b) by community; (c) CIP organized by water body type and (d) by community; (e) ERY organized by water body type and (f) by community; (g) ERY organized by water body type and (h) by community etc. p, statistical significance of these factors from one-way ANOVA

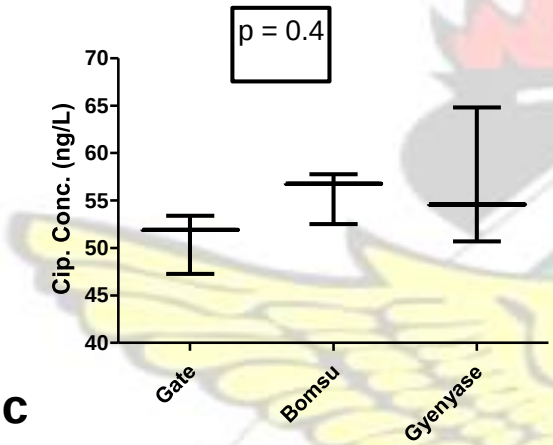
Tukey HSD post test revealed that there is no significant difference between irrigation water and river for most antibiotics (p values were all above 0.05 indicated in Fig. 4.2a-l) except cefuroxime ($p < 0.05$) and ampicillin ($p < 0.05$). Considering the data sorted by community (Fig. 4.2a-l), antibiotics determined were generally higher in Asafo (where untreated wastewater located) than Ahensan and Chirapatre. One- way ANOVA indicated statistically differences (p values were below 0.05 indicated in Fig. 4.2m-x) among communities. The Turkey HSD test reveal that the difference was actually among samples from Asafo and Ahensan; and Asafo and Chirapatre. There is no significant difference between antibiotic concentration in all samples from Chirapatre and Ahensan.

Fig. 4.3, shows the box and Whisker plots of water used for irrigation at six vegetable farms along the river receiving effluent from WSPs studied. They are organized by Ahensan and Chirapatre community. There is also no significant difference in concentrations of most antibiotics detected in the various vegetable farms from Ahensan and Chirapatre. However, Ampicillin showed significant difference between irrigation water from farms at Ahensan and Chirapatre communities. Considering the upstream, midstream and downstream river concentrations for Ahensan, Asafo, and Chirapatre, there is no significant difference in concentrations of antibiotics detected in the various points (p-value = 0.46, 0.33 and 0.77) respectively.

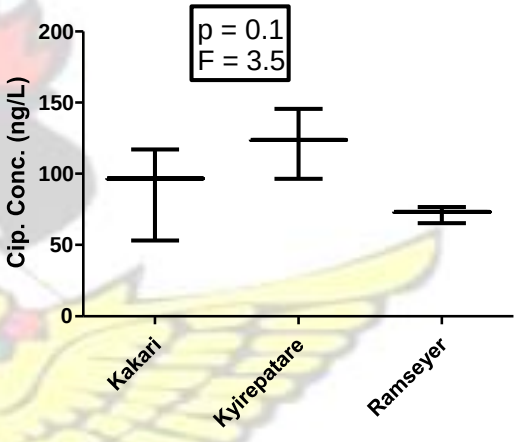


a

b



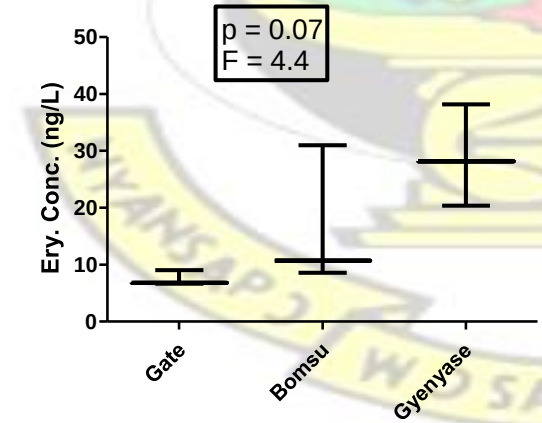
c



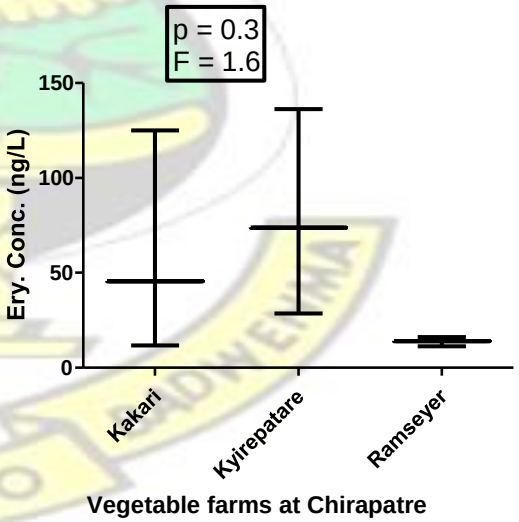
d

F =

1.2

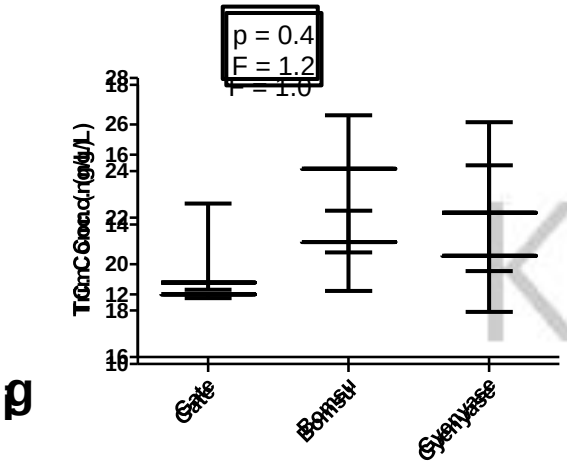


e Vegetable farms at Ahensan



Vegetable farms at Chirapatre

f



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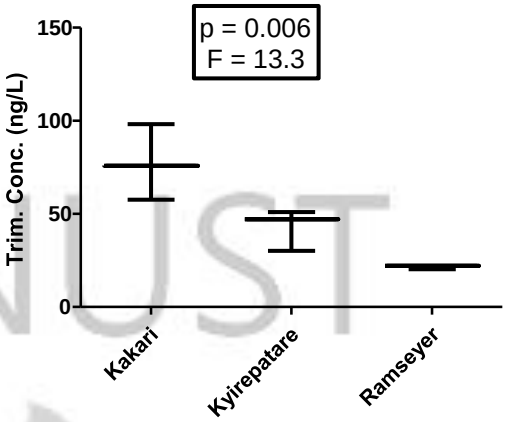
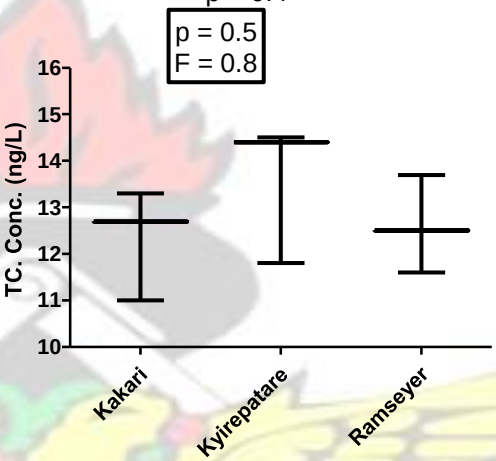


h

j

p = 0.4



Vegetable farms at Ahensan

Vegetable farms at Chirapatre

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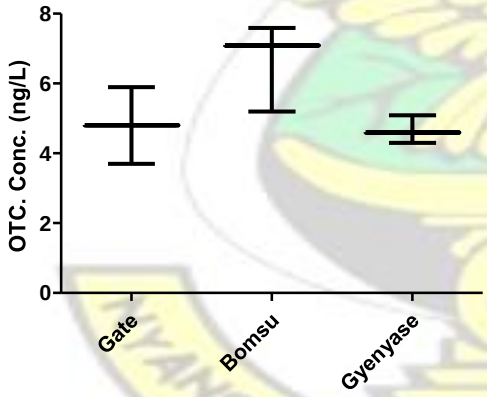

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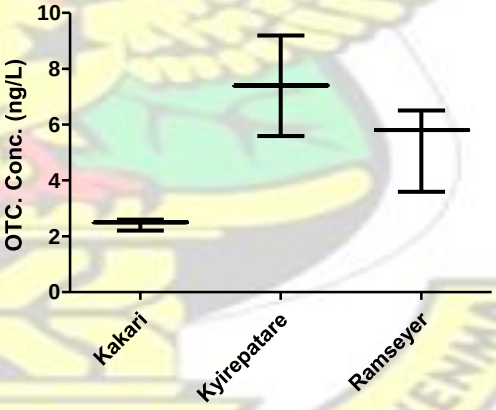

l

Vegetable farms at Ahensan

Vegetable farms at Chirapatre

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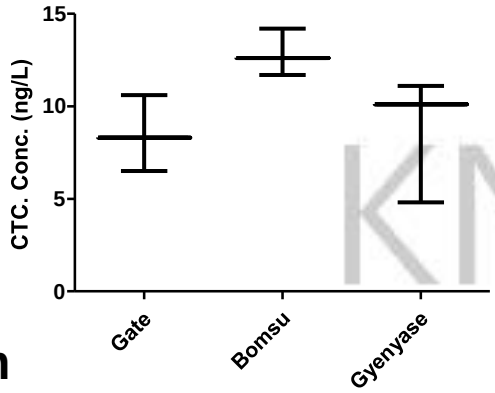

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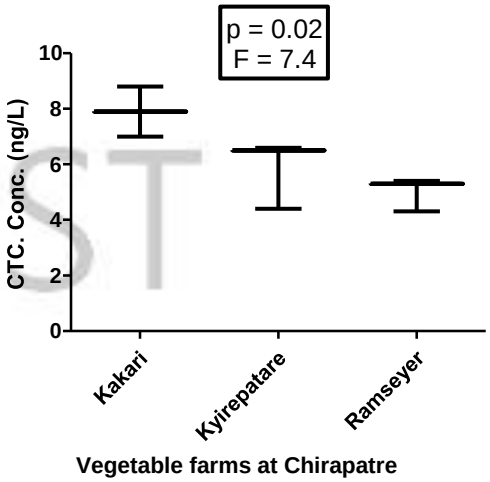

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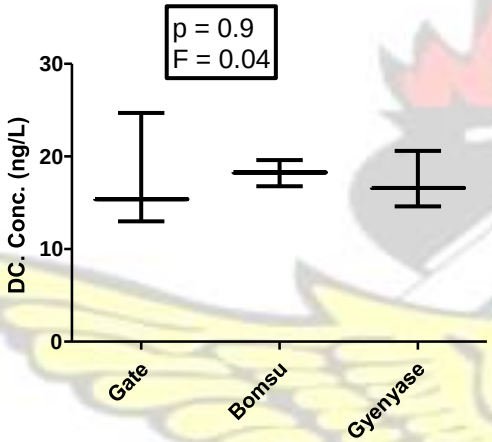

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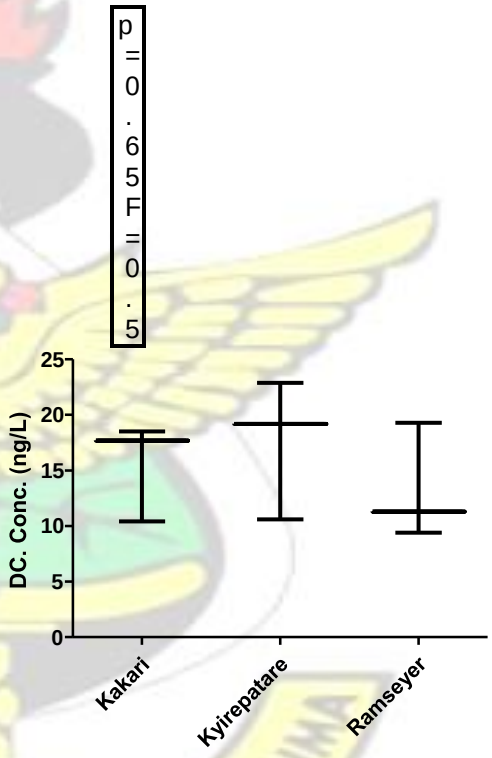
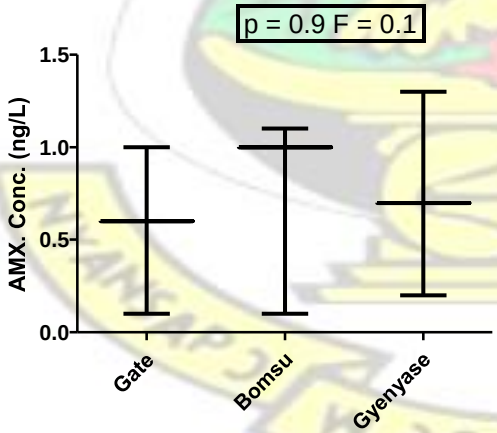
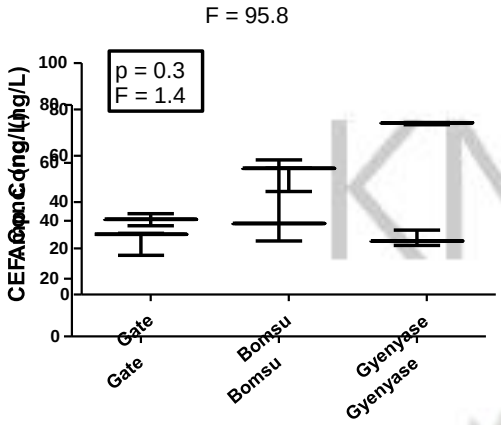


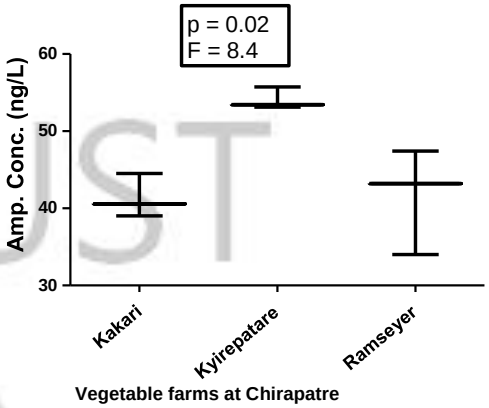
p

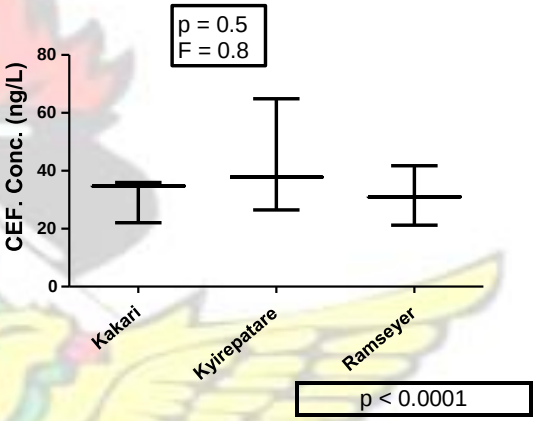


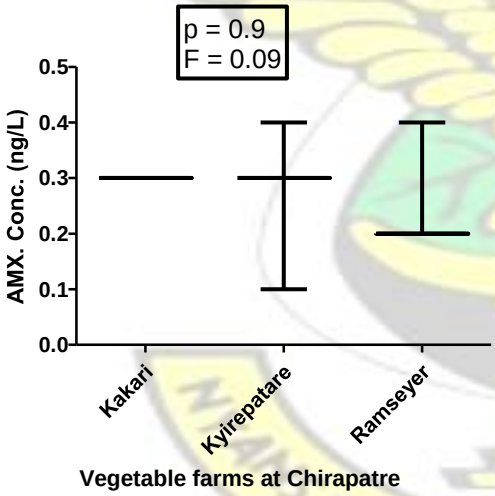
q

r









Vegetable farms at Ahensan

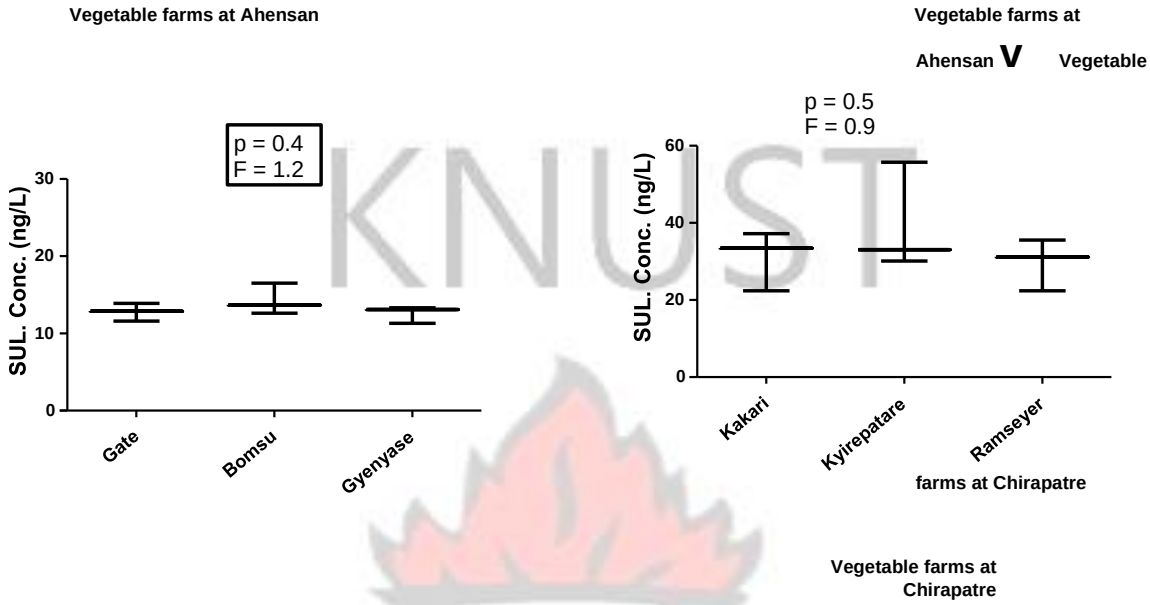
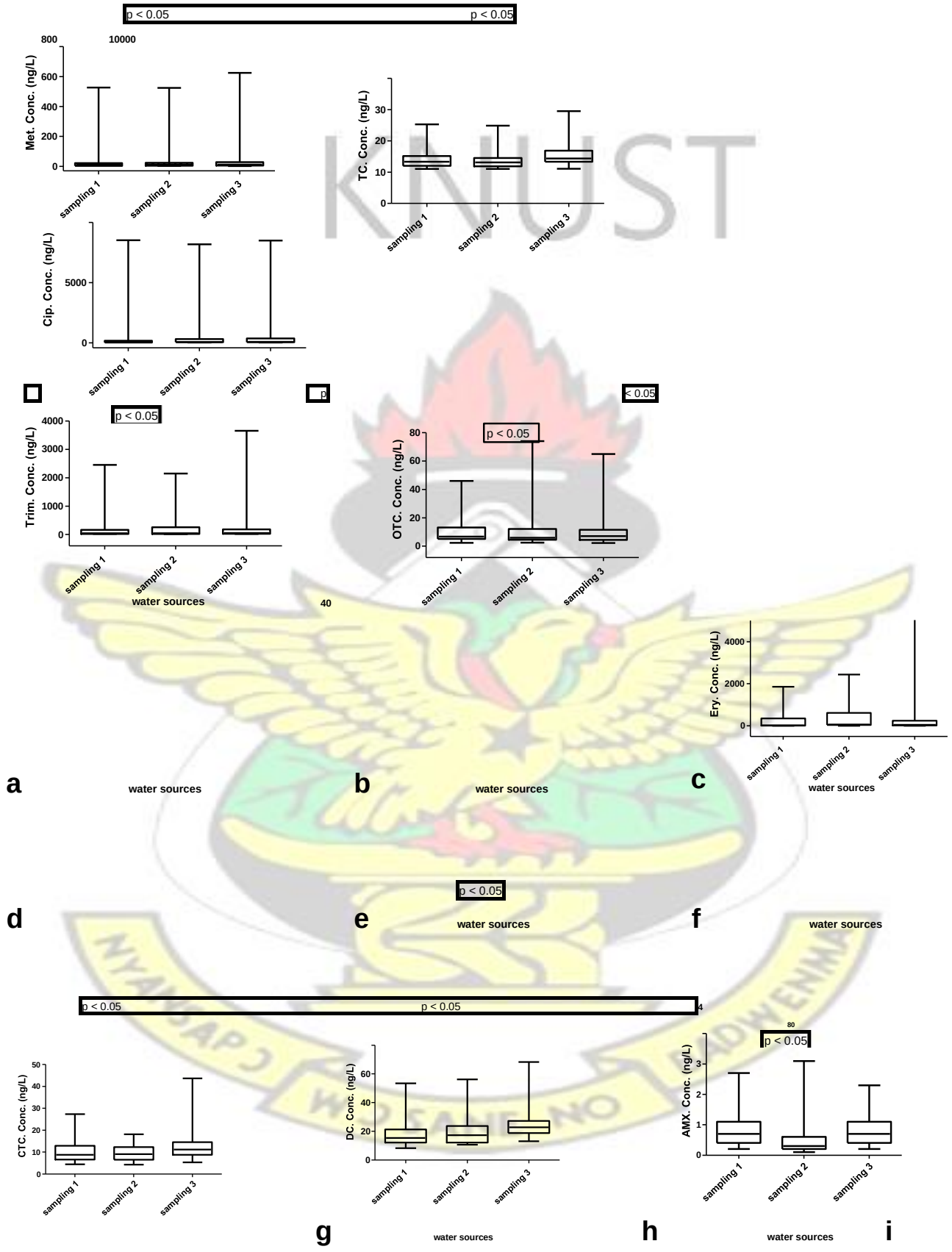


Fig. 4.3: Box-and-whisker plots of irrigation water
(a) MET organized by vegetable farms at Ahensan sampling sites and (b) by vegetable farms at Chirapatre sampling sites; (c) CIP organized by vegetable farms at Ahensan sampling sites and (d) by vegetable farms at Chirapatre sampling sites etc. p, statistical significance of these factors from one-way ANOVA

4.4.3 Variation in sampling period

The mean concentrations of antibiotics measured in various sampling period were quite similar (Fig. 4.4). The Wilks' lambda was 0.61, and the p value was 0.57. The chi-square statistics corresponding to Wilks' lambda was 20.56. The results indicate that the concentrations at various sampling period are not significantly different. One-way ANOVA performed showed p values below 0.05 (Fig. 4.4).


p < 0.05
p < 0.05
Met. Conc. (ng/L)
TC. Conc. (ng/L)
Cip. Conc. (ng/L)
sampling 1
sampling 2
sampling 3
p < 0.05
Trim. Conc. (ng/L)
p < 0.05
OTC. Conc. (ng/L)
p < 0.05
Ery. Conc. (ng/L)
water sources
a
b
c
d
e
f
p < 0.05
p < 0.05
CTC. Conc. (ng/L)
DC. Conc. (ng/L)
p < 0.05
AMX. Conc. (ng/L)
g
h
i
water sources

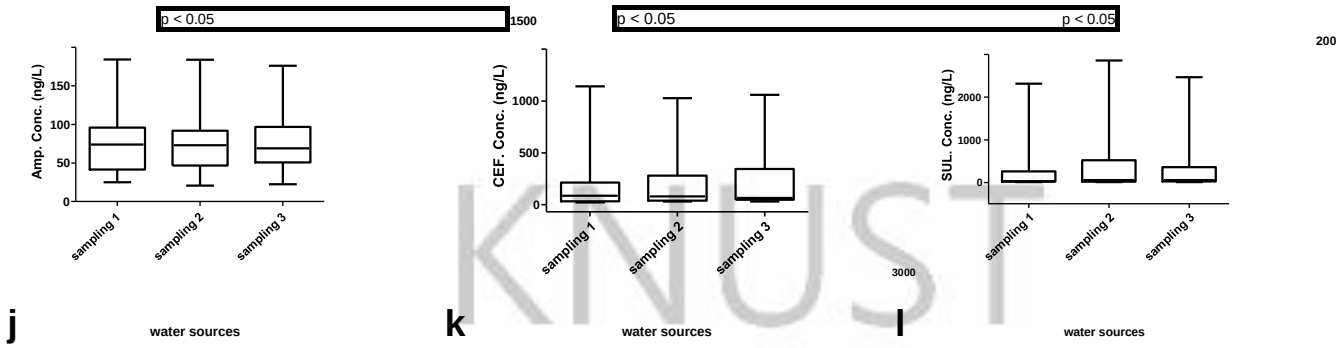


Fig. 4.4: Box-and-whisker plots of antibiotics organized by sampling period
(a) MET; (b) CIP and (c) ERY etc. p, statistical significance of these factors from one-way ANOVA

4.4.4 Correlation analysis

Correlation analysis of the concentration of antibiotics (Table 4.3) showed moderate to strong correlations. Sulfamethoxazole, tetracycline, trimethoprim, erythromycin, metronidazole, doxycycline and cefuroxime were positively correlated with all the antibiotics (Table 4.3). Amoxicillin, ciprofloxacin ampicillin, and oxytetracycline were positively correlated with all antibiotics except CTC; CTC was positively correlated with MET, TC, CEF and SUL, except CIP and OTC, AMX and AMP.

Table 4.3 Correlation matrix for the antibiotics concentrations in water

	MET	CIP	ERY	TRIM	TC	OTC	CTC	DC	AMX	AMP	CEF	SUL
MET												
CIP	0.887											
ERY	0.812	0.85										
TRIM	0.939	0.946	0.941									
TC	0.571	0.471	0.553	0.567								

OTC	0.782	0.884	0.84	0.861	0.629						
CTC	0.538	0.336	0.456	0.483	0.662	0.388					
DC	0.54	0.521	0.589	0.587	0.649	0.667	0.477				
AMX	0.577	0.677	0.642	0.662	0.598	0.757	0.354	0.622			
AMP	0.502	0.553	0.567	0.583	0.684	0.713	0.333	0.72	0.671		
CEF	0.769	0.723	0.71	0.787	0.8	0.784	0.627	0.8	0.732	0.773	
SUL	0.866	0.7	0.681	0.795	0.681	0.665	0.706	0.649	0.563	0.576	0.847

4.4.5 Principal Component Analysis

Distribution of the antibiotics in water sources and sampling area were analyzed using PCA which showed a significant separation between Ahensan irrigation water (A), grouped on one side with few remaining in the center, and the Chirapatre irrigation water (E), clustered on another side (Fig. 4.5a). Interestingly, erythromycin, ciprofloxacin and trimethoprim made one cluster or group in irrigation water toward Chirapatre sampling site (Fig. 4.5b). Ahensan irrigation water was correlated with amoxicillin and oxytetracyline (Fig. 4.5b).

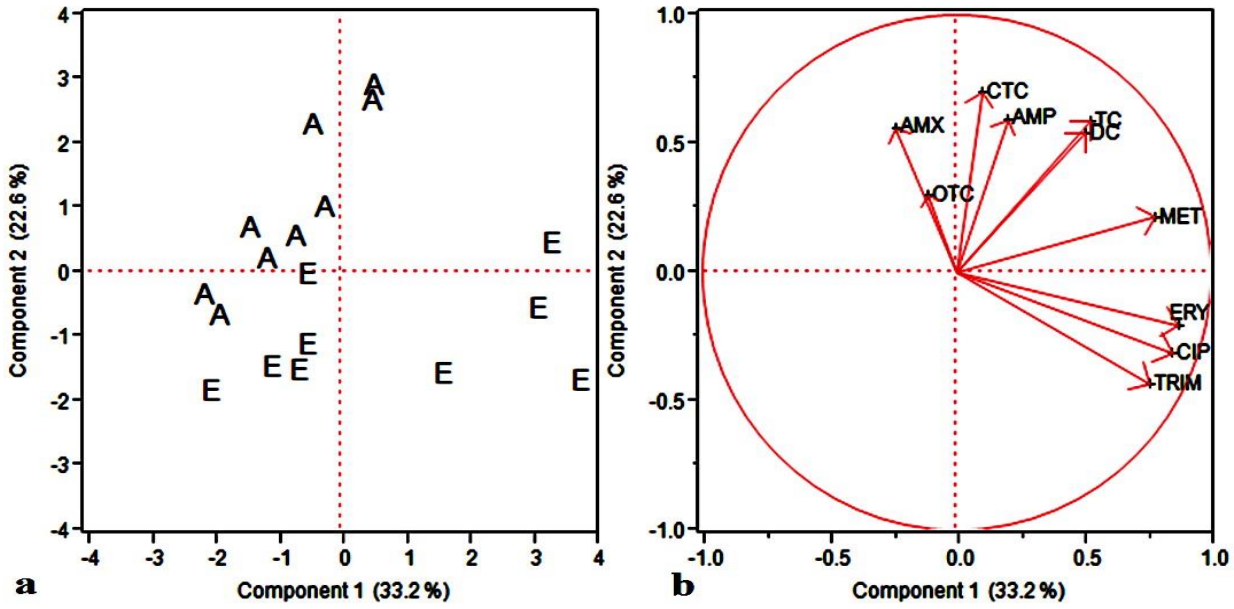


Fig. 4.5**:** Distribution pattern of antibiotics in irrigation water characterised by PCA (A: Ahensan irrigation water; and E: Chirapatre irrigation water), (a) scoreplot showing each sources and (b) biplot showing the direction of correlation.

The PCA result between Ahensan samples (B), and Chirapatre samples (F) were not clearly separated (Fig. 4.6).

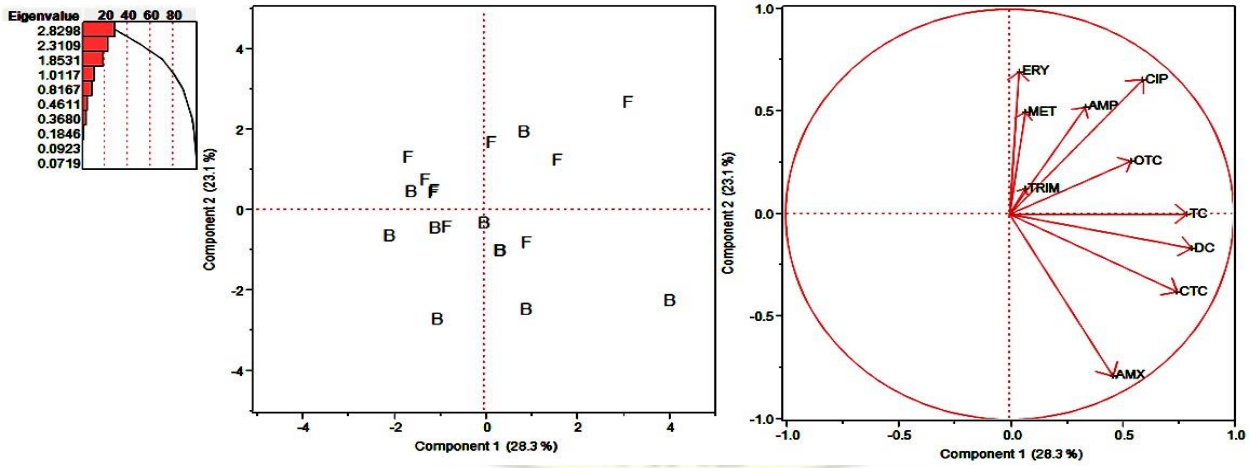


Fig. 4.6**:** Distribution pattern of antibiotics in rivers characterised by PCA (B: Ahensan rivers; and F: Chirapatre rivers)

4.4.6 Environmental risk assessment

A realistic evaluation of hazards in low quality water collected from Kumasi, Ghana was performed using long-term data, with both standard and non-standard organisms. Highest concentrations of antibiotics in the water samples (to set in the worst-case scenario), PNEC values and risk quotients for each analyte are shown in Table 4.4. The RQ for algae for all antibiotics ranged from 4.6×10^{-9} to 1.3×10^{-1}, RQ for daphnids ranged from 4.1×10^{-10} to 1.5×10^{-3} and RQ for fishes ranged from 7.6×10^{-11} to 3.3×10^{-4} (Table 4.4).

Table 4.4: Maximum environmental concentrations (MEC) of antibiotics in waters. PNEC and RQ for fish, daphnids (all species belonging to their trophic level) and algae (or bacteria) for the studied antibiotics.

Antibiotics	MEC (µg/L)	PNEC algae (µg/L)	RQ algae	PNEC daphnid (µg/L)	RQ daphnid	PNEC fishes (µg/L)	RQ fishes
MET	3.0E-01	2.9E+04	1.0E-05	3.2E+04	9.4E-06	8.8E+04	3.4E-06
CIP	8.2E-01	6.1E+00	1.3E-01	9.9E+03	8.3E-05	2.5E+06	3.3E-07
ERY	7.5E-01	1.2E+03	6.2E-04	1.3E+03	5.7E-04	2.2E+03	3.3E-04
TRIM	8.0E-01	1.1E+02	7.3E-03	5.5E+02	1.5E-03	1.0E+03	8.0E-04
TC	2.6E-02	9.1E+05	2.9E-08	1.0E+06	2.6E-08	3.6E+06	7.3E-09
OTC	2.1E-02	1.7E+07	1.3E-09	1.9E+07	1.1E-09	8.9E+07	2.4E-10
CTC	3.0E-02	6.5E+07	4.6E-10	7.2E+07	4.1E-10	3.9E+08	7.6E-11
DC	5.1E-02	9.8E+05	5.2E-08	1.1E+06	4.8E-08	3.8E+06	1.3E-08
AMX	1.7E-03	1.0E+04	1.7E-07	1.1E+04	1.6E-07	2.5E+04	6.8E-08
AMP	1.8E-01	3.9E+03	4.6E-05	4.2E+03	4.3E-05	8.9E+03	2.0E-05
CEF	8.2E-01	4.2E+04	1.9E-05	4.6E+04	1.8E-05	1.2E+05	6.8E-06
SUL	2.4E+00	1.7E+04	1.4E-04	1.9E+04	1.3E-04	4.8E+04	5.0E-05

4.5 Discussion

4.5.1 Occurrence of antibiotics in untreated wastewater

Ciprofloxacin is often used as one of the first line drugs at hospitals in Ghana and recorded the highest concentration in untreated wastewater (Table 4.2). Its difference between other water bodies are statistically significant ($p < 0.0001$). Concentrations of antibiotics studied were generally higher in untreated wastewater than the other water sources sampled. This could be due to the fact that the untreated wastewater sampled was a broken sewage line linking the main regional hospital serving the Ashanti region and northern parts of Ghana, the Komfo Anokye Teaching Hospital (KATH) and the 4BN soldier Barracks. The broken drain enters directly into the tributary of Subin river. As antibiotic is often associated with hospital wastewater, it is not surprising that high concentrations of all the 12 antibiotics studied were seen in untreated wastewater, which contains Komfo Anokye Teaching Hospital wastewater.

4.5.2 Occurrence of antibiotics in WSPs effluent

Metronidazole concentrations found in effluent samples in this study ranged from 2.9 to 19 ng/L (Table 4.2), which were comparable to concentrations recorded in literature. Two conventional activated sludge plants situated in the Po Valley, northern Italy, with their corresponding population served, expressed as number of inhabitants being 138 000 and 5000 respectively recorded metronidazole concentration range of 9- 21 and 14-21 ng/L respectively (Aukidy et al., 2012). The concentrations found in this study are within the range reported in Italy. However, K'oreje et al. (2012), analysed effluent sample from the Dandora sewage plant, Kenya and detected no metronidazole. Ciprofloxacin, a quinolones class of antibiotics was not detected in effluent

samples from New Mexico (Brown et al., 2006), but found up to 499 ng/ L in Two conventional activated sludge plants situated in the Po Valley, northern Italy, (Aukidy et al., 2012) The ciprofloxacin concentrations, found in effluent samples in this study ranged from 27 to 262 ng/L (Table 4.2), indicating that the maximum concentration found in this study is lower than that reported in Italy.

The maximum concentration of erythromycin being 882 ng/L (Table 4.2) found in effluents in this study was higher than the maximum of 287 ng/L found in STP at Switzerland by Giger et al., (2003), but lower than 6000 ng/L found by Hirsch et al., (1999) in STP effluent at Germany. The β-lactam class of antibiotics are the most frequently prescribed class of antimicrobials in Ghana, particularly amoxicillin, followed by ampicillin and cefuroxime (Tagoe and Attah 2010). In this study, amoxicillin concentrations were below the LOQ in effluents samples (Table 4.2). Amoxicillin is chemically unstable due to the β-lactam ring which readily undergo hydrolysis. This could account for the low concentration found in the samples. Amoxicillin was not detected in STP effluent sample in Germany (Hirsch et al., 1999). However, Andreozzi et al., (2004), found amoxicillin concentrations ranging from <1.8 ng/L to 120 ng/L in Italian STPs effluents. The lower concentrations detected in STPs effluents in Ghana as compared with those in Italy probably reflect differences in prescription patterns for macrolide antibiotics in the two countries. Unfortunately, information on prescription rates of antibiotics in Ghana are not available, so it is not possible to confirm this hypothesis.

Among the sulphonamides, sulfamethoxazole and trimethoprim are the two most abundant antibiotics in the samples analysed, with concentrations ranging from 103 to 320 ng/L and 31 to 225 ng/L respectively (Table 4.3). The highest concentration of sulfamethoxazole detected in the

STPs effluent analysed were lower than the 6000 ng/L reported in Dandora STP effluent in Kenya (K'oreje et al., 2012). Trimethoprim was not detected in effluent from Kenya but up to 180 ng/L was found in STP effluent from New Mexico (Brown et al., 2006). The highest concentration of trimethoprim detected in the STPs effluent analysed were higher than the concentration reported in STP effluent from New Mexico (Brown et al., 2006).

Although sulfamethoxazole/trimethoprim is administered as one drug (co-trimoxazole) at a mass ratio of 1:5, the measured aqueous concentration of sulfamethoxazole exceeds that of trimethoprim by a factor of 3. This may be explained by their different fates both in the human body and in the environment (Madureira et al., 2010). Although the fraction that is excreted in unchanged form by humans is lower for sulfamethoxazole (15%) than for trimethoprim (60%), the latter is more readily removed in STPs (Madureira et al., 2010) and via photolytic degradation (up to 99%) (Abellán et al., 2009).

The Turkey HSD test which revealed no significant difference between antibiotic concentrations in all samples from Chirapatre and Ahensan community, indicate similar features among these communities. The Chirapatre and Ahensan communities have waste stabilization ponds connected to residential houses of 1800 and 1500 population respectively. There are no small or large industrial activities in these communities which would introduce pharmaceutical waste into the rivers. On the other hand, Asafo community waste stabilization pond which is connected to the residence and offices in the city center of over 35000 populations also receives waste from Golden Tulip Hotel and Kumasi Polytechnic which operate a polyclinic for the community.

4.5.3 Occurrence of antibiotics in rivers

K'oreje et al. (2012), analysed river samples from Kenya and detected metronidazole up to 700 ng/L. The concentrations found in this study were up to 363 ng/L (Table 4.2) and were lower than those reported from Kenya. Ciprofloxacin, was not detected in river samples from New Mexico (Brown et al., 2006) but detected in US streams with concentrations up to 30 ng/L (Kolpin et al., 2002). Calamari et al., (2003) also reported concentrations of ciprofloxacin up to 26.2 ng/L in surface water samples. The high concentrations up to 1168 ng/L (Table 4.2) of ciprofloxacin found in river sample in this study could be due to rate of consumption in the country. Ciprofloxacin is the second most purchased drug in the country (Tagoe and Attah 2010) and a survey in Kumasi, Ghana, shows that rivers or streams in the country are heavily polluted with raw sewage (Cornish and Aidoo, 2000; Cornish et al., 2001) because sewage is either discharged directly into rivers and streams or is collected from septic tanks and then disposed-off to waterways (Keraita et al., 2008). Hirsch et al., (1999) and Kolpin et al., (2002), found erythromycin in streams with concentrations up to 1700 ng/L. In this study, erythromycin, was detected with concentrations ranging from 7.0 to 1149 ng/L (Table 4.3). The maximum concentration found in this study was slightly lower than maximum reported elsewhere.

Tetracyclines are a large family of antibiotics, characterized by persistence and low mobility which account for their paucity in surface water. Tetracyclines are relatively inexpensive, hence they tend to be used as first–line antibiotics. Kolpin et al., (2002), found tetracycline, chlortetracycline, and oxytetracycline in streams with concentrations up to 110 ng/L, 690 ng/L and 340 ng/L respectively. These concentrations were higher than those found in the present study. Yan et al., (2013), found doxycycline up to 5.63 ng/L in Yangtze Estuary, China. The maximum concentration of doxycycline found in the present study (68 ng/L) (Table 4.2) was higher than that reported in China.

Hirsch et al., (1999) found sulfamethoxazole up to 480 ng/L in surface water. K'oreje et al. (2012), also found sulfamethoxazole and trimethoprim in Kenya river samples up to 20,000 ng/L and 5,000 ng/L respectively which were higher than those recorded in the current study being 2,861 and 868 mg/L respectively (Table 4.2). Sulfamethoxazole and trimethoprim has also been detected by some other researchers (Zhang and Zhou, 2007; Zuccato et al., 2010), but at much lower concentrations, i.e. lower than 100 and 20 ng/L, respectively.

In the river receiving the effluent from Ahensan STP, upstream sampling points showed lower concentrations of selected antibiotics studied than the midstream, downstream and effluent samples (Fig. 4.6a). This could suggest less pollution of the river upstream. However, the concentrations of antibiotics in the downstream were higher than those of upstream and midstream (Fig. 4.6a). This could be due to pollution of streams that join the river that was sampled downstream. Through field survey, it was realized that, untreated sewage sludge was being discharged into the stream that join the sampling point downstream.

4.5.4 Occurrence of antibiotics in irrigation water

Calamari et al., (2003) also registered ciprofloxacin concentration up to 26.2 ng/L in surface water samples in Italy. The concentrations of ciprofloxacin found in surface water samples which are mostly used for vegetable irrigation in the present study were up to 146 ng/L, which was higher than ciprofloxacin concentration of up to 30 ng/L found in US surface water (Kolpin et al., 2002). Hirsch et al., (1999) and Kolpin et al., (2002), found erythromycin in surface water with concentrations up to 1700 ng/L. In this study, erythromycin, was detected with concentrations ranging from 6.7 to 136 ng/L, indicating that the maximum found in this study was lower than maximum reported elsewhere.

(Kolpin et al., 2002), found tetracycline, chlortetracycline, and oxytetracycline in streams with concentrations up to 110 ng/L, up to 690 ng/L and up to 340 ng/L respectively. These concentrations were higher than ones found in this study (Table 4.4 and Figs. 5a and 5b). Yan et al., (2013), found doxycycline concentration in Yangtze Estuary, China up to 5.63 ng/L. The maximum concentration of doxycycline found in this study was higher than that reported in China. Hirsch et al., (1999) found sulfamethoxazole in surface water up to 480 ng/L. This level found in is extremely higher than the ones recorded in this study (Table 4.4 and Figs. 5a and 5b). The detection of these compounds in surface water has been reported by other authors as well (Zhang and Zhou, 2007; Zuccato et al., 2010), but at much lower concentrations, i.e. lower than 100 and 20 ng/L respectively.

No significant differences between irrigation water and river implies that their concentrations do not vary. This could be true because the surface water used by the vegetable farmer is only a few meters from the river. In fact, most of them are the rivers that have been directed into the farms. Interestingly, during the dry season the farms are moved closer to the rivers for proximity to water and when rain set in the whole area is flooded. Eventually, there could be contamination of surface water used for vegetable production.

PCA between Ahensan irrigation water (A), and Chirapatre irrigation water (E), revealed correlation of erythromycin, ciprofloxacin, trimethoprim and metronidazole to irrigation water from Chirapatre sampling site (Fig. 4.5). These antibiotics are used to treat sexually transmitted infections and skin infections. Unfortunately, there is lack of data on antibiotics usage in Ghana, which could be used to explain the correlation of these antibiotics with Chirapatre community.

There could be likelihood of vegetables exposure to these antibiotics in Chirapatre community and warrant further study into the possibility of the vegetables grown in these areas being contaminated with antibiotics.

Risk quotients calculated in this study were all below 1. According to risk ranking criterion (Hernando et al., 2006), except ciprofloxacin antibiotics (RQ = 0.13) which showed median risk to aquatic algae, rest of the antibiotics study showed minimal risk. The result in this study was lower to a study focused on the occurrence of pharmaceuticals in water from Pego-Oliva Marsh, Spain, which found ciprofloxacin RQ to be 6.9 in algae (Vazquez-Roig et al., 2012). Previously, other studies have reported high RQs in surface water due to the presence of high concentrations of sulfamethoxazole (García-Galán et al., 2011), amoxicillin and oxytetracycline (Jones et al., 2002). This risk evaluation has its limitations, such as the lack of long-term toxicological studies and the unfeasibility to carry out chronic studies during the lifespan of the organisms (especially in fishes), but on the other hand since mixture of compounds with the same pharmacological mechanism is present in waters, synergistic effects could be expected (Gros et al., 2010), hence the real hazard may be greater than that calculated. Nevertheless, the presence of antibiotics in the environment is not limited only to an ecological problem but most importantly to antibiotics resistance. Antibiotics resistance due to bacteria exposure to sub-MIC could occur in the study area, since Gullberg et al., (2011), found minimum selective concentration (MSC) for antibiotic resistant mutant with ciprofloxacin to be 100 ng/L and 15 µg/L for tetracycline. The fact that antibiotic levels several hundred-fold below the MIC of the susceptible strains can select resistant bacteria means that the ciprofloxacin concentrations recorded in this study being much higher than the traditional selective window could cause antibiotics resistance. Another route of exposure could be through indirect ingestion of low quality water by vegetable farmers. Vegetable farmers in Ghana, uses water cans

for irrigation and obviously wade in the surface water when fetching (Drechsel and Keraita, 2014), these could likely expose them to ingesting low quality water contaminated with antibiotics. Further work would be required to determine the extent of exposure and the risk associated. Since the low quality water is used for vegetable irrigation, and greenhouse study performed in Ghana on vegetables irrigated with antibiotics spiked water were able to take up tetracycline and amoxicillin up to respectively 36.8 and 45.2 ng/g (Azanu et al., 2016b), there is a need to investigate the possibility of contamination of vegetables with antibiotics grown in the study area.

4.6 CONCLUSION

This current study clearly revealed the presence of 12 antibiotic compounds in environmental water samples from Kumasi, Ghana. All antibiotics indicate a distinct difference between untreated wastewater and other water bodies, with no significant differences among other water bodies. The occurrence of antibiotics in the environment could most likely be as a result of misuse and waste disposal challenges of human medicine in Ghana. The antibiotics found with high concentrations in all the samples are sulfamethoxazole, erythromycin, ciprofloxacin, cefuroxine and trimethoprim, hence could also be considered as or add to the list of potential critical compounds from an environmental risk point of view in Ghana. Low quality water used for vegetable irrigation in Kumasi, Ghana has been found to contain antibiotics up to 0.2 ppb, there is a need for further work to determine if these antibiotics have not been taken by the vegetables.

CHAPTER FIVE

5. GREENHOUSE UPTAKE STUDIES OF ANTIBIOTIC BY PLANTS

5.1 SUMMARY

This study investigates the capacity of carrot (*Daucus corota* L.) and lettuce (*Lactuca sativa* L.), to uptake tetracycline and amoxicillin from irrigated water. This would help assess indirect human exposure to antibiotics through consumption of uncooked vegetables. Antibiotics in potted plants were extracted using accelerated solvent extraction and analyzed on a liquid chromatographtandem mass spectrometer. The plants took up the antibiotics from water in all tested concentrations between 0.1 and 15 mg/L. Tetracycline was detected in all samples, at concentrations ranging from 4.4 to 28.3 ng/g in lettuce and 12.0 to 36.8 ng/g in carrots. Amoxicillin showed absorption with concentrations ranging from 13.7 ng/g to 45.2 ng/g for all the plant samples. The mean concentration of amoxicillin (27.1 ng/g) in all the samples was significantly higher ($p = 0.0003$) than that of tetracycline (20.2 ng/g) indicating higher uptake of amoxicillin than tetracycline. The concentrations of antibiotics found in carrot were higher than lettuce and was significant different ($p = 0.003$). Long-term toxicity due to exposure to sub-minimal inhibitory concentration antibiotics have been shown to promote resistance. This suggests that the low antibiotic concentrations found in plants are important for enrichment and maintenance of resistance in bacterial populations.

5.2 OBJECTIVES

The objective of the present study was therefore to investigate the capacity of carrots and lettuce, two plants that are usually eaten raw, to uptake tetracycline and amoxicillin (two commonly used

antibiotics) from irrigated water to help identify indirect human exposure to antibiotics through consumption of uncooked vegetables.

5.3 METHODOLOGY

5.3.1 Chemicals and reagents

Tetracycline hydrochloride (CAS #: 60-54-8, >96% pure) was obtained from Sigma-Aldrich (Steinheim, Germany) while amoxicillin trihydrate (CAS #: 267-87-780, 98% pure) was obtained from Fluka (Steinheim, Germany). The relevant physicochemical properties of the studied antibiotics are presented in Table 2.1. The deuterated standard (internal standard), d_3-trimethoprim, was purchased from Qmx Laboratories (Thaxted, UK). Methanol (HPLC grade) was obtained from Lab-Scan (Gliwice, Poland), formic acid (98-100% pure, Ph Eur) was from Merck KGaA (Darmstadt, Germany). Milli-Q water was produced in-house with a Milli-Q water gradient system (Millipore, Bedford, Massachusetts, USA). Stock solutions were prepared with methanol and stored in freezer at -18 °C.

5.3.2 Test Soils

Test soil used was sterilized soil obtained from an arable vegetable farmland (GPS point: 1.57755 E, 6.65638 N) in Chirapatre township in Kumasi, Ghana (Fig. 5.1). Soils were air-dried and passed through a 2 mm screen and mixed thoroughly prior to characterization and use in the uptake studies. Soil pH was determined using the ratio of 1: 2.5 (w/v) soil: water (Thomas, 1996). Particle size

distribution was determined using hydrometer method (Gee and Bauder, 1986). Organic matter was determined by dichromate oxidation method (Nelson and Sommers, 1996).

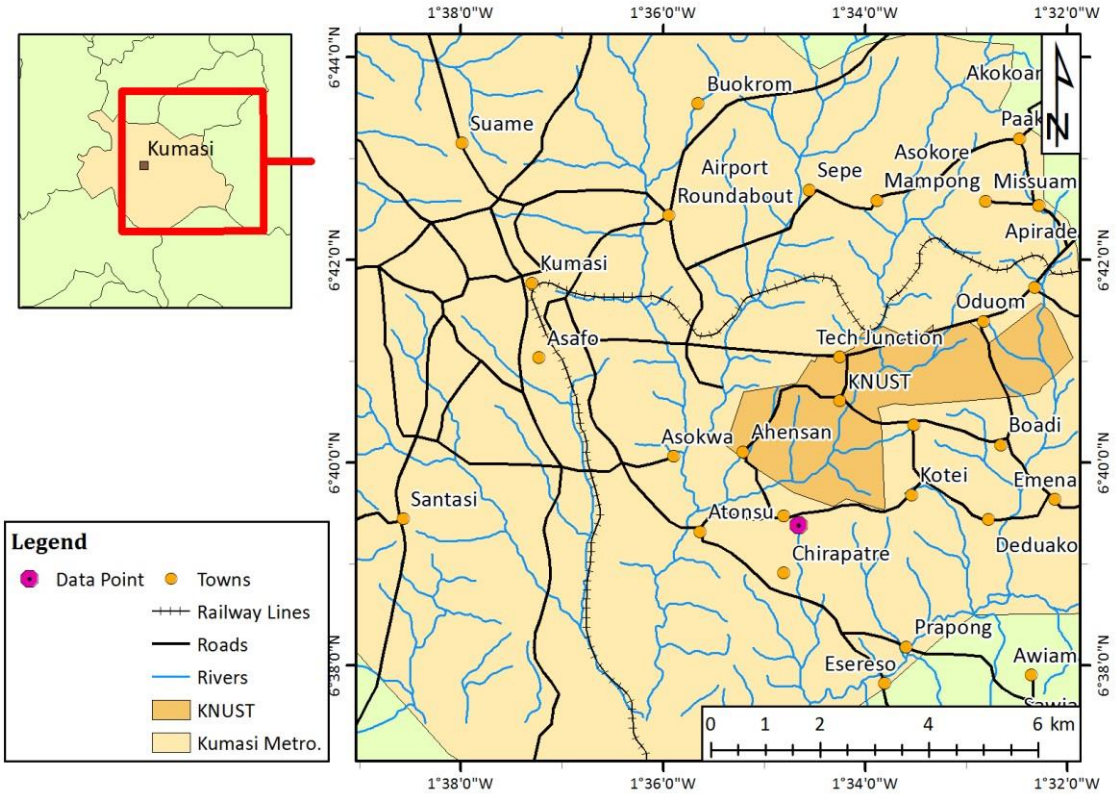


Fig. 5.1 Map of Kumasi showing soil sampling area

5.3.3 Uptake Studies

One-week old seedlings of lettuce and seeds of carrot (16 each) were planted into 4 kg aliquots of soil placed in porous plastic pots (15 cm diameter × 14 cm deep). The plants were grown in a greenhouse under controlled conditions: 50% relative humidity and a temperature of 31 °C and 28 °C during the 12:12 light: dark regime. The transplanted lettuce seedlings were watered for 3 days with tap water to get adjusted to the new soil. The carrot seeds were watered for 60 days for the seeds to germinate and the seedlings to grow to the point of forming the tubers. During the 30 days

preceding maturation and harvesting, the plants were watered twice a day with 420 mL of spiked distilled water (210 mL in the morning and 210 mL in the evening). Each pot was separately irrigated with either 0.1, 1.0, 10.0 or 15.0 mg/L of the antibiotics. The spiked water was poured directly onto the soil at the base of the plant. Four replicates were done for each of the four concentration points and for each of the antibiotics. A control batch was only irrigated with distilled water. At maturation (40 days for lettuce and 90 days for carrot), all plant samples were harvested, washed with distilled water and dried on an adsorbent paper according to Dolliver et al.
(2007). The plant samples were then bulked and kept in a refrigerator at -4 °C before shipping to Denmark where they were stored at -18 °C until use.

5.3.4 Extraction of compounds for chemical analysis

The tubers of carrot and leaves of lettuce samples separately chopped and frozen at -18°C were freeze-dried for 48 h using a Heto FD3 lyophilisor (Heto Lab equipment, Allerød, Denmark) coupled to a roughing pump (Edwards, UK). The system was operated between 0.090 to 0.100 torr at - 48 °C. The freeze-dried samples were then ground thoroughly with a sterile pestle. Pressurized liquid extraction (PLE) was performed on an Accelerated Solvent Extractor, ASE 200 from Dionex (Sunnyvale, CA, USA). A Dionex cellulose filter paper (1.98 cm diameter) was introduced inside the 11 mL extraction cell and gently located by a plunger into the cell's base to avoid sample aggregation, prevent clogging of the extraction cell and allow a greater exposure surface area and thereby improved contact between solvent and matrix. A 0.1 g aliquot of sample was blended gently in a 5 g Ottawa sand (20-30 mesh, AppliChem, Darmstadt, Germany) and placed inside the extraction cell. Another Dionex cellulose filter paper was placed above the packing and 50 µL of 0.4 µg/mL d_3–trimethoprim (internal standard) was added to the filter prior to extraction. Internal

standard was spiked in all PLE extraction cells equivalent to a final amount of 20 ng to correct for any losses during sample preparation and for any variability in injection volume. The extracting solvent was 100% Milli-Q water and the extractor was operated at 70 °C with one cycle for a static period of 10 min. At the end of each extraction a total extract volume of approximately 30 mL was obtained.

The PLE extracts were cleaned up on a reversed phase solid-phase extraction (SPE) using Oasis HLB cartridges (hydrophilic-lipophilic balance, 200 mg sorbent, 30 µm, 6 cm^3) purchased from Waters Oasis (Massachusetts, USA). The SPE cartridges were conditioned with 3 mL MeOH followed by 3 mL distilled water. A 30 mL portion of extract was loaded on the SPE the columns at a flow rate of 1.5 mL/min and then were washed with 3 mL of 5% MeOH in water. The sorbents were allowed to dry for a couple of minutes under vacuum before the antibiotics were eluted with 3 mL MeOH acidified with 0.1% formic acid at a flow of approximately 1 mL min^{-1}. Eluates were evaporated to dryness under a gentle flow of nitrogen at 30 °C and then reconstituted in 1 mL 1% MeOH into brown flat-cap HPLC-vials for analysis.

5.3.5 LC-MS Analysis

The liquid chromatographic system consisted of an Agilent 1290 Infinity Binary System (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a degasser, a cooled autosampler (4 °C), and a column oven (30 °C). The chromatographic separations were achieved by use of a reversedphase column (Kinetex® Biphenyl 100Å column, 2.1 mm x 50 mm x 2.6 µm), coupled to a guard column (Ultra cartridges BP, 2.1 mm x 2.6 µm), both from Phenomenex ApS, (Milford, MA, USA) and application of binary gradient flow rate of 400 µL/min at 30 °C. The injection

volume was set at 5 µL. The mobile phase A consisted of water acidified with 0.1% formic acid whereas mobile phase B consisted of MeOH acidified with 0.1% formic acid. The initial proportion between the mobile phases was 90% A and 10% B. This gradient was changed linearly in 2.5 min to 60% A and 40% B. This proportion was soon changed in a 1 min linear gradient from 2.5 to 3.5 min to 5% A and 95 % B. This gradient was maintained for 1 min followed by a 1 min linearly gradient ending with the initial conditions. This gradient was then maintained for 2 min resulting in a total analysis time of 7 min.

Mass spectrometry was performed with an AB SCIEX QTRAP® 4500 System with CEM detector (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source (Turbo Ionspray). For MS detection, electrospray ionization was performed in positive mode (ESI+) and the temperature was set to 300 ˚C with a nebulizer gas flow of 8 L/min, curtain gas flow of 12 L/min and collision gas flow of 6 L/min and ionspray voltage set to 5000 V.

5.3.6 Optimization of LC-MS/MS

Optimization of the mass spectra included enhancement of the potential settings by direct infusion of all the individual compounds and for some analytes selection of new product ions by product ion scan. The improved parameters for the final LC-MS/MS method including retention times, and mass-to-charge values for precursor ions and product ions are all listed in Table 5.1. During mass spectrometry (MS) determination, parameters such as declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were optimized. The entrance potential (EP) was set at 10 V for all compounds.

Table 5.1. MS instrument parameters

Compound	Retention time, t_r (min)	Precursor ion (m/z)	Product ion quantifier /qualifier (m/z)	CE (V)	CXP (V)	DP (V)	EP (V)
Tetracycline	2.85	445.1	410.05/427.1	26/18	21/18	50/50	10/10
Amoxicillin	2.12	398.3	159.9/348.8	27/22	12/14	45/45	10/10
d_3-Trimethoprim	2.31	294	123/230	44/44	13/13	75/75	10/10

The MS system was set to operate in the multiple reaction monitoring (MRM) mode with the MS chromatogram. Collection and treatment of data were performed using Analyst v. 1.4.2 software (Applied Biosystems) and a Savitzky-Golay smoothing factor at 3 on a Windows XP platformbased data-processing system.

5.3.7 Validation of analytical Procedure

The analytical method was validated in compliance with the requirements in standard guidelines (European Medical Agency 2012; ICH Harmonised Tripartite Guideline 2005; U.S. Food and Drug Adminstration 2001). The linear calibration curves were constructed by analyzing standard solutions ranged from 0.01 to 1000 ng/mL followed by calculating the ratios of analyte peak area to that of the internal standards. Precision was determined by injecting a 0.01 ng/mL tetracycline and amoxicillin standard solution for 8 times. The limit of detection (LOD) and limit of quantification (LOQ) of the HPLC-MS/MS system was determined from the standard deviation (σ) of the response from the lowest calibration standard (0.01 ng/mL) injected 6 times and by the slope (S) of the calibration curve. Matrix effect (recovery) was studied. Matrix used was organic Iceberg lettuce (Lot. Number: ES-ECO-024-MU) from Spain and organic carrot (Lot. Number: DK-ØKO-100) from Denmark. These were bought from a supermarket in Copenhagen, Denmark,

washed and freeze dried overnight. The temperature and pressure before the samples were removed from the freeze drier are -48° C and 0.075T. The experiment for each matrix included twelve blank samples, i.e. matrix sample which is expected not to contain the analytes of interest. A 0.1 g sample was weighed and mixed with 5 g of Ottawa sand. The samples were packed in PLE extraction cells. The first four samples were spiked (before the run of PLE) with 20 µL 5 µg/mL TC, and spiked with 50 µL 0.4 µg/mL d3 – Trim before LC determination. The subsequent four samples were post-spiked (before the LC determination) with 20 µL 5 µg/mL TC, 50 µL 0.4 µg/mL d3 – Trim and 50 µL 0.4 µg/mL Trim. The last four samples were pre-spiked with 50 µL of 0.4 µg/mL d3 – Trim before the PLE and spiked with 20 µL 5 µg/mL TC before the SPE and finally with 50 µL 0.4 µg/mL Trim before the LC determination. The concentration of tetracycline and amoxicillin in the samples were determined on calibration curves constructed for each individual analyte. Quality control (QC) samples were run in parallel during the quantification process. Positive controls consisting of matrix spiked (fortified) with antibiotic-mix and internal standard were used whereas negative controls consisting of matrix and internal standards were used to exclude possible procedural contaminations.

5.3.8 Statistical analyses

The results obtained were subjected to statistical evaluation. Parameters (mean, standard deviation and coefficient of variation were evaluated with Microsoft Office Excel 2013 (Version 15, Microsoft, USA). One-way ANOVA with Tukey's Honesty Significant Difference (HSD) were performed to test the differences between the plants, and antibiotics. The P-value was considered statistically significant at $P<0.05$. Pearson's correlation coefficient was performed as a measure of

dependence between antibiotics studied and soil properties. The closer the coefficient is to either −1 (decreasing linear relationship) or 1 (direct linear relationship), the stronger the correlation between the variables. The statistical analysis was performed by using GraphPad Prism version 5.01 for Windows (GraphPad Software Inc., USA).

5.3.9 Estimation of Daily Intake

The estimated daily intake (EDI) of antibiotics was dependent on both the antibiotics concentration in vegetables and the amount of consumption of the respective food. The food consumption rates for lettuce and carrot in Ghana are estimated to be 0.013 g/kg body weight/day and 0.010 g/kg body weight /day respectively (Amoah et al., 2007). The EDI was obtained by multiplying the median concentration (ng/g) of various antibiotics in lettuce and carrot with their respective food consumption rate (Darko and Akoto, 2008).

5.3.10 Estimation of bioconcentration factor (BCF)

Bioconcentration factor (ratio of contaminant concentration in an organism to contaminant concentration in the surrounding medium) was determined by a linear regression at different concentration levels (Trapp and Legind, 2011). The slope of the regression between soil concentration as predictor variable and plant concentration as estimated variable can be interpreted as the BCF plant to soil, while the y-axis intercept can be interpreted as the constant background concentration due to uptake from air (Trapp and Legind, 2011).

5.4 RESULTS

5.4.1 Quality controls

The precision expressed as coefficient of variation of amoxicillin was 2% and that of tetracycline was 3%. The detection limits were 0.1 ng/L for both tetracycline and amoxicillin. The absolute recovery for tetracycline was 76 % for lettuce and 77.6% for carrot with relative recoveries of 91.8% for lettuce and 92.6 % for carrot. For amoxicillin the absolute recovery was 70.8% for lettuce and 75.6 % for carrot. Their relative recoveries are 86.1 % for lettuce and 91.8% for carrot (Table 5.2).

Table 5.2. Result for validation of analytical procedure for determination of TC and AMX

Analyte	Linearity (r^2)	LOD (ng/L)	LOQ (ng/L)	Accuracy (%CV)	Precision (%CV)	Matrix Effect			
						AR ± RSD (%)		RR ± RSD (%)	
						Lettuce	Carrot	Lettuce	Carrot
TC	0.99	1	2	3	3	76.0±24.9	77.6±10.0	91.8±7.4	92.6±17.6
AMX	0.99	1	2	4	2	70.8±3.4	75.6±3.8	86.1±7.3	91.8±7.4

Where limit of detection (LOD), and limit of quantification (LOQ) were reported in ng/L. Precision and accuracy were reported in percent coefficient variation (%CV), absolute recovery (AR) and relative recovery (RR) were reported with their relative standard deviation (RSD)

5.4.2 Uptake of antibiotics by plants

5.4.2.1 Tetracycline uptake by plants

A bar chart showing the concentrations of tetracycline in carrot and lettuce watered with various spiked concentrations is shown in Fig. 5.2. The highest concentration of tetracycline (34.10 ng/g)

was found in carrot plant irrigated with 15.0 ppm tetracycline spiked solution. Lettuce plant irrigated with 0.1 ppm tetracycline spiked solution recorded the least concentration of 4.4 ng/g. In general tetracycline concentrations found in carrot plants were higher and statistically significant ($p< 0.0001$) than concentrations found in lettuce plant. A Turkey's HSD test of significance among lettuce and carrot irrigated with the same spiked concentration reveals that there is significant differences ($p <0.005$) in concentrations found in various plant.

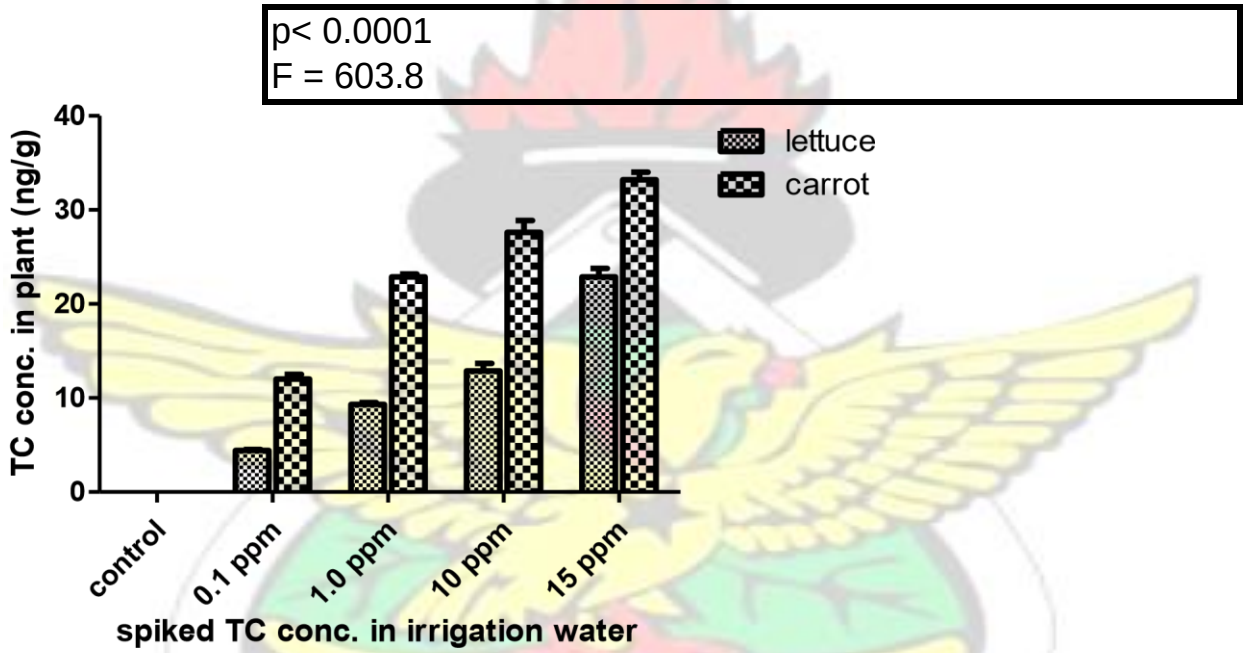


Fig. 5.2: Concentrations of tetracycline found in lettuce and carrot plants grown on Soil 1 using spiking concentration of 0.1, 1.0, 10 and 15 mg/L. The error bars are standard deviation.

5.4.2.2 Amoxicillin uptake by plants

The uptake of amoxicillin by lettuce and carrot irrigated, increased with serial increment of concentration in spiked water (Fig. 5.3). Generally, the concentration of amoxicillin found in carrot were higher and statistically significant ($p < 0.0001$) than that found in lettuce plants. This trend is similar to what was found with tetracycline, however Turkey's HSD test among various spiked

concentrations showed that the difference in concentrations of lettuce and carrot for spiked concentrations of 0.1 and 1.0 ppm were not statistically significant ($p>0.05$).

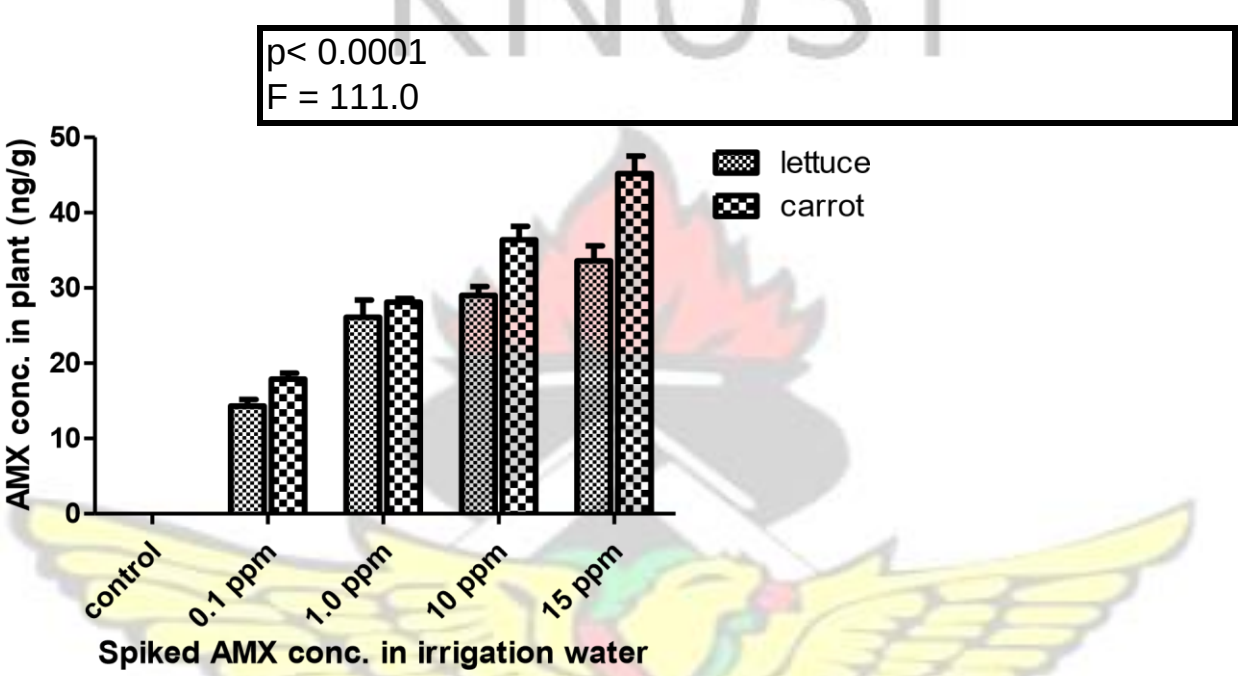


Fig. 5.3: Concentrations amoxicillin found in lettuce and carrot plants grown on Soil 1 using spiking concentration of 0.1, 1.0, 10 and 15 mg/L. The error bars are standard deviation.

5.4.3 Antibiotics effect on plant growth

Amoxicillin was taken up more than tetracycline. Student t-test performed indicated that there is significant difference ($p = 0.0003$, $t = 3.9$) between the mean concentration of tetracycline and amoxicillin (Fig. 5.4a). In addition, carrot plant accumulated more antibiotics than lettuce. The difference between their mean concentrations was statistically significant ($p = 0.003$, $t = 3.1$) (Fig. 5.4b).

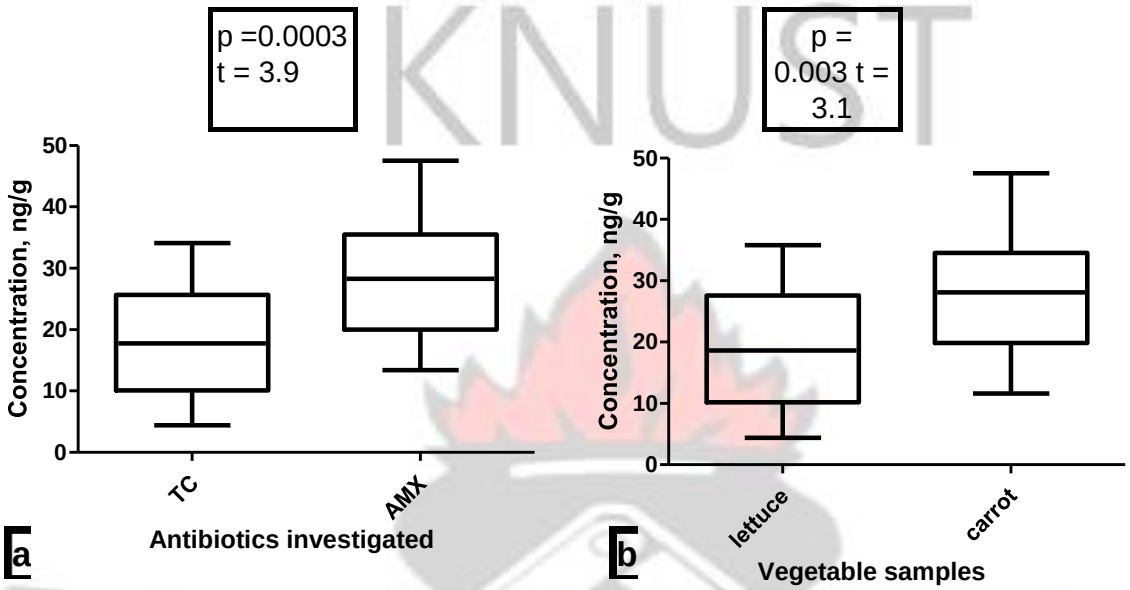


Fig. 5.4 Box-and-whisker plots of uptake studies
(a) concentrations of tetracycline and amoxicillin found in plants studied; and (b) concentrations of antibiotics found in lettuce and carrot plants. p, statistical significance of these factors from ttest.

5.4.4 Effect of soil properties on antibiotics uptake

The soil pH was 7.25. Taxonomic classification of the soil sample was silty clay loam. The % clay, % silt and % sand was 26.5, 51.3 and 14.1respectively. The available phosphorus concentration in the soil was 5.4 mg/L and % organic carbon was 0.88.

To understand if there is any relationship between the soil properties and antibiotics uptake, correlation matrix was performed. Pearson's correlation analysis (Table 5.3) revealed that, tetracycline and amoxicillin concentration in plant, have a strong positive correlation (0.581) and was statistically significant ($p < 0.05$). Tetracycline concentration in plants also showed weak

positive correlation with % sand and cation exchange capacity (CEC). Amoxicillin on the other hand, correlated positively with % clay, % silt and available phosphorus but correlated negatively with % sand, % OC and CEC. However, these correlations were weak and not statistically significant ($p > 0.05$).

Table 5.3: Pearson correlation matrix of soil properties and antibiotics

	TC (ng/g)	AMX (ng/g)
TC (ng/g)		
AMX (ng/g)	0.581*	
% Sand	0.216	-0.220
% clay	-0.216	0.220
% silt	-0.216	0.220
%OC	0.216	-0.220

pH	-0.216	0.220
Available P (mg/L)	-0.216	0.220
CEC (mg/L)	0.216	-0.220

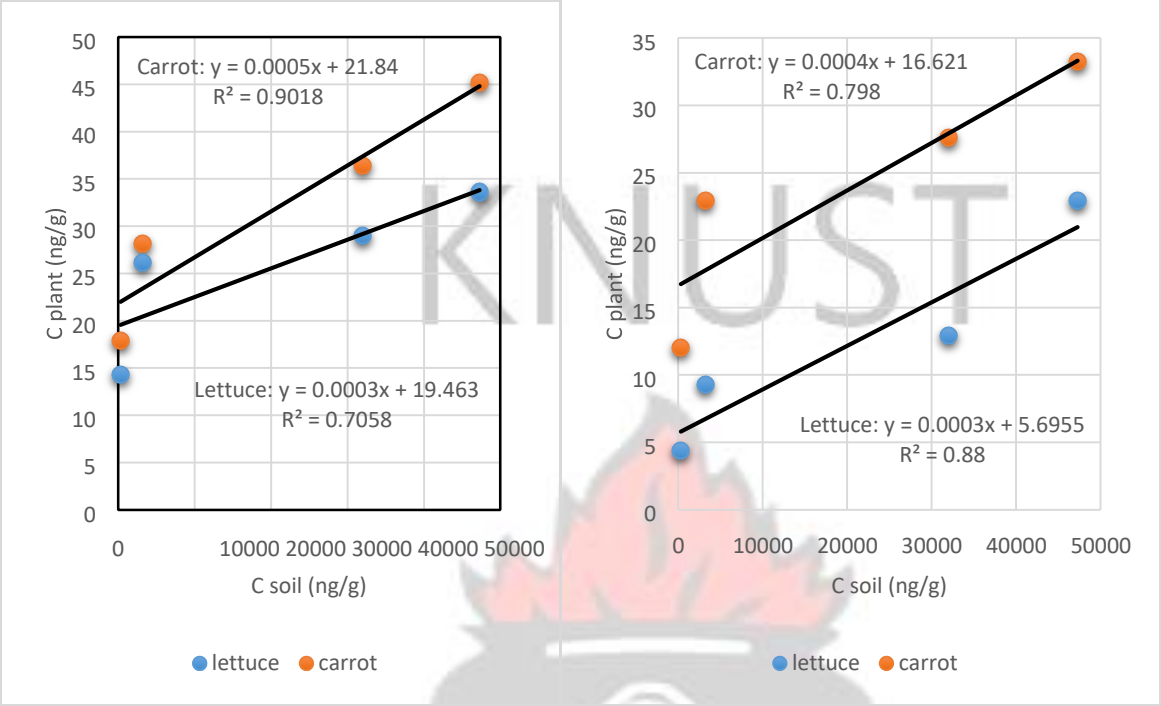
* represent correlation values that are significant at $p < 0.05$

5.4.5 Health risk estimation

The acceptable daily intake (ADI) values for tetracycline and amoxicillin had been established by (JECFA, 2010) and are defined as 0−30 μg/kg body-weight (bw)/d for tetracycline and 0− 0.7 μg/kg bw/d for amoxicillin. The ADI value indicates the level of a chemical that can be ingested daily over a lifetime without health risk. In this study, estimated daily intakes of tetracycline for the consumption of lettuce and carrot plants were 0.0002 μg/kg bw/d and 0.0003 μg/kg bw/d respectively. On the other hand, EDIs of amoxicillin were 0.0004 μg/kg bw/d and 0.0003 μg/kg bw/d for the consumption of lettuce and carrot plants respectively.

5.4.6 Uptake processes: Bioconcentration factor

The relationship between concentrations in plant and soil was quantified by a linear regression at different concentration levels. The BCF recorded for lettuce was 0.0003 for tetracycline and amoxicillin antibiotics, while as BCF for carrot was 0.0005 for amoxicillin (Fig. 5.5 a) and was 0.0004 for tetracycline (Fig. 5.5 b).



a b

Fig. 5.5: Graph of concentrations in soil and plants tissues (a) Amoxicillin (b) tetracycline

5.5 DISCUSSION

5.5.1 Antibiotics uptake by plants

Earlier studies have shown that plants take up less than 2% of the pharmaceuticals applied to soil (Dolliver et al., 2007; Kumar et al., 2005). In this study, the plants took up to 0.02% of the antibiotics applied to the soil and this is less than the uptake values reported applied. Boxall et al., (2006) found that the total accumulation of sulfamethazine in plant tissue after 45 days of growth was less than 0.1% of the amount applied to soil in manure. Dolliver and colleagues (2007), found sulfamethazine taken up by lettuce leaves, corn seeds and potato tuber tissue, with concentrations ranging from 100 to 1200 ng/g (Dolliver et al., 2007). The present study found the concentrations of tetracycline and amoxicillin in carrot tubers and lettuce leaves tissues ranging from 4.4 to 45.2

ng/g. This range, are lower and could be due to difference in the treatment concentrations used. Dolliver and colleagues applied up to 100 mg/L of sulfamethazine, while in this study, we applied up to 15 mg/L of tetracycline and amoxicillin. Dolliver and colleagues (2007) applied 0, 50, and 100 mg/L of sulfamethazine to a manure at a rate of 56,000 L/ha on corn, lettuce and potato plants and observed in their study that concentrations of sulfamethazine increased with increasing amounts of antibiotics present in the manure (Dolliver et al., 2007). In this current study, lettuce and carrot uptake of tetracycline and amoxicillin antibiotics followed a dose-response effect. This suggests that if plants are exposed to higher concentrations there would be likely to take up higher amounts of antibiotics.

Carrot samples (Figure 5.4b) showed higher average concentration of amoxicillin and tetracycline than lettuce samples (p=0.003). This is interesting because during carrot growth, nutrients and water are provided to the plant by the roots; therefore, active nutrient or water uptake by the tuber is not necessary for plant growth and is likely not a mechanism for antibiotic accumulation in the tuber. We speculate that diffusion through the peel resulted in higher antibiotic accumulation in the tuber. A study by Boxall and colleagues (2006) also found higher antibiotic (diazinon and enrofloxacin) accumulation in carrot peels than in the whole carrots, indicating that antibiotics could be taken up with the outer layer of the carrot. Therefore, peeling of carrots prior to consumption would greatly reduce the potential for exposure.

In this study, the ratio between the measured concentrations of antibiotics in plants and in soil was high at low soil concentrations, and decrease for higher soil concentration. This was comparable studies performed on p,p'-DDT uptake by radishes (Mikes et al., 2009). A plausible explanation for this pattern could be that plants have a limited sorption capacity for organic contaminant, which becomes saturated at higher soil concentrations. Additional, uptake into plants has been found to

be from two different and independent sources, namely from soil and from air and when antibiotics concentration in soil are very low there still is a background contamination of the plant tissue originating from air (Mikes et al., 2009; Trapp and Legind, 2011).

The BCF recorded in this study were about 1000 times lower than BCF obtained for measured concentrations of p,p'-DDT in radishes and in soil (Mikes et al., 2009). The greater the value of the BCF, the more the chemical accumulates in the plants and the higher the risk of exposure to humans. Hence the tetracycline and amoxicillin would be less taken up by plants and would have lower risk of exposure to humans as compared to p,p'-DDT.

5.5.2 Effect of soil properties on antibiotics uptake

The behaviour of antibiotics in the environment is related to a range of factors including the Hbonding potential, cation exchange, cation bridging at clay surfaces and complexation (Tolls, 2001). Amoxicillin concentrations in the plants were higher than that of tetracycline. This could be due to differences in soil sorption coefficients. The sorption coefficient of amoxicillin is smaller than tetracycline in soil (Boxall et al., 2002; Tolls, 2001), which makes amoxicillin more bioavailable. With the soil pH (7.25) being neutral, the test compounds would be predominantly in their neutral forms. The neutral forms of ionisable organic compounds generally favour root uptake and ionisation can reduce their bioaccumulation in plants (Rabølle and Spliid, 2000). The uptake of tetracycline is pH dependent, which is known to be lowest at pH 5.0 and the highest at pH 7.0 (Chitescu et al., 2013). Amoxicillin is in neutral form at the test soil's pH. This means that absorption and transport of the antibiotics in plant could be a passive transport following the partitioned model. Again, tetracycline can form strong complexes with metal cations through its N-functional groups, thus influencing its sorption to the soil components (minerals and organic

matter) (Andreu et al., 2009; Jones et al., 2005). Once adsorbed onto the soils, tetracycline is hardly desorbed (suggested by the soil organic carbon-water partitioning coefficient, Koc value), with only 0.5% to 2.3% released from sandy loam and loamy sand soils (Rabølle and Spliid, 2000). Sorption coefficients of amoxicillin, on the other hand, are very low in soil (Boxall et al., 2002; Tolls, 2001), which indicates that amoxicillin is more bioavailable.

5.5.3 Antibiotics effect on plants growth

The significant difference between the fresh weights of vegetables at various treatments was due to the vast difference in weight of the control to the rest of the spiked samples. This indicates that antibiotic did have a negative effect on the growth of these vegetables. The uptake and effects on plants varies considerably between reports and depends on the antibiotic substance and plant species (Migliore et al., 1995; Patten et al., 1980; Trapp and Legind, 2011).

5.5.4 Human health risk due to estimated daily intake of antibiotics

Comparison of the estimated daily intakes (EDIs) with the ADIs reveals that the EDIs are several thousand times lower than respective ADI for amoxicillin and tetracycline. This suggests that for the studied compounds exposure of consumers to antibiotics via plants is likely to be considerably below the ADI and that the risk to human health in terms of toxicity is probably low. This simplistic risk assessment may, however, to some extend be misleading, because toxicity is not the major health concern related to antibiotics. The main health concern with regards to antibiotics is resistance. Gullberg et al., (2011), found minimum selective concentration (MSC) for antibiotic resistant mutant for tetracycline to be 15 ng/ml. The concentration of tetracycline in carrot (irrigated

with 0.1 mg/l tetracycline solution) samples were up to 15.6 ng/g. This is much higher than the tetracycline MSC observed by Gullberg et al (2011) thus, antibiotic-resistant bacteria could be found in humans exposed to carrot at these concentrations. This simplistic deduction is very conservative, because it assumes that plant material consumed in the diet is derived from crops grown with irrigation water containing 0.1 mg/l antibiotics. Nonetheless, antibiotics concentrations up to 0.2 mg/l for ciprofloxacin has been found in sewage water used for plant irrigation from Mafisa, Tanzania (Møller et al., 2015). This demonstrates that the lowest concentration used in this study is in fact environmentally relevant and exposure via the plant material consumed in the diet could potentially be a significant source of antibiotic resistance. It is possible that chronic exposure to low levels of antibiotics via the plants could select for resistance in the human intestine. Or perhaps, antibiotic-resistant bacteria may be found in soils due to the low concentrations of antibiotics found in the surface water and could potentially be transferred to vegetables which are in close contact with soil.

5.6 CONCLUSION

We have investigated the accumulation of tetracycline and amoxicillin in lettuce and carrot plants. Tetracycline and amoxicillin were found taken up by all the plant in a dose-response scenario. Amoxicillin antibiotics was taken up more than tetracycline which could be due to very low sorption coefficients of amoxicillin in soil and indicates that amoxicillin are more bioavailable. Vegetables irrigated with contaminated water are a potential source for exposure to sub-MIC levels of antibiotics.

CHAPTER SIX

6. SIMULATION OF ANTIBIOTICS UPTAKE BY LETTUCE AND CARROT PLANTS

6.1 SUMMARY

The goal of this study was to construct a simulation model for predicting the uptake of antibiotics by vegetable plants, using STELLA. This model was developed for uptake of tetracycline and amoxicillin antibiotic by lettuce and carrot plants for a simulation period of 30 days. The result of calibration gave a satisfactory correlation with slope approximately being 1, indicating an acceptable calibration. Validation of the model showed an appreciable agreement between simulated and measured data with standard deviation below 40%. Sensitivity analysis identified leaching and evaporation as the most sensitive parameters in the model. These parameters could be determined accurately hence, makes the model easily adoptive for other antibiotics. The uptake model used to predict amoxicillin uptake, gave an acceptable standard deviation. As it is not possible to cover the costs of such a comprehensive analytical program in all cases where these antibiotics are applied or where antibiotics are applied generally, it is assumed that the model due to its transparency can be applied generally by changing the parameters describing the physicalchemical properties of the antibiotics and the site specific parameters and forcing functions.

6.2 OBJECTIVES

The goal of this study was to construct a simulation model for predicting the uptake of antibiotics by vegetable plants, using the commercial available software package STELLA. This model was

conducted to investigate the uptake of antibiotics by lettuce and carrot plants for a simulation period of 30 days. The specific objectives of this study were to: (1) develop a dynamic model for predicting uptake of tetracycline by lettuce and carrot plant; (2) calibrate the model using available experimental data using soil 1; (3) validate the model with experimental data from different soil (soil 2) (4) predict amoxicillin antibiotics uptake by lettuce and carrot and compare with experimental data.

6.3 METHODOLOGY

6.3.1 Experimental uptake study

6.3.1.1 Test Soils

Two test soils were used. The first test soil (Soil 1) was sterilized soil obtained from the Department of Horticulture, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The second test soil (Soil 2) was collected from Karikari Vegetable Farmland (GPS point: 1.57755 E, 6.65638 N) in Kumasi, Ghana. Soils were air-dried and passed through a 2 mm screen and mixed thoroughly prior to characterization and use in the uptake studies. The soil pH was 7.01 and 7.25 for Soil 1 and Soil 2 respectively. Taxonomic classification of the soil samples was loamy fine sand for Soil 1 and silty clay loam for Soil 2. The % clay content was 7.36 for Soil 1 and 26.5 for Soil 2.

6.3.1.2 Greenhouse Uptake Studies

One-week old seedlings of lettuce and seeds of carrot (16 each) were planted into 4 kg aliquots of soil placed in porous plastic pots (15 cm diameter × 14 cm deep). The plants were grown in a

greenhouse under controlled conditions: 50% relative humidity and a temperature of 31 °C and 28 °C during the 12:12 light: dark regime. The transplanted lettuce seedlings were watered for 3 days with tap water to get adjusted to the new soil. The carrot seeds were watered for 60 days for the seeds to germinate and the seedlings to grow to the point of forming the tubers. During the 30 days preceding maturation and harvesting, the plants were watered twice a day with 420 mL of spiked distilled water (210 mL in the morning and 210 mL in the evening). Each pot was separately irrigated with either 0.1, 1.0, 10.0 or 15.0 mg/L of the antibiotics. The spiked water was poured directly onto the soil at the base of the plant. Four replicates were done for each of the four concentration points and for each of the antibiotics. A control batch was only irrigated with distilled water. At maturation (40 days for lettuce and 90 days for carrot), all plant samples were harvested, washed with distilled water and dried on an adsorbent paper according to Dolliver et al. (2007). The plant samples were then bulked and kept in a refrigerator at -4 °C before shipping to Denmark where they were stored at -18°C until use. The antibiotics were extracted using accelerated solvent extraction and analyzed on a liquid chromatograph-tandem mass spectrometer. The details of the methodology could be found in greenhouse uptake study in chapter 5.

6.3.2 Model development

6.3.2.1 Conceptual diagram

Schematic diagram showing processes involved in antibiotics transport/uptake in the soil–plant system with a compartment model for antibiotic transport within a plant system (Fig. 6.1a) and overview of crop-specific plant uptake processes (Fig. 6.1b) were constructed

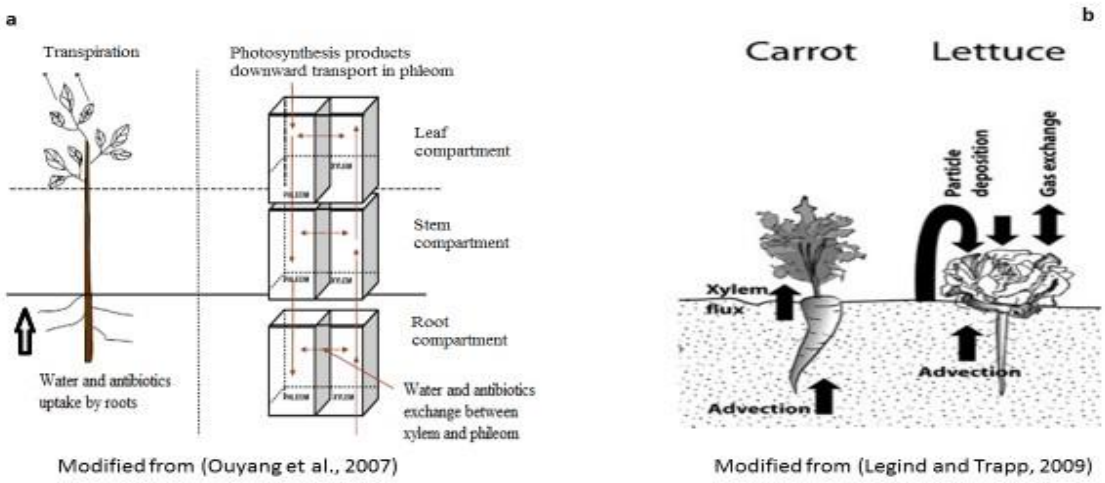


Fig. 6.1: Antibiotics transport and uptake process

(a) Schematic diagram showing processes involved in antibiotics transport/uptake in the soil-plant system with a compartment model for antibiotic transport within a plant system, and (b) overview of crop-specific plant uptake processes

To establish a simulation model with STELLA®, the first step is to develop a conceptual diagram to capture the main processes involved in the uptake of tetracycline antibiotics by plants (Fig. 6.1a, b). The conceptual diagram constructed in STELLA® (isee Systems) (Fig. 6.2), shows the uptake of water and antibiotics processes as forcing functions and the reservoirs (water in soil, antibiotics in soil, antibiotics in soil water and antibiotics in plant) as state variables (Jørgensen and Fath, 2011).

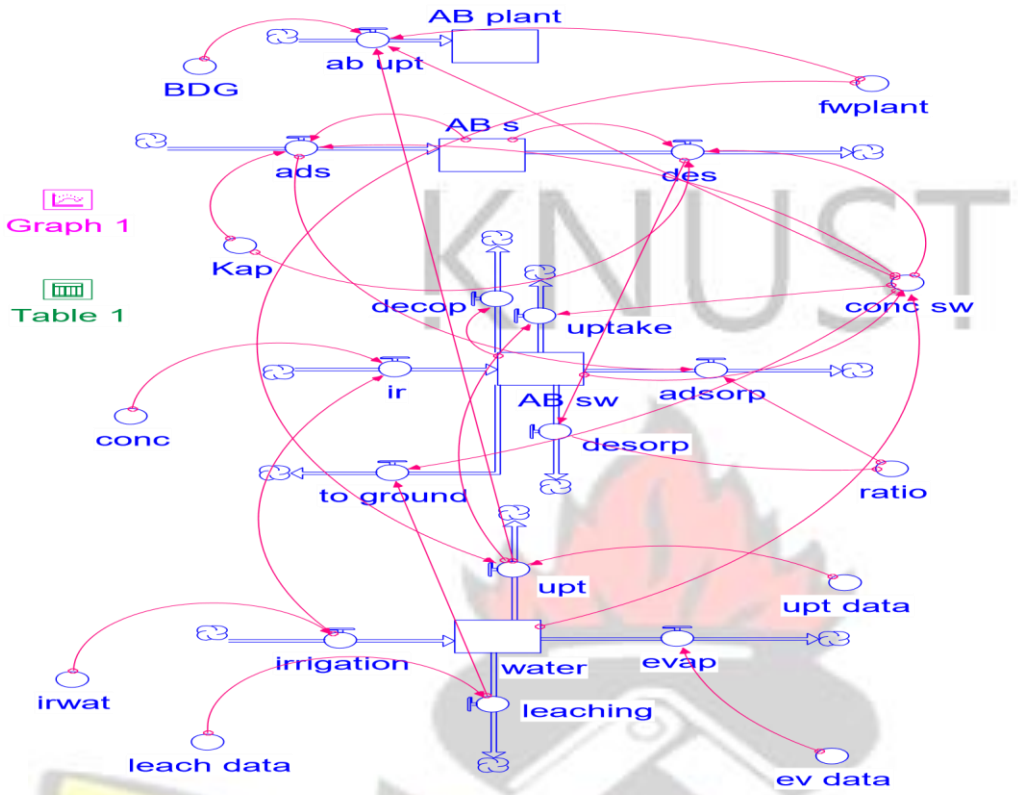


Fig. 6.2: The conceptual diagram constructed in STELLA®

The model was run for 30 days, with the time step (DT) set to 0.02 days. DT refers to the time intervals between calculations in STELLA® (Jørgensen and Fath, 2011; Kumar et al., 2011). Solutions to the differential equations were obtained using the fourth-order Runge-Kutta 4 method when running the model.

6.3.2.2 Model Equations

The basic equations for the state variable and the processes were defined, after setting up the conceptual diagram in STELLA®. The concentration of antibiotics in plant at time 't' was defined in STELLA® as a mass balance differential equation (Jørgensen and Fath, 2011):

AB_plant(t) = AB_plant(t - dt) + (ab_upt) * dt (1)

INFLOW: ab_upt = BDG+(conc_sw*upt*1000/fwplant)

where AB_plant(t) is the concentration of in plant at time 't'. The process of inflow to the plant was defined by multiplying concentration in soil water by root uptake rate and normalizing with fresh weight. The background correction factor (BDG) was added to account for anomalies. The antibiotics in soil at time 't' was defined in STELLA® as a mass balance differential equation:

AB_s(t) = AB_s(t - dt) + (ads - des) * dt

Inflows: ads = conc_sw*Kap-AB_s

Outflows: des = conc_sw/Kap-AB_s

The antibiotics in soil water at time't' was characterized by the following equations in STELLA®

AB_sw(t) = AB_sw(t - dt) + (ir - adsorp - uptake - desorp - to_ground - decop) * dt

INFLOWS: ir = irrigation*conc

OUTFLOWS: adsorp = ads*ratio

uptake = conc_sw*upt desorp =

des*ratio to_ground =

leaching*conc_sw decop =

AB_sw*3.5

The water in soil at time't' was estimated by the following equations:

water (t) = water (t - dt) + (irrigation - evap - leaching - upt) * dt

6.3.2.3 Model parameters

The variables influencing the forcing functions are the evaporation rate (ev data), leaching data (leach data), uptake rate (upt data), volume of irrigation water (irwat), volumetric soil moisture (ratio), concentration of antibiotics in irrigation water (conc), soil adsorption capacity (Kap) and fresh weight of plants (fwplant). The parameters used to construct the model are summarized in Table 6.1.

Table 6.1: List of parameters and data used for calibration

State variables	Items (abbreviation)	Description	Unit	Source
Water	Water	Amount of water in the pot before irrigation	mL	measured
	Irwat	Amount of water used	mL	measured
	Leach data	Amount of leached/seepage	mL	measured
	Ev data	Water evaporated	L/d	calibrated
	upt data	Water uptake by plant rate	mL/d	calibrated
Antibiotics in soil water	Ratio	Soil moisture content	mg/mg	measured
	Ir	Amount of antibiotics in irrigation water	µg	measured
	conc	Concentration of antibiotics in irrigation water	mg/L	measured
	conc sw	Concentration of antibiotics in soil water	µg/ml	measured
Antibiotics in soil	Kap	adsorption capacity	kg/g	calibrated
Antibiotics in plant	Fwplant	Fresh weight of plant	g	measured
	ab upt	Antibiotics uptake	ng/g	calibrated
	BDG	Background correction factor	ng/g	calibrated

The hydrolysis and photolysis rate constants were found experimentally. Eight test solutions (3 replicates), each with a concentration of 1000 ng/mL, were prepared by diluting stock solution of the antibiotics in 100 mL Milli-Q water. Stock solutions of tetracycline were purchased from Fluka. The test solutions were transferred to Erlenmeyer flasks and placed in a climate-controlled cabinet. The temperature of the cabinet was set to 30° C and the samples were exposed to light in a 12:12 h light: dark regime. 1 mL test solution was transferred to a LC-MS vial for analysis at 0, 1.4; 4.5, 23, 67, 73, 94.5, and 119 h. The sample preparation procedure for analysis is described chapter 3. Hydrolysis and photolysis follow first order kinetics and can be described according to $dx/dt = K_1 (\alpha-x)$

where K_1 is the first order rate constant, α is the initial concentration and x is the concentration at time t (Florence, 2006). The first order rate constant for hydrolysis and photolysis combined was found by Eq. (x). From the first order rate constant, the half -life was found from $t_{1/2}= 0.693/K_1$

Tetracycline degraded with a $t_{1/2}$ of 30 minutes by hydrolysis and photolysis.

6.3.2.4 Sensitivity analysis

The parameters influence on the state variables were evaluated by a sensitivity analysis. The analysis was carried out to aid in the model calibration. Changing the value of the parameters by ±10% and then running the model to obtain change in model output. The sensitivity (S) was calculated as the relative change in model output divided by the relative change in the value of the parameter tested.

$S = \Delta(simulated)s/(simulated)c/\Delta P/P$

where Δ(simulated)s is the difference between simulated concentration at changed parameter and simulated concentration at calibrated parameter (simulated)c. ΔP is the change in the value of parameter and P is the calibrated parameter. A parameter with a high S greatly influences the outcome of simulated concentration.

6.3.2.5 Calibration, validation and application

The model was calibrated by adjusting root uptake rate (upt_data) and background correction factor (BDG) so that the modelled concentrations of tetracycline were similar to experimentally measured concentrations for plants grown in the soil 1. The upt. data and BDG are related to plants and the environment and are not compound specific. Validation of the model was performed using experimental data collected from uptake of lettuce and carrot on soil 2 and calibrated parameters. The adsorption capacity, which is soil specific was changed accordingly. The optimized parameters used in validation of the model are listed in Table 6.2.

Table 6.2. Optimized parameters used for validation

Symbol	Tetracycline validation value		Amoxicillin model value	
	Lettuce	Carrot	lettuce	Carrot
Kap	500	500	300	300
Ev data	250	250	250	250
upt data	0.001	0.003	0.001	0.003
Ratio	0.17	0.17	0.17	0.17

The model was used to simulate the uptake of amoxicillin antibiotics uptake by lettuce and carrot. The experimental data on amoxicillin uptake by lettuce and carrot on soil 1 used in model application are also listed in Table 6.3.

Table 6.3. Experimental data used for model calibration and validation of tetracycline, and data for amoxicillin model

Conc. in irrigation water	Tetracycline				Amoxicillin	
	Soil 1		Soil 2		Soil 1	
	Carrot (ng/g)	lettuce (ng/g)	carrot (ng/g)	lettuce (ng/g)	(ng/g)	(ng/g)
0.1 ppm	9.5	10.59	4.4	12	13.7	14.3
1.0 ppm	11.3	11.35	9.3	22.9	25.4	23.7
10 ppm	22.6	19.02	12.9	27.6	27.6	27.5
15 ppm	28.3	23.28	22.9	33.2	33.2	37.1

6.3.3 Statistical Analysis

Calibration and validation data were evaluated by the correlation between the simulated values given by the simulated and measured. Simulated was plotted against measured and fitted to a linear regression described as: $Y(t) = c + mX(t)$ where X is measured, Y is simulated, c is the intercept and m is the slope. A perfect correlation between simulated and measured will have $m = 1$ and c being 0. To test if simulated and measured were significantly different, a two-tailed t-test was performed with a confidence interval of 95%. The standard deviation of the differences between measured and simulated validation result was determined to evaluate the capacity of the model to simulate the uptake of the antibiotics. All statistical analysis was performed using Graphpad Prism version 6.00 for Windows 10 (GraphPad Software, La Jolla California, USA).

6.4 RESULTS

6.4.1 Model calibration

The best calibration result, using concentrations of tetracycline measured in plants grown on soil 1 has been shown in Fig. 6.3 (a-b).

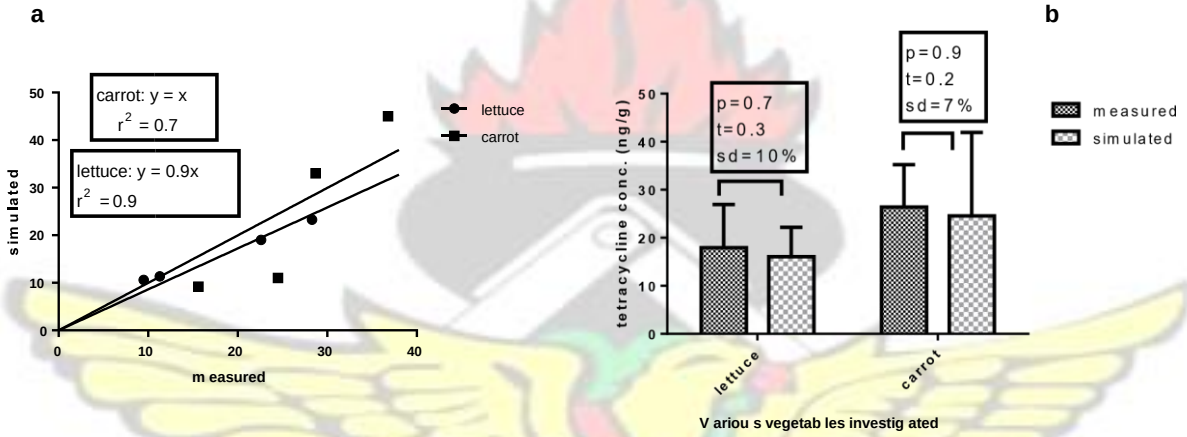


Fig. 6.3: Graphs of calibrated result
(a) scatterplot of showing regression line of antibiotics uptake model calibration result, (b) bar chart of simulated and measured concentration of tetracycline indicating t-test result and standard deviation (s)

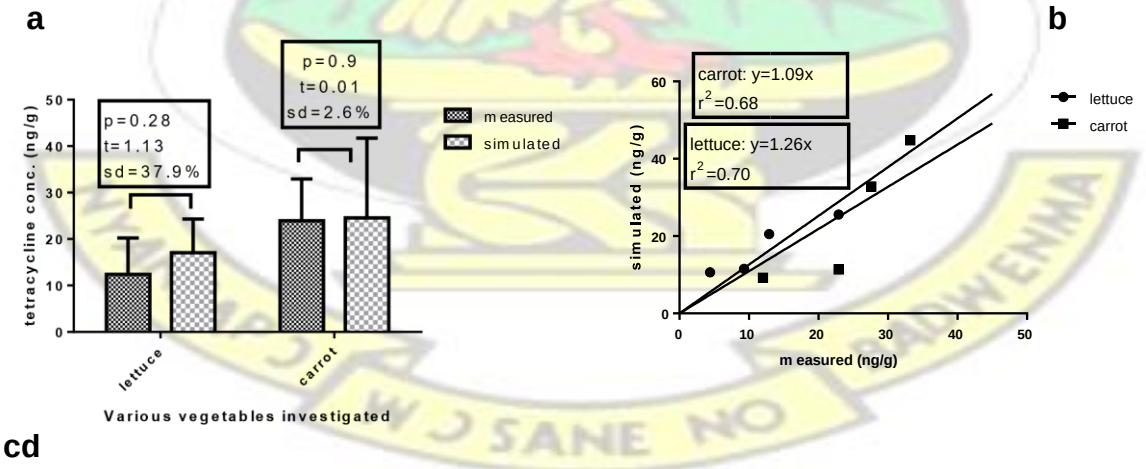
The calibration for tetracycline in carrot gave a satisfactory correlation as the slope was 1 and regression coefficient (r^2) = 0.7 (Fig. 6.3a, Table 6.4). A two tailed t-test on measured and simulated tetracycline in carrot results, with a confidence interval = 95% showed that there was no significant difference between simulated and measured for the calibration (p=0.9) (Fig. 6.3b) and the standard deviation (sd) was = 7 %, giving an acceptable calibration. For the case of tetracycline in lettuce plants, correlation between simulated and measured gave slope to be 0.9 and regression coefficient (r^2) = 0.9 (Fig. 6.3b, Table 6.4). Once again, two tailed t-test on measured and simulated

tetracycline in lettuce results at a confidence interval = 95% showed that there was no significant difference between simulated and measured for the calibration (p=0.7) and standard deviation = 10 %, giving an acceptable calibration also for lettuce. **Table 6.4:** Statistical analysis results

	Calibration			Validation			Amoxicillin model		
	r^2	P	SD (%)	r^2	p	SD (%)	r^2	p	SD (%)
Lettuce	0.9	0.7	10	0.7	0.3	37.9	0.7	0.2	33.4
Carrot	0.7	0.9	7	0.7	0.9	2.6	9.7	0.9	3.4

6.4.2 Validation of model

In order to use the antibiotics uptake model as a tool for prediction, the model must be validated using different set of experimental data. In this study, the model was validated using experimental data (Table 6.2) from a greenhouse studies. The calibrated parameters were used coupled with Kap and ratio values specific to the soil. The scatterplot and bar graphs using concentrations of tetracycline measured in plants grown on soil 2 and simulated data for validation have been shown in Fig. 6.4 (a-d).



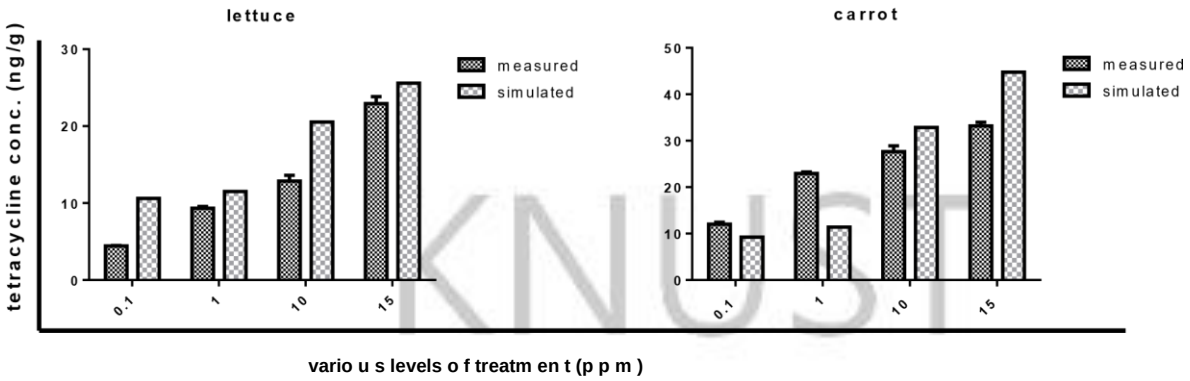


Fig. 6.4 Graph of validated result
(a) shows a bar chart of the validation data indicating, two tailed t-test on measured and simulated tetracycline in carrot and lettuce at a confidence interval = 95% to have no significant difference ($p > 0.05$), (b), shows simulated and measured scatterplot as well as regression line of the correlation between simulated and measured (c) bar graphs of tetracycline measured and simulated in lettuce under various levels of treatment (d) bar graphs of tetracycline measured and simulated in carrot under various levels of treatment.

Fig. 6.4a shows a bar chart of the validation data indicating, two tailed t-test on measured and simulated tetracycline in carrot and lettuce at a confidence interval = 95% to have no significant difference ($p > 0.05$). The mean standard deviation for carrot and lettuce was 2.6 % and 37.9 % respectively (Fig. 6.4a). The Fig. 6.4b, shows simulated and measured scatterplot as well as regression line of the correlation between simulated and measured. The slopes for tetracycline in carrot and lettuces are 1.09 and 1.26 respectively. The regression coefficient (r^2) was 0.7 for carrot and lettuce.

6.4.3 Model sensitivity analysis

Table 6.5 shows the sensitivity of the parameters used in the model by changing the value of the parameters by ±10% and then running the model to obtain change in model output. The sensitivity was performed for six parameters (upt_data, BDG, ratio, Kap, ev_data and leach_data) and ranged

from 0 – 2.48 for tetracycline in lettuce model at -10% change in parameters. However, at +10% change in parameters the lettuce model sensitivity ranged from 0 - 16.34. In the case of tetracycline in carrot model, the sensitivities ranged from 0.002 to 3.35 and 0 to 21.92 for – 10% and +10% change in parameters respectively.

Table 6.5: Results of sensitivity analysis

Parameter	$(simulated)_s$		Sensitivity	
	(-10%)	(+10%)	(-10%)	(+10%)
Lettuce				
upt_data	24.07	27.08	0.59	0.59
BDG	24.52	26.62	0.41	0.41
Ratio	25.57	25.57	0	0
Kap	25.57	25.57	0	0
ev_data	19.24	67.36	2.48	16.34
leach_data	21.26	35.77	1.69	3.99
carrot				
upt_data	41.18	48.33	0.80	0.80
BDG	43.85	45.65	0.20	0.20
Ratio	44.76	44.75	0	0
Kap	44.76	44.75	0	0
ev_data	29.78	142.84	3.35	21.92
leach_data	34.57	68.79	2.28	5.37

6.4.4 Model application: amoxicillin uptake by lettuce and carrot plants

The results from simulating the uptake of amoxicillin antibiotics by lettuce and carrot has been shown in Fig. 6.5 (a-b). The Fig. 6.5a, shows the scatterplot as well as regression line of the correlation between simulated and measured. The slope of the regression lines were 1.0 and 0.7 for carrot and lettuce respectively, while regression coefficient (r^2) was 0.7 for both. A two tailed t-test on measured and simulated amoxicillin in plants, with a confidence interval = 95% showed that there was no significant difference between simulated and measured for carrot (p=0.92) and

lettuce (p=0.17) (Fig. 6.5b). The standard deviation for carrot was SD = 4.3 %, and that of lettuce was 33.4%.

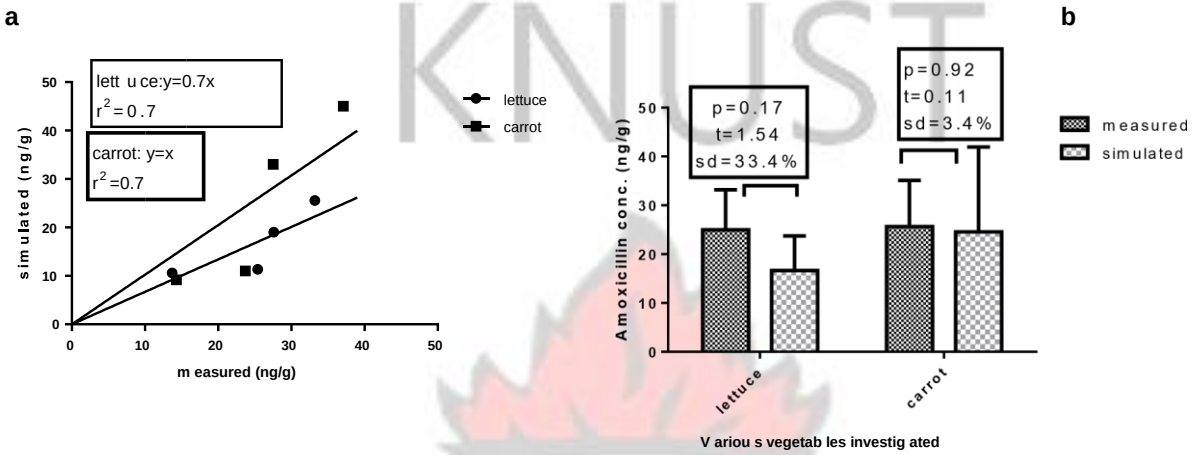


Fig. 6.5 Graphs amoxicillin simulation result
(a) scatterplot of simulated and measured showing regression line for amoxicillin antibiotics, (b) bar chart of simulated and measured indicating t-test result and standard deviation (sd)

6.5 DISCUSSION

With the idea behind a model being iterative development of a pattern, it will never be able to contain all the features of the real system (Jørgensen and Fath, 2011). The antibiotics model developed focus on the major processes occurring in the soil-water and soil water-plant interface. Best agreement between the simulated and measured data was achieved with slope being 1 and 0.9 for carrot and lettuce respectively. The regression coefficients were 0.7 and 0.9 also for carrot and lettuce respectively. The calibration results were comparable to the simulation carried out by (Møller et al., 2015) on the transport and removal of antibiotics, which recorded a slope of 1.01 and regression coefficient of 0.89 for calibrating antibiotics model with trimethoprim.

Validation of the model, showing how well the model output fits experimental data gave standard deviation of 2.6% for carrot and 39% for lettuce. Because toxic substance models are simple and use parameter estimation methods, as far from all parameters, are determined experimentally and high assessment factors are used, standard deviations of up to 50% are acceptable (Jørgensen and Fath, 2011). Since the standard deviations of this antibiotics uptake model were below 40%, it is concluded that the model give results with an acceptable proximity.

The model postulated that, the antibiotics in the edible portion of the plant could represent the concentration found in the plant. Experimental studies on the distribution of antibiotics in plants have provided diverging views about the distribution in plant, due to the limited understanding of the interactions of antibiotic concentrations in soil, antibiotic chemical characteristics, specific crops, plant growth stage, and plant physiology (Dolliver et al., 2007). Ahmed et al., (2015) found the distributions of TCs and SAs accumulated in plant to be roots > leaves > fruits. Similarly, Liu et al., (2013) concluded that the distribution of ciprofloxacin, oxytetracycline and sulfamethazine antibiotics in the wetland plant (*Phragmites australis*) followed the sequence root > leaf > stem. On the other hand, Hu et al., (2010) found that the distribution of antibiotics in various tissues of the vegetables was leaves > stems > roots. Hu et al also insisted that the types and growth stages of vegetables would affect the distribution of antibiotics.

Antibiotics being organic pollutant are mostly sorbed by soil organic matter and minerals. The adsorption process is not only dependent on the soil, but also on the compounds. The "chemistry" so to say between the soil and the chemical determines the adsorption and therefore also the adsorption capacity. Therefore, in this study, adsorption capacity was changed when performing the validation. Sensitivity analysis revealed striking patterns which are worth-noting. Sensitivity result for -10% change in parameter were generally similar to +10% result expect ev_data and

leach_data (which were higher at + 10%). Again, sensitivity result for adsorption capacity and water potential were zero (0) for both carrot and lettuce, indicating that these parameters on the model are insensitive. The water potential and background correction factor had sensitivity values within 0.2 and 0.8, suggesting they could be less sensitive parameters in the model. However, evaporation data and leaching data were all above 1 and ranged from 1.7 to 21.9, these parameters could be very sensitive to the model and hence there is a need for a more accurate data. Humans have always used models as tools to solve complex problems and complex systems. The model was used to identify or affirm a phenomenon which was imbedded in the experimental uptake data.

Although the concentration of tetracycline in plant increased with higher concentration in irrigation water, the increment did not follow perfect linearity. Again, concentration ratio of tetracycline in plant to concentration in irrigation water (eventually in soil) was high at low soil concentrations, and decreases for higher soil concentrations. This experimental anomaly (pattern) have been reported in other researchers and explained that, when soil concentrations are very low there still is a background contamination of the plant tissue originating from air (Mikes et al., 2009). Again, a plausible explanation for this pattern is that plants have a limited sorption capacity for organic contaminants, which becomes saturated at higher soil concentrations. However, a more likely interpretation is that the uptake into plants is from two different and independent sources, namely from soil and from air. Interestingly, when background correction factor (BDG) was introduced to the model, there was a good agreement between the experimental (measured) and simulated data.

The uptake model applied to amoxicillin data, gave an acceptable agreement with standard deviation being 4.3 % for carrot and 33.7 % for lettuce. Therefore, the model could confidently be used to predict other antibiotics.

6.6 CONCLUSION

The model presented here illustrate the details of antibiotics model developed for lettuce and carrot plants on basis of a comprehensive experimental data. The result of calibration gave a satisfactory correlation indicating an acceptable calibration. Validation of the model showed an appreciable agreement between simulated and measured data with standard deviation below 40%. Sensitivity analysis identified leaching and evaporation as the most sensitive parameters in the model. The uptake model used to predict amoxicillin uptake gave an acceptable standard deviation. These parameters could be determined accurately hence, makes the model easily adoptive for other antibiotics. As it is not possible to cover the costs of such a comprehensive analytical program in all cases where these antibiotics are applied or where antibiotics are applied generally, it is assumed that the model due to its transparency can be applied generally by changing the parameters describing the physical-chemical properties of the antibiotics and the site specific parameters and forcing functions.

CHAPTER SEVEN

7. OCCURRENCE OF ANTIBIOTICS IN LETTUCE FROM FARMS AND MARKETS IN GHANA

7.1 SUMMARY

The role of vegetables as a reservoir and carrier of antibiotic contaminants is quite clear, however identifying antibiotics in actual vegetables consumed has not yet been studied. The occurrence of 12 antibiotics in lettuce vegetables irrigated with low quality water was determined by collecting lettuce from vegetable farms and markets in Kumasi, Ghana. Antibiotics were extracted using accelerated solvent extraction and analyzed on a liquid chromatograph-tandem mass spectrometer. Seven out of 12 antibiotics investigated being; metronidazole, ciprofloxacin, erythromycin, trimethoprim, ampicillin, cefuroxime and sulfamethoxazole were detected in at least one sample. Metronidazole concentration in the lettuce plants range from 13.5 to 44.0 ng/kg. Ciprofloxacin, one of the commonly administered antibiotics in Ghana ranged between 28.5 and 92.8 ng/kg. Erythromycin, in all the samples ranged from 41.4 to 56.7 ng/kg. Trimethoprim concentration in the lettuce plants studied are within the range of 32.7 and 104.3 ng/kg. Pearson correlation analysis performed also revealed, a positive linear correlation between sulfamethoxazole and ciprofloxacin which was statistically significant ($p<0.05$). The acceptable daily intake (ADI) for erythromycin and sulfamethoxazole are 0−7μg/kg body-weight/ d and 0−50 μg/kg body-weight/ d respectively. In this study, estimated daily intakes of erythromycin and sulfamethoxazole for the consumption of lettuce were 6.4×10^{-7} μg/kg body-weight/ d and 2.0×10^{-7} μg/kg body-weight/ d respectively.

Consumer antibiotic exposure through food of plant origin is occurring from the result of this study. For food safety reasons, it needs to be investigated in further research, whether low levels of antibiotics in food plants could contribute to development of bacterial resistance.

7.2 OBJECTIVES

Lettuce from vegetable farms were tested for occurrence of 12 commonly prescribed human and veterinary antibiotics. Furthermore, vegetables on the Kumasi markets were analyzed for remnants of antibiotics and possible risk they pose to consumers were estimated.

7.3. MATERIALS AND METHODS

7.3.1 Chemicals

A total of 12 antibiotics were studied. These were ciprofloxacin (quinolone), erythromycin (macrolide), trimethoprim and sulfamethoxazole (sulphonamides), amoxicillin, ampicillin and cefuroxime (β-lactams), metroimidazole (nitroimidazole) as well as doxcycline, tetracycline, chlorotetracycline and othrotetracycline (tetracyclines). Bases of selection are presented in chapter 3 section 3.3.1 and relevant physicochemical properties of the studied antibiotics are presented in Table 2.1.

7.3.2 Study area

The study was conducted in Kumasi. For easy comparison, the 10 out of 20 farming sites were selected and group under four sampling area (Fig. 7.1). These are Ahensan, Chirapatre, KNUST

and University of Education Winneba-Kumasi (UEW-K) sampling areas. Three vegetable farms in Ahensan sampling area were identified. The first farm (Ahensan Gate farming site) is about 100 m upstream (north) to where the effluents from Ahensan WSP enters, the second farm (Bomso farming site) is 50 m after the entry of the effluent of Ahensan WSP. The Gyenyase farm site (third farm) is about 1 km downstream (southward).

Three vegetable farms identified at Chirapatre are; Karikari farms (about 200 m upstream to where the Chirapatre WSP enters), Chirapatre farms (1 km downstream from the entry point of the effluent) and Ramseyer farms (about 1.5 km downstream). At KNUST campus sampling area, the vegetable farms identified are; Engineering gate site (located about 400 m upstream from KNUST Business School), Business School farm site (behind KNUST Business School) midstream to the Wiwi tributary and Guss Hostel farm site (about 200 m away from KNUST Guss Hostel) downstream of the Wiwi River. UEW-K campus sampling area is located at Kwadaso sub-metro of Kumasi. UEW-K farm land is the major vegetable farm land in this sampling area, occupying about 19 acres. The farmers uses the Tano River that flow through the campus for irrigation.

Sampling was also done at the three large markets (Central Market, Railway Market and Asafo Market) and two neighbourhood markets (Bantama market sampling site, Ayigya market sampling sites) (Fig. 7.1).

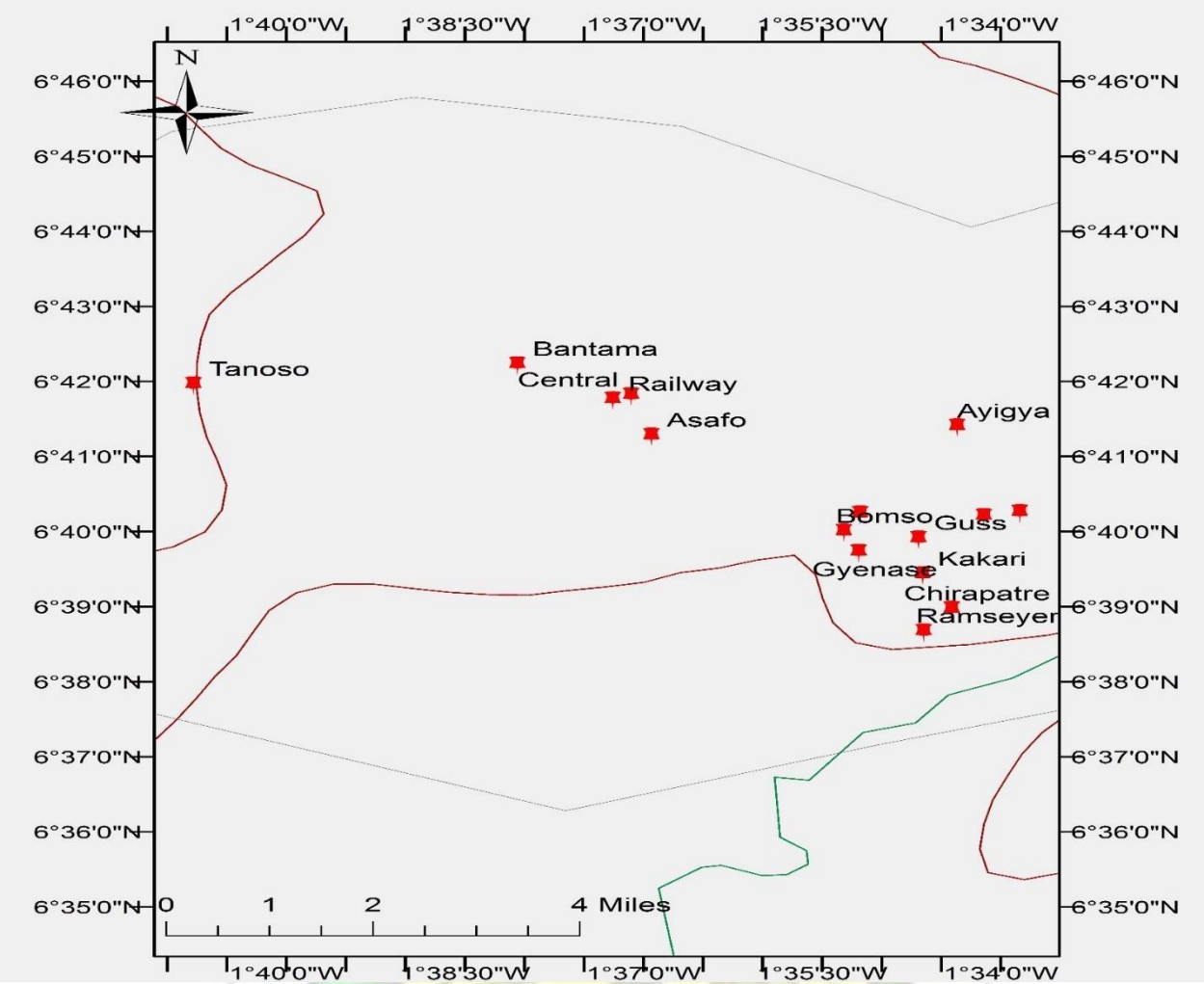


Fig. 7.1: Map of sampling area showing the various sampling sites as pins

7.3.3 Sampling

Three ready to harvest lettuce beds were selected at each farm site and 6 whole lettuce picked randomly from each bed. For the markets, 3 permanent retailers were randomly selected at each market, and six whole lettuce purchased from each of them. Forty- five sampling points consisting

of thirty farm beds and 15 market retailers were identified in the sampling area. Sampling was conducted in two sampling periods; 18th – 22nd August, 2015 (first), 22nd – 26th September, 2015 (second). In all, 90 lettuce samples were collected and transported to the laboratory. All plant samples were washed with distilled water and dried on an adsorbent paper according to Dolliver et al. (2007). The leaves of lettuce samples were chopped and three replicate composite samples for each sampling point were bulked and kept in a refrigerator at 4 °C before shipping to Denmark where they were stored at -18 °C until use.

7.3.4 Extraction of compounds for chemical analysis

The leaves of lettuce samples, chopped and frozen at -18 °C were freeze-dried for 48 h using a Heto FD3 lyophilisor (Heto Lab equipment, Allerød, Denmark) coupled to a roughing pump (Edwards, UK). The system was operated between 0.090 to 0.100 torr at - 48 °C. The freeze-dried samples were then ground thoroughly with a sterile pestle. Pressurized liquid extraction (PLE) was performed on an Accelerated Solvent Extractor, ASE 200 from Dionex (Sunnyvale, CA, USA). A Dionex cellulose filter paper (1.98 cm diameter) was introduced inside the 11 mL extraction cell and gently located by a plunger into the cell's base to avoid sample aggregation, prevent clogging of the extraction cell and allow a greater exposure surface area and thereby improved contact between solvent and matrix. A 0.1 g aliquot of sample was blended gently in a 5 g Ottawa sand (20-30 mesh, AppliChem, Darmstadt, Germany) and placed inside the extraction cell. Another Dionex cellulose filter paper was placed above the packing and 20 µL of 2.5 µg/mL IS (internal standard) mixture was added to the filter prior to extraction. Internal standard mixture was spiked in all PLE cells equivalent to a final amount of 50 ng to correct for any losses during sample preparation and for any variability in injection volume. The extracting solvent was Milli-Q water

and the extractor was operated at 70 °C with one cycle for a static period of 10 min. At the end of each extraction a total extract volume of approximately 30 mL was obtained.

The extracts were cleaned up on a reversed phase solid-phase extraction (SPE) using Oasis HLB cartridges (hydrophilic-lipophilic balance, 200 mg sorbent, 30 µm, 6 cm^3) purchased from Waters Oasis (Massachusetts, USA). The SPE cartridges were conditioned with 3 mL aliquot of MeOH followed by 3 mL of distilled water. A 30 mL portion of extract was loaded on the SPE the columns at a flow rate of 1.5 mL/min and then were washed with 3 mL of 5% MeOH in water. The sorbents were allowed to dry for about 60 min under vacuum before the antibiotics were eluted with 3 mL MeOH acidified with 0.1 % formic acid at a flow of approximately 1 mL/min. Eluates were evaporated to dryness under a gentle flow of nitrogen at 30 °C and then reconstituted in 1 mL 1% MeOH into brown flat-cap HPLC-vials for analysis.

7.3.5 LC-MS Analysis

The liquid chromatographic (LC) system consisted of an Agilent 1290 Infinity Binary System (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a degasser, a cooled autosampler (4 °C), and a column oven (30 °C). The chromatographic separations were achieved by use of a reversed-phase column (Kinetex® Biphenyl 100Å column, 2.1 mm x 50 mm x 2.6 µm), coupled to a guard column (Ultra cartridges BP, 2.1 mm x 2.6 µm), both from Phenomenex ApS, (Milford, MA, USA) and application of binary gradient flow rate of 400 µL/min at 20 °C. The injection volume was set at 10 µL. The mobile phase A consisted of water acidified with 0.1% formic acid whereas mobile phase B consisted of MeOH acidified with 0.1% formic acid. The initial proportion between the mobile phases was 99% A and 1% B. This gradient was held for 1 minute followed by

a 5 min linear gradient ending with 50% A and 50% B. This proportion was soon changed in a 1 min linear gradient from 6 to 7 min to 1% A and 99% B. This gradient was maintained for 2 min followed by a 1 min linear gradient ending with the initial conditions. This gradient was then maintained for 2 min resulting in a total analysis time of 12 min.

Mass spectrometry was performed with an AB SCIEX QTRAP® 4500 System with channel electron multiplier detector (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source (Turbo Ionspray). For MS detection, electrospray ionization was performed in positive mode (ESI+) for metronidazole, ciprofloxacin, erythromycin, trimethoprim, tetracycline, oxytetracycline, chlorotetracycline, doxycycline, amoxicillin, ciprofloxacin-d_8 and trimethoprim-d_3. The negative mode (ESI-) was performed for ampicillin, cefuroxime, sulfamethoxazole and sulfamethoxazole-d_4. The temperature was set to 300 ˚C with a nebulizer gas flow of 8 L/min, curtain gas flow of 12 L/min and collision gas flow of 6 L/min. The ionspray voltage was set to 5000 V. The MS system was set to operate in the multiple reaction monitoring (MRM) mode with the parameters listed in Table 3.1. Collection and treatment of data were performed using Analyst v. 1.4.2 software (Applied Biosystems) and a Savitzky-Golay smoothing factor at 3 on a Windows XP platform-based data-processing system.

7.3.6 Validation of analytical Procedure

The analytical method was validated in compliance with the requirements in standard guidelines (European Medical Agency 2012; ICH Harmonised Tripartite Guideline 2005; U.S. Food and Drug Adminstration 2001). The linear calibration curves were constructed by analyzing standard solutions ranged from 0.001 to 1000 ng/mL followed by calculating the ratios of analyte peak area

to that of the internal standards. Precision was determined by injecting a 1 ng/mL tetracycline and amoxicillin standard solution for 8 times.

The limit of detection (LOD) and limit of quantification (LOQ) of the HPLC-MS/MS system was determined from the standard deviation (σ) of the response from the lowest calibration standard (0.001 ng/mL) injected 6 times and by the slope (S) of the calibration curve. The LOD and LOQ were calculated according to ICH requirement as 3.3σ/S and 10σ/S respectively (ICH Harmonised Tripartite Guideline 2005; U.S. Food and Drug Adminstration 2001).

Matrix effect (recovery) was studied. The matrix used was organic Iceberg lettuce (Lot. Number: NL-310-01) from Holland. This was bought from a supermarket in Copenhagen, Denmark, washed and freeze dried overnight. The temperature and pressure before the samples were removed from the freeze drier are -48 °C and 0.075 T. The experiment for each matrix included twelve blank samples, i.e. matrix sample which is expected not to contain the analytes of interest. 0.5 g sample was weighed and mixed with 5 g of Ottawa sand. The samples were packed in PLE extraction cells. The first four samples were spiked (before the run of PLE) with 20 µL of 5 µg/mL antibiotics-mix, and spiked with 20 µL of 2.5 µg/mL IS-mixture before LC determination. The subsequent four samples were post-spiked (before the LC determination) with 20 µL of 5 µg/mL antibioticsmix and 20 µL of 2.5 µg/mL IS-mix. The last four samples were pre-spiked with 20 µL of 2.5 µg/mL IS- mix before the PLE and finally with 20 µL of 5 µg/mL antibiotic - mix before the LC determination.

The concentration of antibiotics in the samples were determined on calibration curves constructed for each individual analyte. Quality control (QC) samples were run in parallel during the quantification process. Positive controls consisting of matrix spiked (fortified) with antibiotic-mix

and internal standard were used whereas negative controls consisting of matrix and internal standards were used to exclude possible procedural contaminations.

7.3.7 Statistical analyses

The results obtained were subjected to statistical evaluation. Mean, standard deviation (SD) and coefficient of variation (CV %) were evaluated with Microsoft Office Excel 2013 (Version 15, Microsoft, USA). Pearson's correlation coefficient was performed as a measure of dependence between antibiotics studied. The closer the coefficient is to either −1 (decreasing linear relationship) or 1 (direct linear relationship), the stronger the correlation between the variables. One-way ANOVA was performed to test the differences in various treatment, with a Turkey's Honesty Significant Differences (HSD) as a post test. The P-value was considered statistically significant at $P<0.05$. The statistical analysis was performed by using GraphPad Prism version 6.01 for Windows (GraphPad Software Inc., USA). Principal component analysis (PCA) based on antibiotics concentrations in samples was done, to determine the distribution pattern of antibiotics in sources sampling site, using JMP statistical software v. 10 (SAS Institute). The principal components were extracted with eigenvalues >1.

7.3.8 Estimation of Daily Intake

The estimated daily intake (EDI) of antibiotics was dependent on both the antibiotics concentration in vegetables and the amount of consumption of the respective food. The food consumption rates for lettuce in Ghana has been estimated to be 0.013 g/kg body weight/day (Amoah et al., 2007).

The EDI was obtained by multiplying the median concentration (ng/g) of various antibiotics in lettuce and carrot with their respective food consumption rate (Darko and Akoto, 2008).

7.4 RESULT

7.4.1 Quality control

The regression coefficient (r^2) expressed as percentage for all the 12 antibiotics ranged from 97.1 to 99.5 %. The precision expressed as coefficient of variation ranged from 3% to 9%. The LOD determined for all antibiotics (Table 7.1) using the slope and the standard deviation ranged from 2 to 8 ng/L, while the LOQ ranged from 7 to 23 ng/L (Table 7.1). The matrix absolute recovery (%) ranged from 61.0 to 83.5% and the relative recovery ranged from 64.8 to 112.1% (Table 7.1). They were all above the minimum acceptable target value of 60% (Venn, 2008)

Table 7.1: Result for validation of analytical procedure for determination of lettuce

Analyte	Linearity	LOD	LOQ	Accuracy	Precision	Matrix Effect

	R^2 (%)	(ng/L)	(ng/L)	(%CV)	(%CV)	AR ± RSD (%)	RR ±RSD (%)
MET	97.8	4	11	3	3	83.2±15.2	111.7±18.6
CIP	98.7	8	23	4	7	62.7±23.9	85.8±27.0
ERY	97.1	6	18	4	5	68.3±17.0	64.8±20.1
TRIM	98.5	3	10	8	4	69.3±7.7	92.9±13.2
TC	99.1	6	17	5	4	79.2±21.8	106.3±24.3
OTC	99.3	3	9	8	9	79.8±19	107±21.8
CTC	99.5	8	23	6	8	66.2±21.2	75.4±23.7
DC	99.2	2	7	5	6	83.5±20.0	112.1±22.7
AMX	99.5	2	6	4	4	65.1±10	87.3±14.7
AMP	99.3	6	20	4	3	61.0±12.4	66.1±16.1
CEF	98.8	4	12	7	4	70.4±8.5	94.5±13.7
SUL	98.7	7	21	4	9	69.2±17.2	104.1±21.7

7.4.2 Occurrence of antibiotics in lettuce

Seven out of 12 antibiotics investigated were detected in at least one sample. Seven antibiotics, including metronidazole, ciprofloxacin, erythromycin, trimethoprim, ampicillin, cefuroxime and sulfamethoxazole were detected. The maximum number of antibiotics detected in a sample was 3, which occurred in 2 sampling point. In 26 sampling points, one antibiotics was detected and finally 2 antibiotics was detected in 5 sampling point (Fig. 7.2a.). Thirty-three out of the 90 sampling points recorded at least one antibiotics. The percentage detection ranged from 2.2 % (ERY) to 7.8

% (TRIM) in all the lettuce samples. In the first sampling session, 19 samples recorded at least one antibiotics, while 14 samples detected at least one antibiotics in the second sampling session. Total load of all the antibiotics was 1138.8 ng/kg while the highest total load was found to be 394.4 ng/kg. Trimethoprim and ampicillin showed the lowest total load of 51.1 ng/kg in all the samples. The concentrations of antibiotics in lettuces ranged from 12.0 to 104.3 ng/kg with mean concentration of 38.1 ± 24.0 ng/kg.

Metronidazole concentration in the lettuce plants range from 13.5 to 44.0 ng/kg (Fig. 7.2b). The concentration of ciprofloxacin ranged between 28.5 and 92.8 ng/kg. Erythromycin, in all the samples ranged from 41.4 to 56.7 ng/kg. Trimethoprim concentration in the lettuce was within the range of 32.7 and 104.3 ng/kg. The highest concentration of ampicillin found in all the lettuce plants was 22.0 ng/kg and the lowest concentration of ampicillin recorded was 12.0 ng/kg. Cefuroxime was found to range from 11.0 to 27.3 ng/kg in all the samples. The highest concentration of sulfamethoxazole in all the samples was 21.4 ng/kg and the lowest was 11.2 ng/kg. The mean concentrations of the various antibiotics detected in all samples are shown in Fig. 7.2b. The mean concentrations could be arranged in the following increasing order:

SUL<AMP<CEF<MET<CIP<ERY<TRIM. There was significant difference ($p = 0.001$) between the total concentrations of various antibiotics found in all the lettuces samples (Fig. 7.2b).

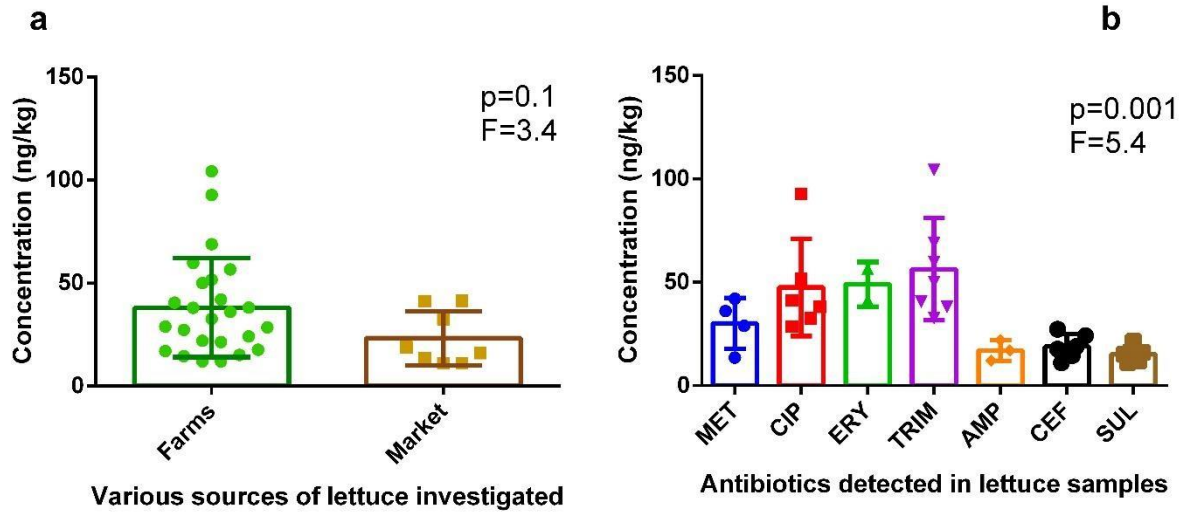


Fig. 7.2: Concentrations of all antibiotics in lettuce
(a) various sources (b) various antibiotics detected

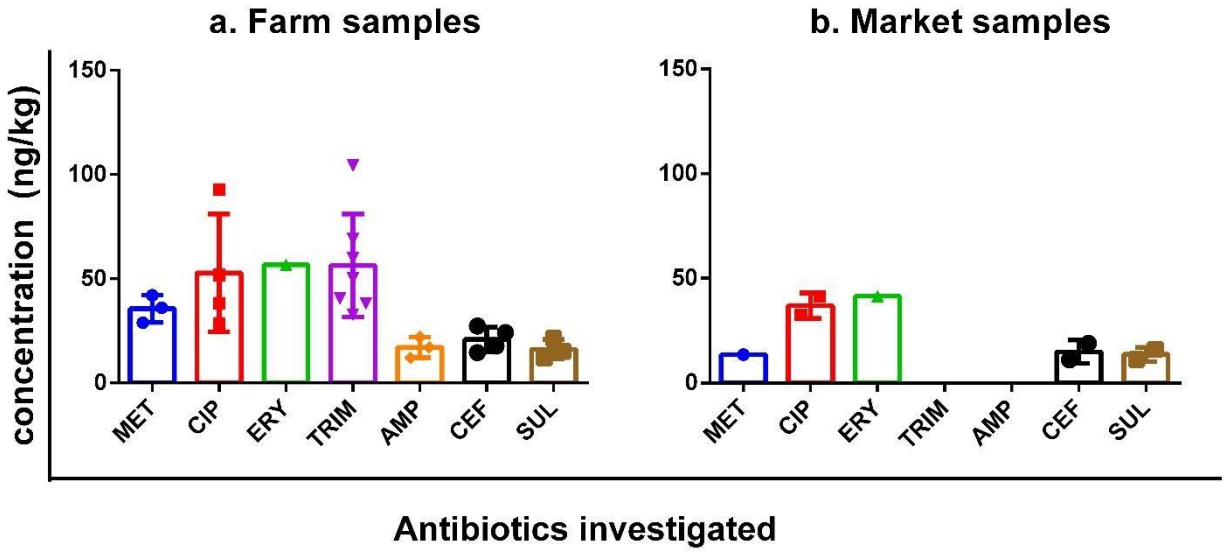


Fig. 7.3: Concentrations of various antibiotics in lettuce
(a) farm sources (b) market sources

7.4.2.1 Occurrence of antibiotics in farm samples

All the seven antibiotics detected were reported in farm samples (Fig. 7.3a) while five (metronidazole, ciprofloxacin, erythromycin, cefuroxime and sulfamethoxazole) antibiotics were detected in market samples (Fig. 7.3b).

Fig. 7.4a, shows that there are significant differences ($p = 0.02$) between the concentrations of all antibiotics in various vegetable farming areas (community). Fig. 7.4b-4d shows the concentrations in various sampling sites in each sampling area. The maximum number of antibiotics detected in a farm sample was three. This sample was collected from Chirapatre farm site (third lettuce bed during first sampling) and UEW-K campus farm site (second lettuce bed during second sampling session). There were no antibiotics detected in Ahensan gate and Bomso sampling site (Fig. 7.4b)

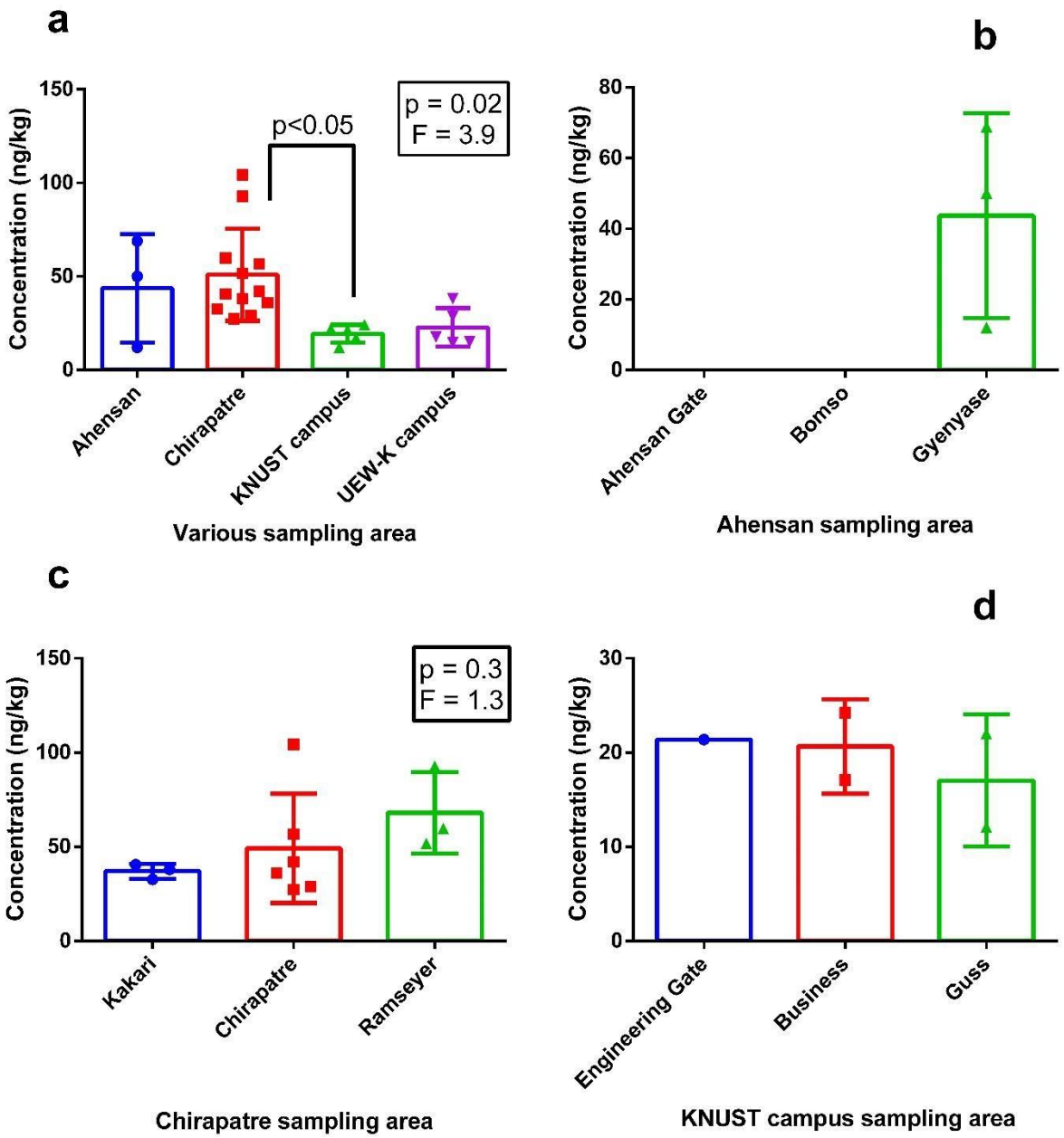


Fig. 7.4: Concentration of antibiotics found in lettuce
(a) various farm sampling area (b) various sampling site in Ahensan sampling area, (c) various sampling site in Chirapatre sampling area and (d) KNUST campus sampling area

7.4.2.2 Occurrence of antibiotics in market samples

Cefuroxime antibiotics recorded the lowest concentration in the market samples (Fig. 7.5) and occurred in Asafo market sample. The maximum recorded concentration for the market samples was 41.4 ng/kg, and mean ± standard deviation was 23.3 ± 13.1 ng/kg. Fig. 7.4, represent the concentrations of antibiotics in various market sampling sites in Kumasi.

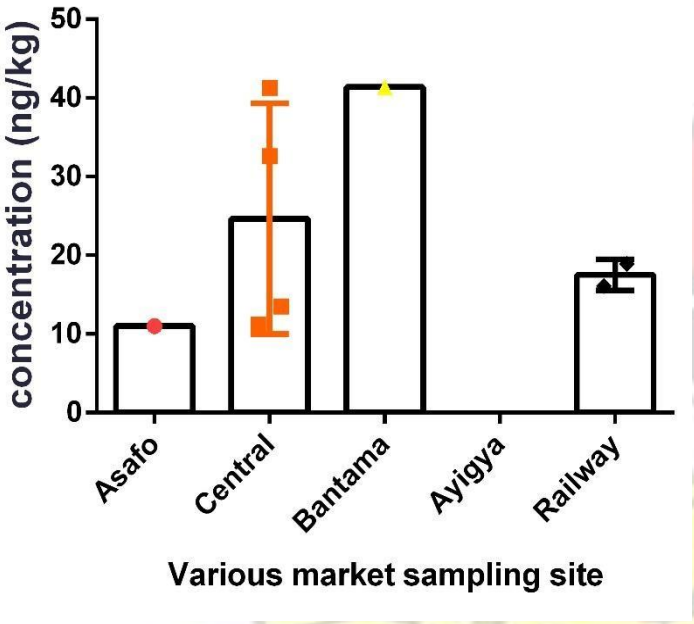


Fig. 7.5: Concentration of antibiotics found in various market sampling sites

The minimum antibiotics concentration in the market samples was 11.0 ng/kg. The maximum recorded concentration for the market samples was 41.4 ng/kg, and the mean was 23.3 ± 13.1 ng/kg. Although there were differences between the mean concentrations of total antibiotics in various sources of lettuce, the difference was not statistically significant ($p = 0.1$).

7.4.3 Pearson correlation analysis

Pearson correlation analysis performed also revealed, a positive linear correlation (Table 7.2) between CIP and MET, CEF and AMP, SUL and CEF, and SUL and CIP. However, only

correlation between SUL and CIP was statistically significant ($p<0.05$). The strong inverse relationship identified between TRIM and MET was not statistically significant.

Table 7.2: Pearson correlation result for antibiotics in lettuce plants

	MET	CIP	TRIM	AMP	CEF	SUL
MET						
CIP	0.71					
TRIM	-0.84	-0.22				
AMP	-0.54	-0.35	0.20			
CEF	0.40	0.55	-0.19	0.73		
SUL	0.44	0.93*	0.09	-0.32	0.61	

*significant at $p < 0.05$

7.4.5 Estimated daily intake of antibiotics

The acceptable daily intake (ADI) values for erythromycin and sulfamethoxazole have been established by (JECFA, 2010) and are defined as 0−7 μgkg−1 body-weight d−1 for erythromycin and 0− 50 μgkg−1 body-weight d−1 for sulfamethoxazole. The ADI value indicates the level of a chemical that can be ingested daily over a lifetime without health risk. In this study, estimated daily intakes of erythromycin and sulfamethoxazole for the consumption of lettuce were 6.4×10^{-7} μgkg−1 body-weight d−1 and 2.0×10^{-7} μgkg−1 body-weight d−1 respectively (Table 7.3). The EDI for metronidazole, ciprofloxacin, trimethoprim, ampicillin and cefuroxime were 4.2×10^{-7}, 5.2×10^{-7}, 6.5×10^{-7}, and 2.2×10^{-7} and 2.4×10^{-7} μgkg−1 body-weight d−1 respectively for the consumption of lettuce (Table 7.3). The acceptable daily intake (ADI) values for metronidazole, ciprofloxacin, trimethoprim, ampicillin and cefuroxime have not been established, hence comparison with EDI was not possible.

Table 7.3: Estimated daily intake result

	%	MED	AV	MAX	EDI (ug/kg bw	ADI (ug/kg bw)	MRL
MET	4.4	32.5	30.1	42	4.225E-07	na	na
CIP	6.7	39.8	47.5	92.8	5.174E-07	na	na
ERY	2.2	49.1	49.1	56.7	6.383E-07	0.7	50
TRIM	7.8	50.1	56.3	104.3	6.513E-07	na	200
AMP	3.3	17.1	17	22	2.223E-07	na	50
CEF	6.7	18.3	18.9	27.3	2.379E-07	na	Na
SUL	5.6	15.2	15.2	21.4	1.976E-07	50	100

7.4.6 Principal Component Analysis

Principal component analysis (PCA) based on antibiotics concentrations in samples was carried out to determine the distribution pattern of antibiotics in farm sampling site. The PCA showed metronidazole, erythromycin and trimethoprim correlating with Chirapatre farming site (Fig. 7.6a). The distribution between market sampling sites showed metronidazole correlating with samples from central markets (Fig. 7.6b).

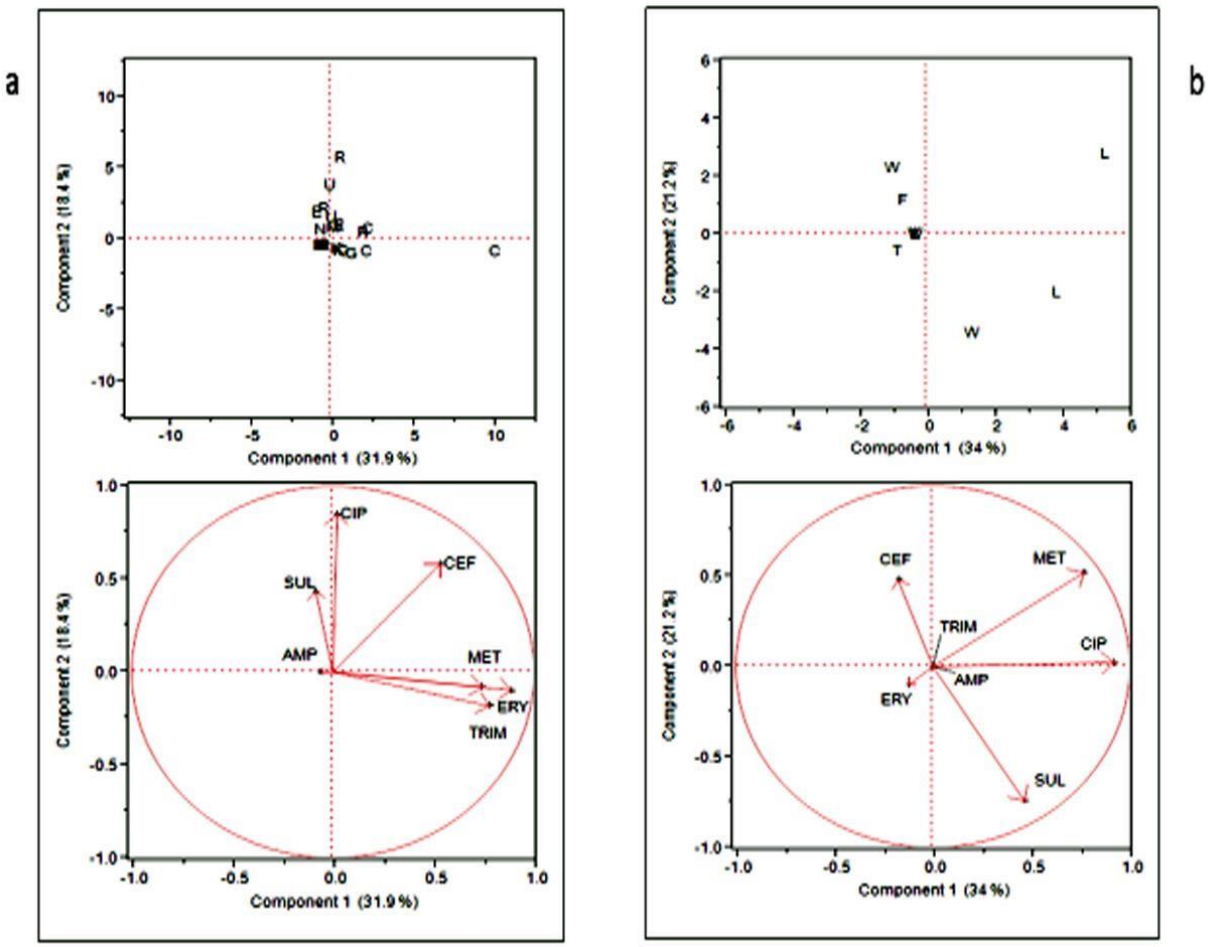


Fig. 7.6: Distribution pattern of antibiotics in lettuce characterized by PCA
(a) sorted by sources (A: Ahensan gate farm, B: Bomsu farm, G: Gyenyase farm, K: Kakari farm, C: Chirapatre farm, L: Ramseyer farms, E: Engineering gate farm, M; Business school farm, N: Guss farm and U: UEW-K campus farm (b) sorted by market sampling sites (W: Railway market, Y: Ayigya market, T: Bantama market, L: Central market, F: Asafo market).

7.5 DISCUSSION

7.5.1 Occurrence

The levels of antibiotics reported in this study were in their ng/kg fresh weight of lettuce. This extremely low levels could be highly anticipated due to low environmental concentrations reported in earlier studies. A wide range of antibiotics have been detected, in various aquatic compartments including municipal sewage (Castiglioni et al., 2006), groundwater (Hirsch et al., 1999) and surface water (Kolpin et al., 2002), usually at concentrations in the ng/L to a few g/L range (Zuccato et al., 2010). Another study conducted in the study area showed that concentrations of antibiotics in wastewater discharges into surface water are generally less than 9 µg/L (or 9000 ng/L), surface water impacted by wastewater discharges are typically less than 3 µg/L (or 3000 ng/L) and concentrations in irrigation water are usually below 0.2 µg/L (or 200 ng/L).

Again, the percentage uptake of antibiotics by plants would affect the levels found. Earlier studies have shown that plants take up less than 2% of the pharmaceuticals applied to soil (Dolliver et al., 2007; Kumar et al., 2005). In tetracycline and amoxicillin uptake study carried out in Ghana, the lettuce and carrot plants took up to 0.02% of the antibiotics applied (Azanu et al., 2016b); Boxall et al., 2006) found that the total accumulation of sulfamethazine in plant tissue after 45 days of growth was less than 0.1% of the amount applied to soil in manure. Antibiotics concentrations in plants reported so far in literature are uptake studies with spiked concentrations mostly higher than what has been reported in environment. Dolliver and colleagues (2007), applied up to 100 mg/L of sulfamethazine to manure at a rate of 56000 L/ha on corn, lettuce and potato plants and found sulfamethazine taken up by lettuce leaves, corn seeds and potato tubers, with concentrations ranging from 100 to 1200 ng/g (Dolliver et al., 2007).

Majority (7 out of 12) of investigated antibiotics were detected. These include; metronidazole, ciprofloxacin, erythromycin, trimethoprim, ampicillin, cefuroxime and sulfamethoxazole. The occurrence of trimethoprim, erythromycin and ciprofloxacin in lettuce plants at levels higher than other antibiotics is not surprising, because another study carried out on the water used for vegetable irrigation in some of these study area showed maximum concentrations of metronidazole, ciprofloxacin, erythromycin, trimethoprim, ampicillin, cefuroxime and sulfamethoxazole to be 33, 146, 136, 98, 74, 65 and 56 ng/L respectively. Metronidazole was mostly recorded in Chirapatre farm site, this was in accordance with the PCA analysis of the low quality water, which showed metronidazole correlating with Chirapatre WSP effluent. The effluent from the Chirapatre WSP discharges into nearby stream which is about 100 m from the Chirapatre farm site. This stream flows adjacent the farm (most tributaries flowing within the farm beds) before going downstream along the Ramseyer farms. This could probably be the reason why metronidazole was recorded in 3 out of 6 samples collected from the farms.

Tetracyclines and amoxicillin were not detected in any of the samples. No uptake of TCs into pinto beans and coconut trees was observed even after direct application (Batchelder, 1982; McCoy, 1976) and Boxall et al., (2006), detected no amoxicillin, when uptake studies with 1 mg/kg amoxicillin in manure was performed. However, tetracycline was detected at concentrations ranging from 4.4 to 28.3 ng/g in lettuce and 12.0 to 36.8 ng/g in carrots when irrigated for 30 days with water spiked up to 15 mg/L. Tetracyclines are not usually expected to be found in the aquatic environment due to the easy precipitation (Boxall et al., 2006) and accumulation of tetracyclines in soil (Kemper, 2008). This could be the plausible reason tetracyclines and amoxicillin were not detected in the samples.

7.5.2 Effect of market dynamics on occurrence of antibiotics in lettuce

The mean concentration of all antibiotics found in the market samples was 23.3 ± 13.1 ng/kg, whiles the mean concentrations of antibiotics in farm lettuces was 38.1 ± 24.0 ng/kg. Even though, lower levels of antibiotics were found in the market samples, as compared to concentrations found in the farms samples, they were not statistically different ($p = 0.1$). There could be the indication of less ageing effect of the antibiotics. Again there could not be further contamination of lettuce from the farm to market process. Knowing that the lettuce is normally washed with water before packaged to the market, it was anticipated that the levels could have increased since studies have revealed that market women often uses the low quality water in the farm to wash the vegetables before sending them to the market. The Central Market recorded the maximum number (3) of antibiotics detected. Four out of the 8 samples which antibiotics was detected in the market samples came from the Central Market. This affirms the fact that most vegetables are sent to Central Market for selling.

7.5.3 Health risk

The MRL for erythromycin, trimethoprim, ampicillin, and sulfamethoxazole are; 50, 200, 50, and 100 µg/kg respectively (European Commission, 2010). The maximum concentration recorded in this study for erythromycin, trimethoprim, ampicillin, and sulfamethoxazole are; 56.7, 104.3, 22.0, 21.4 ng/kg. The concentrations reported in this study is about a thousand times lower their respective maximum residual limit set by EU. Hence concentrations found in lettuce studied could not have any toxic effect on humans when consumed. It is noteworthy that the maximum residual limit mentioned here are for food animals, which consumption rate could be much higher than

vegetables. Metronidazole has been banned for use in Europe and USA as veterinary medicine, hence there is no residual limit set (European Commission, 2010). Metronidazole concentration in the lettuce plants was up to 44.0 ng/kg. Ciprofloxacin, one of the commonly administered antibiotics in Ghana recorded a maximum concentration of 92.8 ng/kg and cefuroxime antibiotics maximum concentration was 27.3 ng/kg in all the samples. There is no maximum residual limit set for ciprofloxacin and cefuroxime yet.

In this study, estimated daily intakes of erythromycin and sulfamethoxazole for the consumption of lettuce were several million times lower than its respective ADI. The EDI for metronidazole, ciprofloxacin, trimethoprim, ampicillin and cefuroxime which ADIs had not be set could also be such much lower in comparison with ADI. The low EDI values indicate that, the levels of the studied antibiotics in lettuce plants found could be ingested daily over a lifetime without health risk in term of toxicity. This simplistic risk assessment is very misleading, because toxicity is not the major health concern related to antibiotics but antibiotic resistance. It has been shown that antibiotic concentrations far below the minimum inhibitory concentration can select for antibiotic resistance in concentration can select for antibiotic resistance in bacteria (Gullberg et al., 2011; Sandegren 2014). Thus, it cannot be concluded that low level concentrations of antibiotics found in the lettuce plants could not contribute to the development of bacterial antibiotic resistance.

Gullberg et al., (2011), found minimum selective concentration (MSC) for antibiotic resistant mutant with ciprofloxacin to be 100 pg/ml. The fact that antibiotic levels several hundred-fold below the MIC of the susceptible strains can select resistant bacteria means that the sub-MIC selective window is much larger than the traditional selective window. In effect this means that concentrations of antibiotics commonly found in the lettuce samples could be high enough to enrich for resistant bacteria. Furthermore, the European Union has set the maximum allowed combined

concentration of ciprofloxacin and enrofloxacin in milk for human consumption to be less than 100 ng/mL (Ashwin et al., 2009), levels up to 1000-fold above the selective concentrations found by Gullberg et al., (2011). Long-term toxicity due to exposure to subminimal inhibitory concentration antibiotics have been shown to promote resistance. This suggests that the low antibiotic concentrations found in plants are important for enrichment and maintenance of resistance in bacterial populations.

7.6 CONCLUSION

Consumer antibiotic exposure through food of plant origin is occurring from the result of this study. For food safety reasons, it needs to be investigated in further research, whether low levels of antibiotics in food plants can contribute to development of bacterial resistance. From current studies, it cannot be concluded that low level concentrations of antibiotics found in these food plants could contribute to the development of bacterial antibiotic resistance. Endangerment of humans via the food chain is possible and toxicity of plant due to low concentration could occur. In order to assess possible consequences with regard to food safety, the role of vegetables as a reservoir and carrier of antibiotic contaminants requires further studies.

LIST OF PUBLICATIONS

1. Azanu, D., Mortey, C., Darko, G., Weisser, J.J., Styrishave, B., 2016. Uptake of Antibiotics from irrigation water by plants. Chemosphere. Accepted
2. Azanu, D., Styrishave, B., Darko, G., Weisser, J.J., Abaidoo, R.C., 2016. Occurrence and risk assessment of antibiotics in low quality water used in vegetable irrigation in Ghana.

Sci. Total Environ. In preparation

3. Azanu, D., Jørgensen, S.E., Styrishave, B., Darko, G., Abaidoo, R.C., 2016. Simulation of antibiotics uptake by lettuce and carrot plants. Ecological Modeling. In preparation
4. Azanu, D., Styrishave, B., Darko, G., Abaidoo, R.C., 2016. Occurrence of antibiotics in lettuce from farms and markets in Ghana. Food Chemistry. In preparation
5. Azanu, D., Styrishave, B., Darko, G., Abaidoo, R.C., 2016. Antibiotics occurrence in waste stabilization ponds and hospital wastewater in Kumasi, Ghana. Environmental Pollution. In preparation

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APPENDIX

CHAPTER THREE

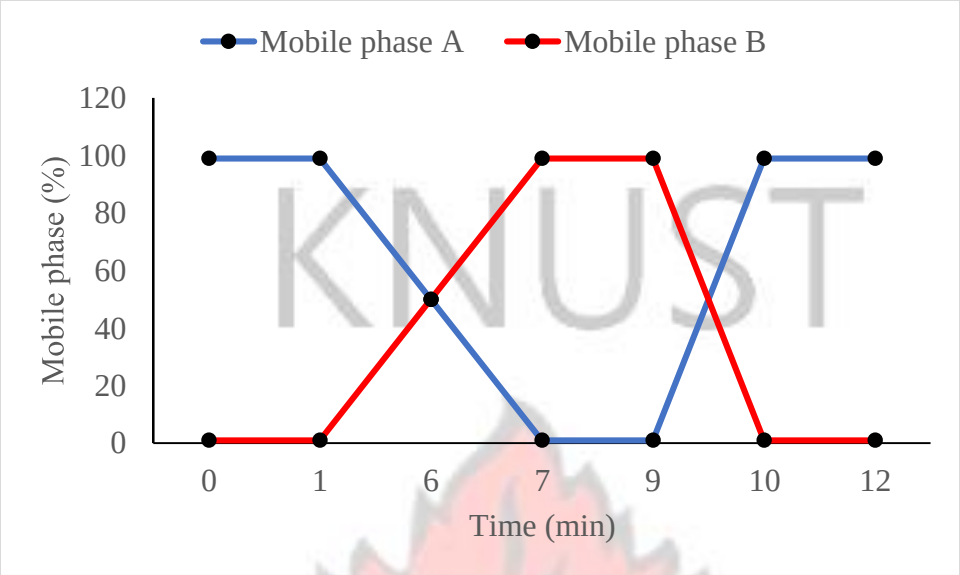
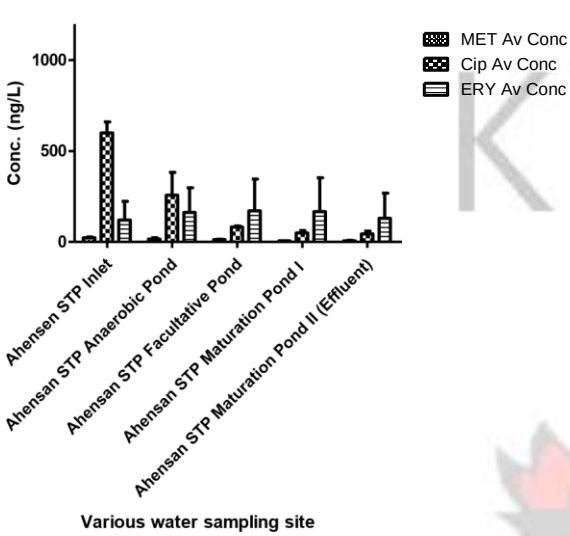
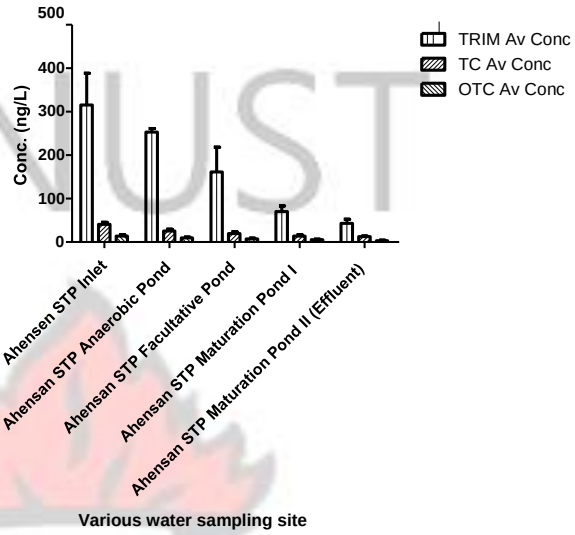


Fig. A3.1 LC Mobile phase gradient for antibiotics in water samples


1000
500
0
Conc. (ng/L)
MET Av Conc
Cip Av Conc
ERY Av Conc
Ahensen STP Inlet
Ahensan STP Anaerobic Pond
Ahensan STP Facultative Pond
Ahensan STP Maturation Pond I
Ahensan STP Maturation Pond II (Effluent)
Various water sampling site


500
400
300
200
100
0
Conc. (ng/L)
TRIM Av Conc
TC Av Conc
OTC Av Conc
Ahensen STP Inlet
Ahensan STP Anaerobic Pond
Ahensan STP Facultative Pond
Ahensan STP Maturation Pond I
Ahensan STP Maturation Pond II (Effluent)
Various water sampling site

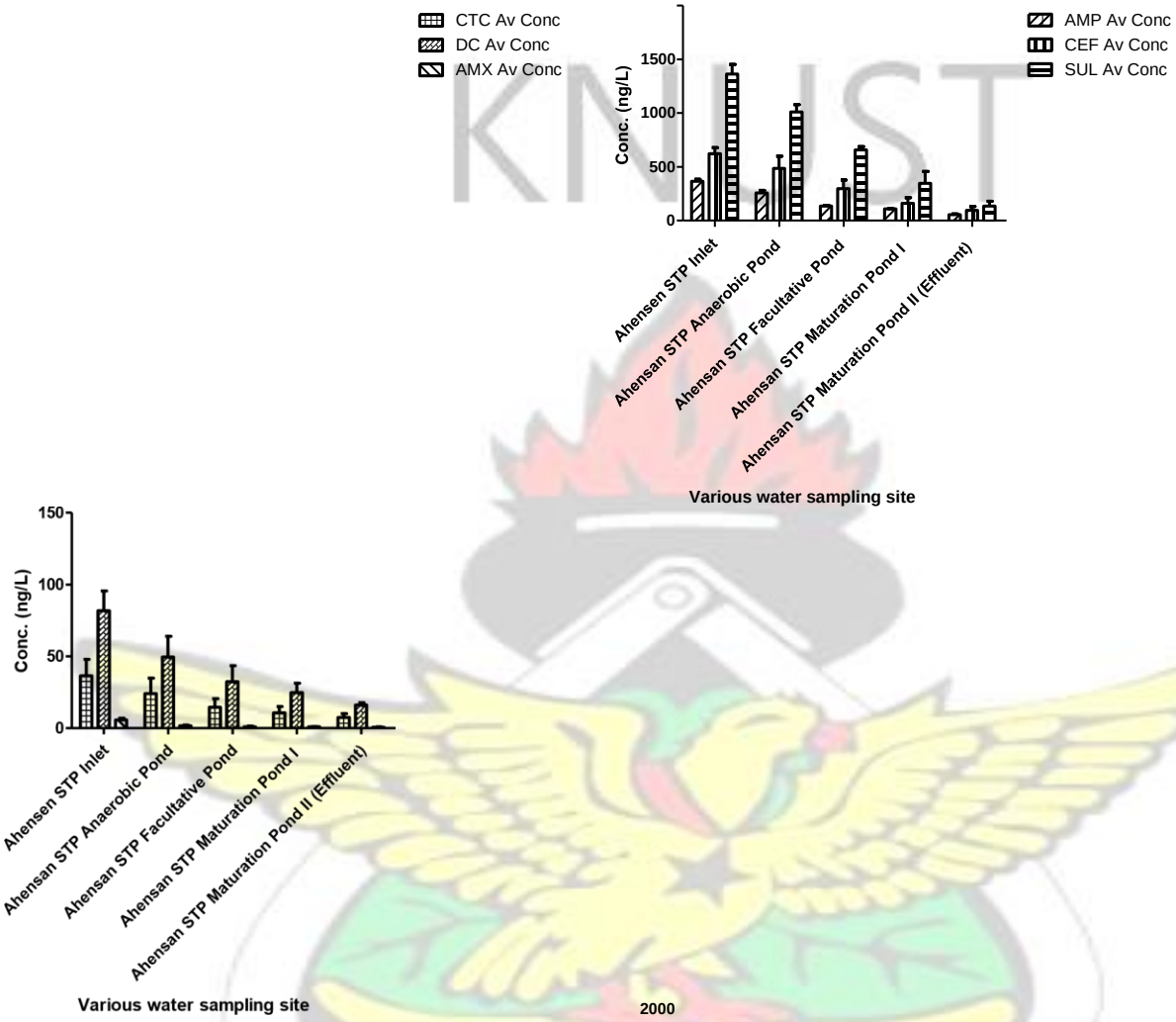


Fig. A3. 2: Ahensan STP

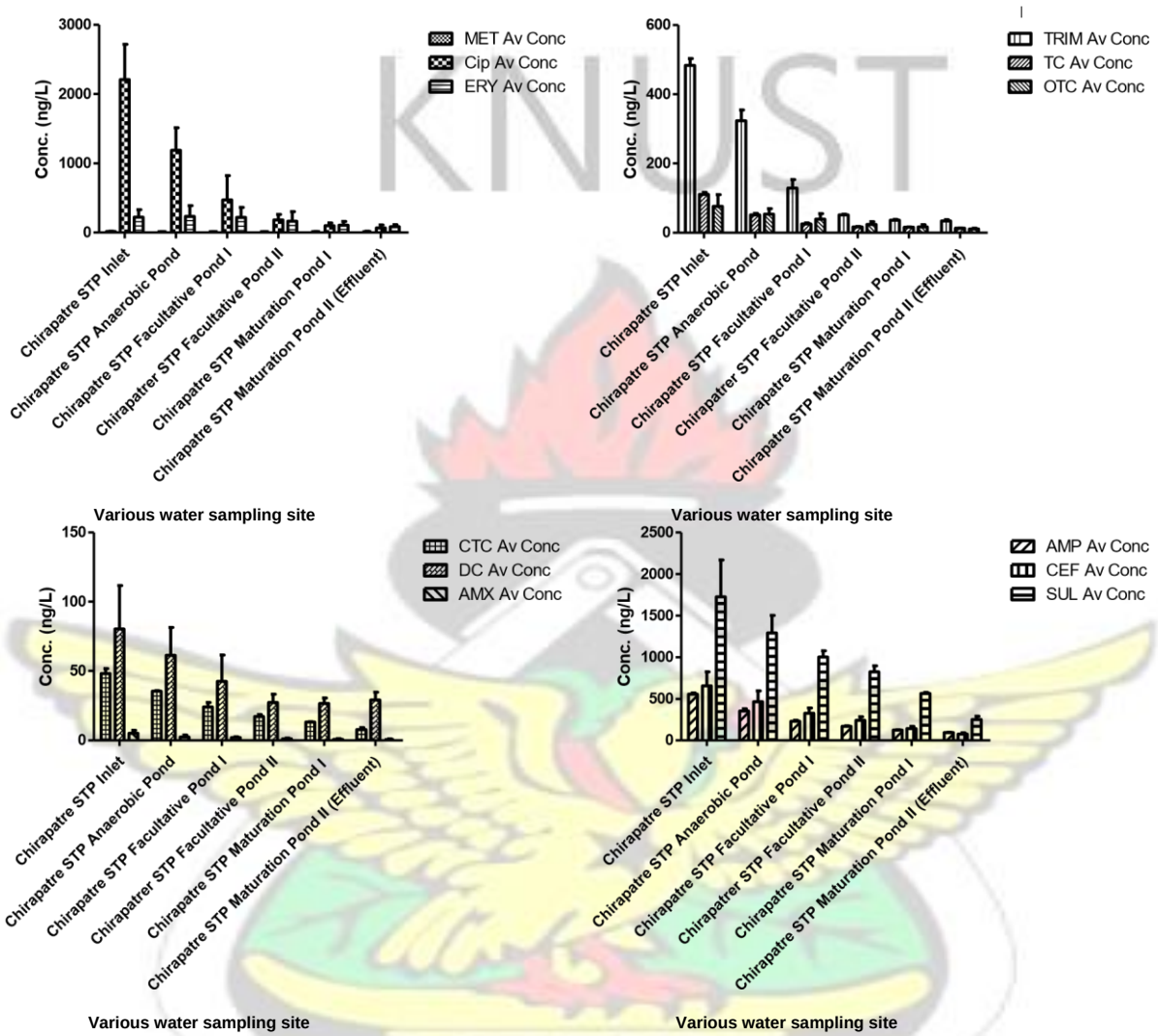


Fig. A3. 3: Chirapatre STP

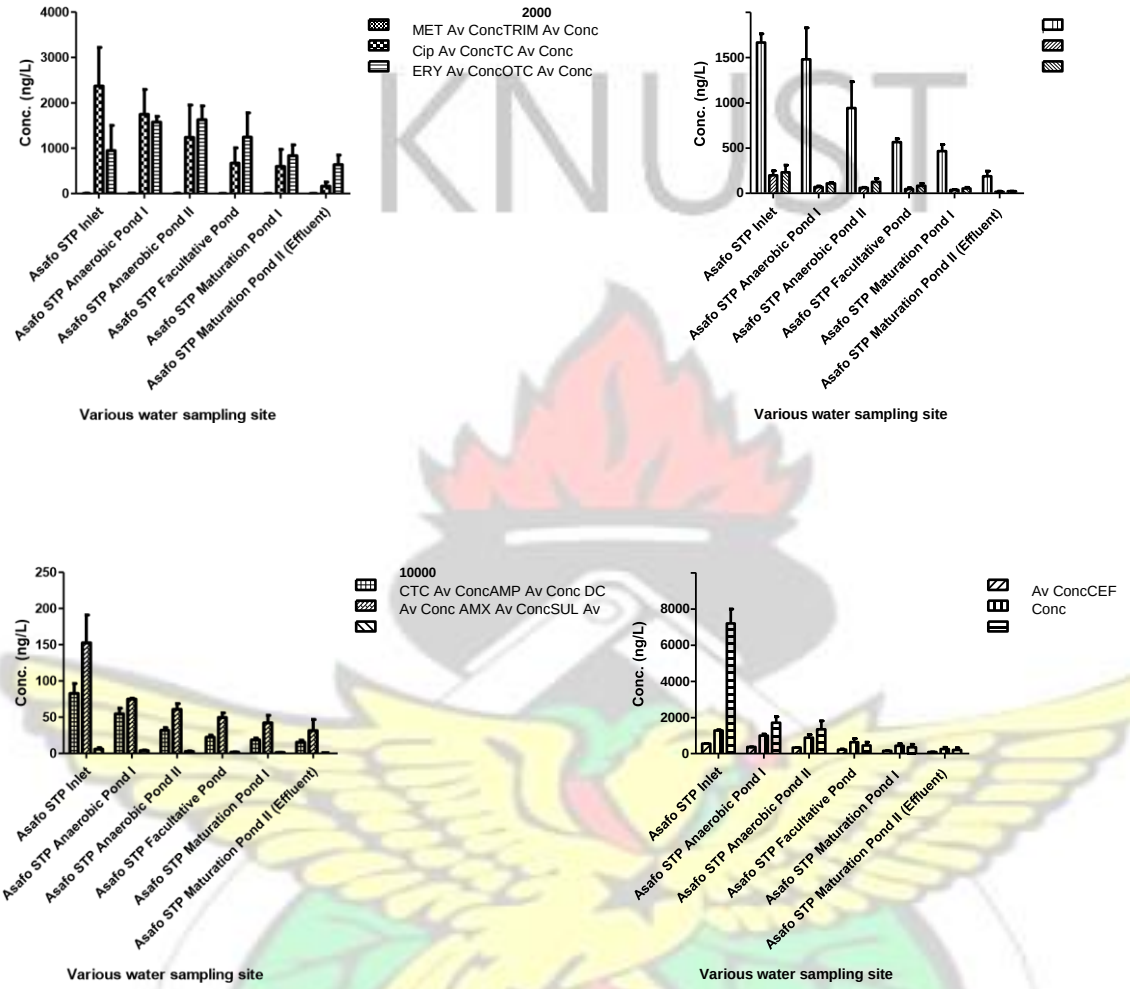


Fig. A3. 4: Asafo STP

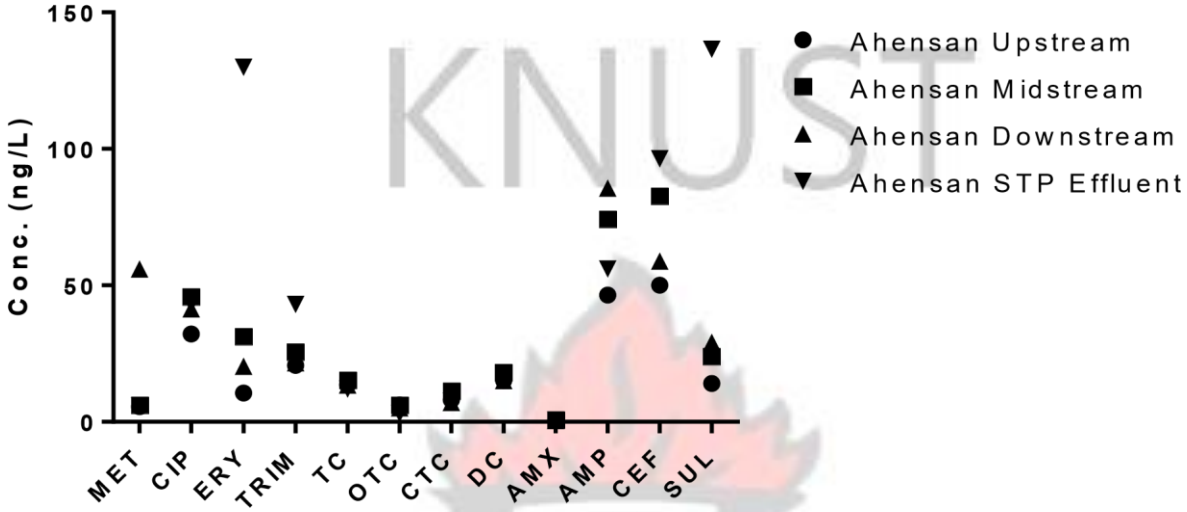


Fig. A4. 1: Distribution of antibiotics in water samples from Ahensan

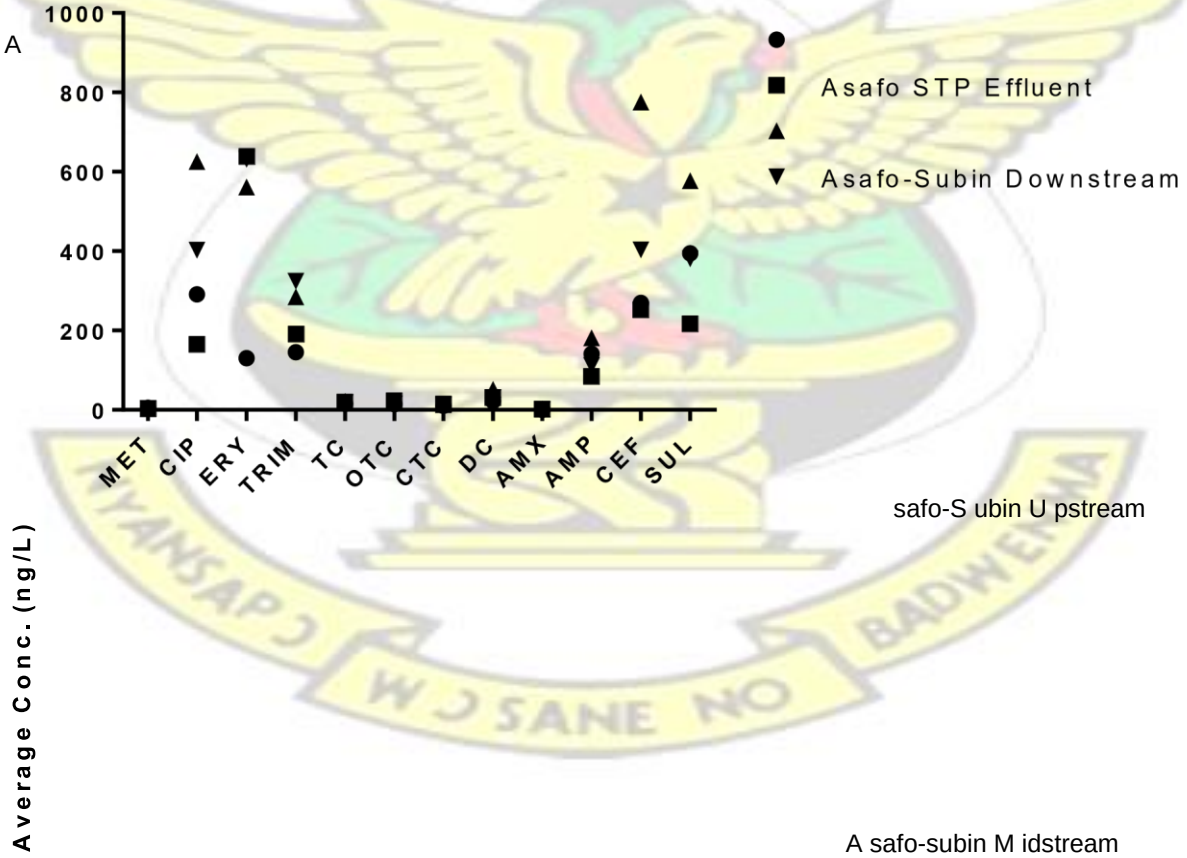


Fig. A4. 2: Distribution of antibiotics in water samples from Asafo

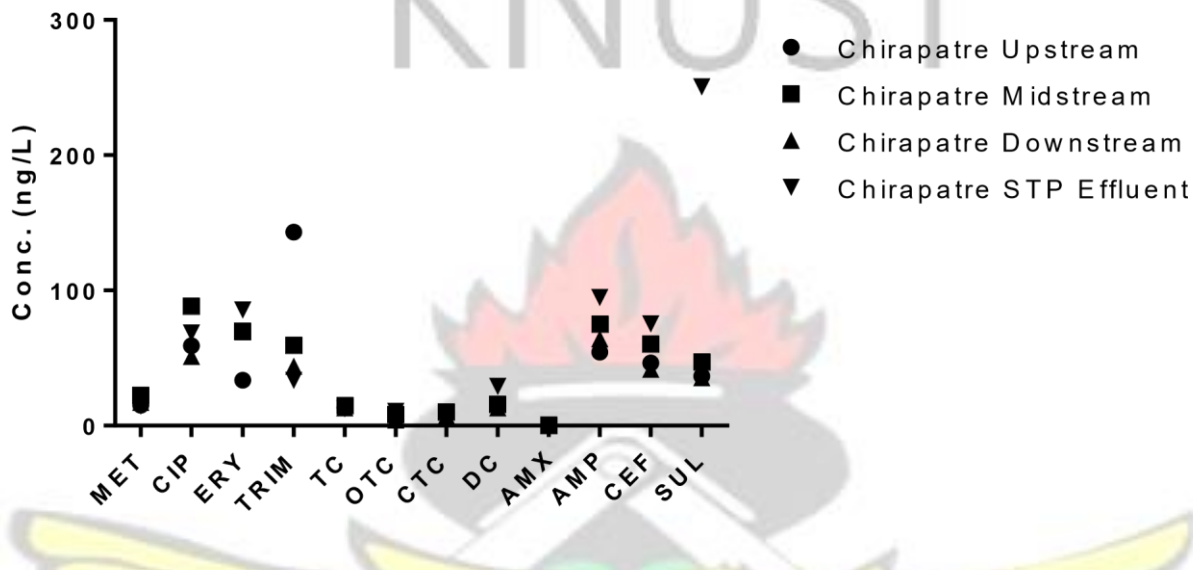
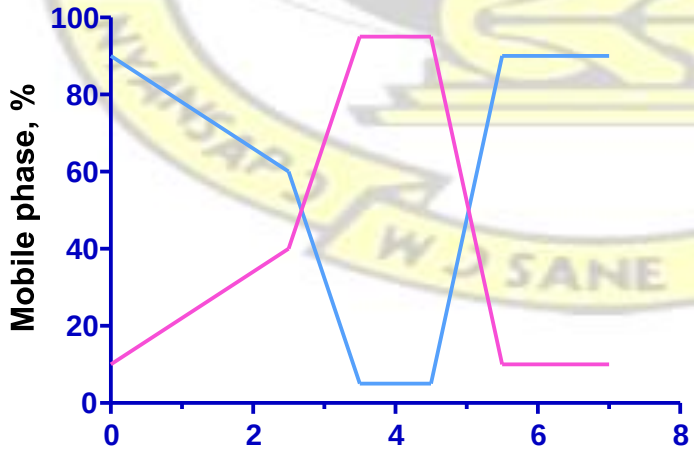


Fig. A4. 3: Distribution of antibiotics in water samples from Chirapatre

CHAPTER FIVE



Time (min)

Fig. A5.1 LC Mobile phase gradient

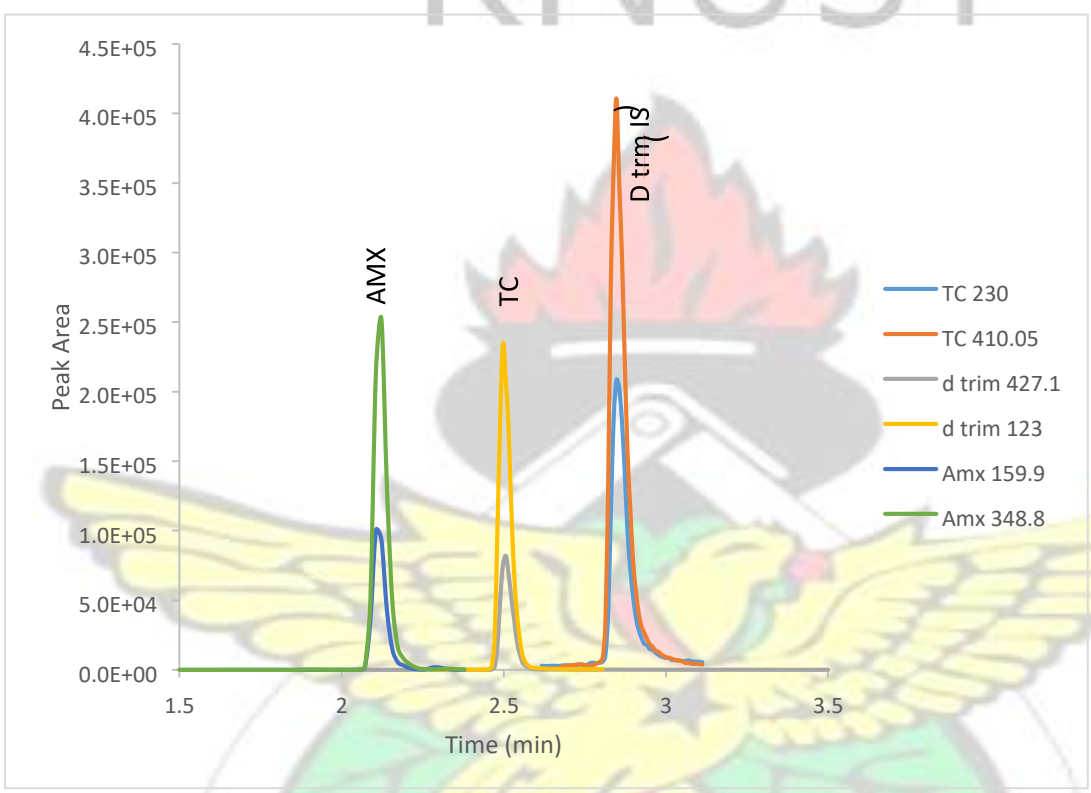


Fig. A5.2 MS chromatogram of the antibiotics of interest CHAPTER SIX

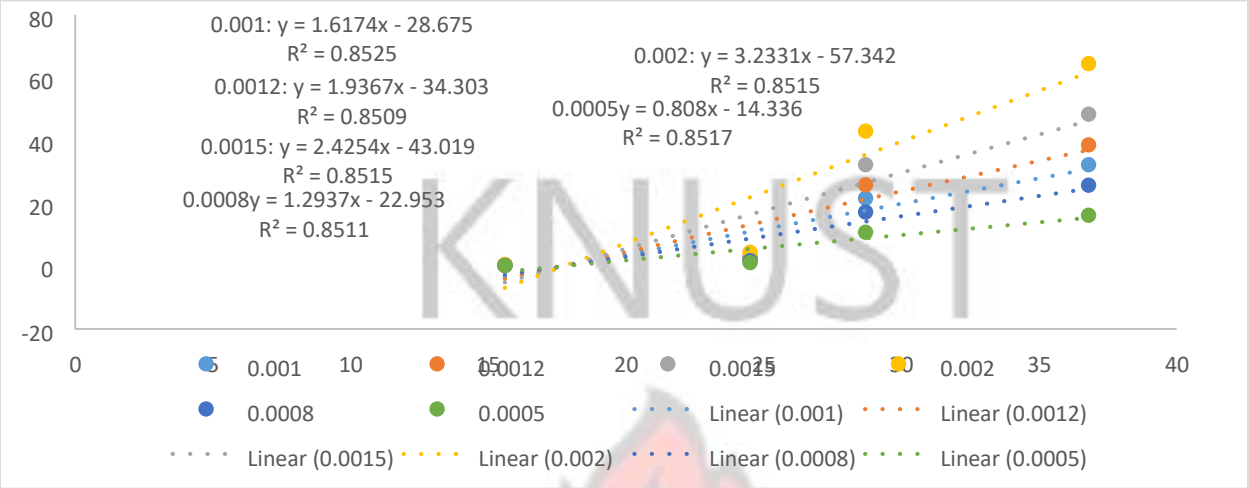


Fig. 6. 1: Graph of calibration result with different uptake data for carrot

Lettuce

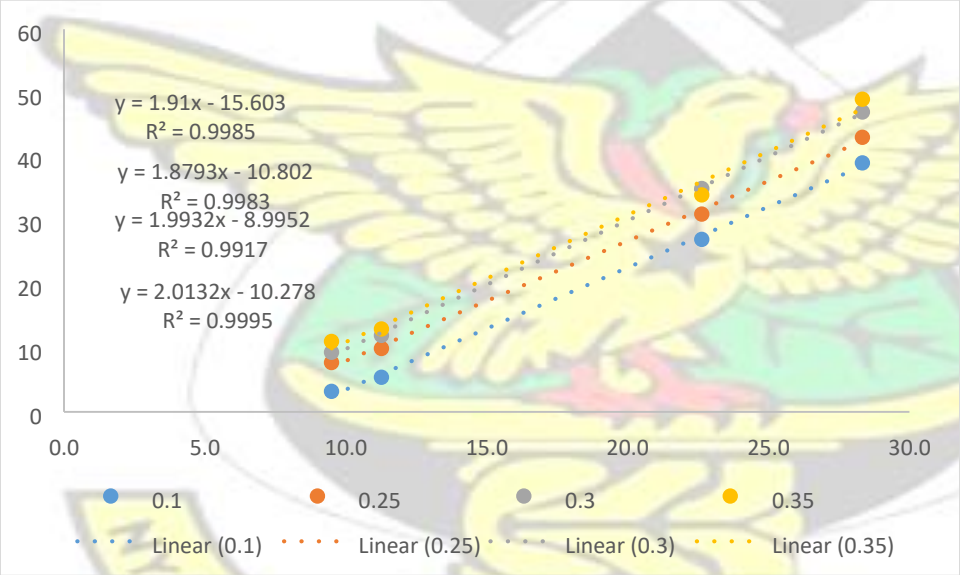


Fig. 6. 2: Graph of calibration result with different uptake data for lettuce CHAPTER SEVEN

Table 7.1: Descriptive result of antibiotics in lettuce samples

	Metronidazole (ng/kg)	Ciprofloxacin (ng/kg)	Erythromycin (ng/kg)	Trimethoprim (ng/kg)	Ampicillin (ng/kg)	Cefuroxime (ng/kg)	Sulfamethoxazole (ng/kg)	TOTALD	MAX	MIN
AGFL								0	0	0

BOFL								0	0	0
GYFL				**68.9**, 50.1	**12**			2	68.9	12
KKFL				40.5, **32.7**, 38.1				1	40.5	32.7
CPFL	42.0, 28.9, 36.1,		56.7	104.3	27.3			4	10 4.3	27.3
RSFL		92.8, **51.7**		59.8				2	92.8	51.7
EGFL						**21.4**		1	21.4	21.4
BUFL					17.1	24.2		2	24.2	17.1
GUFL					22		**12.1**	2	22	12.1
TNFL		28.5, **38.2**				14.5, **17.7**	**15.2**	3	38.2	14.5
ASML						11		1	11	11
CNML	**13.5***	**32.6**, **41.3**					**11.2**	3	41.3	11.2
BTML			41.4					1	41.4	41.4
AYML								0		
RWML						**18.9**	16.1	2	18.9	16.1
TOTAL								24		
D	4.0	6.0	2.0	7.0	3.0	6.0	5.0			
MIN	13.5	28.5	41.4	32.7	12	11	11.2			
MAX	42	92.8	56.7	104.3	22	27.3	21.4			

* the bold fonts are ones detected in second sampling session