AMERICAN UNIVERSITY OF BEIRUT

UPTAKE OF ANTIBIOTICS BY PLANTS FROM IRRIGATION WATER

by
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AN ABSTRACT OF THE THESIS OF

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Antibiotics that are added to the drinking water of poultry and the feed of fattening livestock to promote growth and reduce illness. When manure containing antibiotic residues are used as plant fertilizer they may erode or leach and contaminate soil and water resources. This research was conducted to evaluate the accumulation of gentamicin and oxytetracycline in lettuce leaves, radish bulbs and cucumber fruits in a field experiment and in a pot experiment, where crops are grown in two soils mixed with 0 or 5 % manure and irrigated with water containing 20 mg/L of each of the tested antibiotics. After harvest, plant tissues were analyzed by ELISA in both experiments. Regarding field experiment, concentrations of oxytetracycline and gentamicin used in the field were 30 mg/Kg of dried manure. The antibiotics were mixed in manure and amended in the soil. The results of field experiment showed that oxytetracycline wasn't detected in plant tissue. However, gentamicin accumulated in radish bulb in soil treated with manure and gentamicin. For the pot experiment, the results of analysis indicated that oxytetracycline and gentamicin can be accumulated in radish, lettuce and cucumber and the addition of manure to the potted soil increased the uptake of gentamicin significantly in radish, lettuce and cucumber. The three crops can accumulate oxytetracycline applied in irrigation water in the pot experiment but not in field experiment when oxytetracycline was applied inside manure in the soil. It is recommended that the haphazard use of antibiotics be controlled, and antibiotics be used for the treatment of sick animals only.

Keywords: Gentamicin, Oxytetracycline, irrigation water, field experiment, radish, cucumber, lettuce.

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ABBREVIATIONS

ELISA	Enzyme-Linked Immunosorbent Assay
ng/g	Nanogram per Gram
mg/L	Milligram per liter
FAO	Food and Agriculture organization
FDA	Food and Drug Administration
AUB	American University of Beirut
AREC	Agricultural Research and Educational Center
USA	United States of America
OTC	Oxytetracycline
ug/l	Microgram per Liter
Kd	Distribution coefficient
ug/Kg	Microgram per Kilogram
MRL	Maximum Residual Level
CaCO3	Calcium Carbonate
K	Potassium
P	Phosphorous

Fe	Iron
Zn	Zinc
Cu	Copper
Mn	Manganese
mm	Millimeter
m	Meter
Kg/du	Kilogram per Dunum
°C	Degrees Celsius
FAFS	Faculty of Agricultural and Food Sciences
EC	Electrical Conductivity

CHAPTER I

INTRODUCTION

Antibiotics is very important in our daily life especially because it cures many diseases. According to the food and agriculture organization (FAO)(2017), antibiotics are used to cure livestock and poultry from diseases also used for promoting growth and used against bacteria. It was claimed that 2000 years ago, people tend to use moldy bread to treat wounds in Egypt, China and Serbia (Hutchings et al. 2019). However, the overuse of antibiotics worldwide has led to major health and environmental problems such as the increase of resistant bacteria. Research has shown that antibiotics can be transmitted to plants via animal manure. The increase use of antibiotics contaminated manure in the agriculture sector will contribute to the increase in antibiotic contamination in humans. Research has also shown that antibiotics can be transmitted to plants via irrigation water. Therefore, it is important to test the quality of the irrigation water before using it.

The golden age of antibiotics started when penicillin was discovered in 1928 by Alexander Fleming. The discovery of natural producing antibiotics peaked during the 1950s but then started to decline due to the appearance of certain pathogens that resisted certain drugs, causing the major antimicrobial resistance crisis of today (Hutchings et al. 2019).

The main antibiotics used are Tetracyclines because they have a broad spectrum against gram positive, gram negative bacteria as well as mycoplasma (Önal, 2011). Humans are prone to such antibiotics when they eat crops fertilized with manure containing antibiotics (Pan and Chu, 2016). Sources of antibiotics in the environment are mostly from manure, wastewater, contaminated rivers, surface water, and pharmaceutical wastewater (Du and Liu, 2012). Around 20 million hectares of land are being irrigated with wastewater in the world (Daghrir & Drogui, 2013). Some wastewater treatments don't remove all pollutants such as bioactive chemicals. The treated water is used to irrigate lands; however, it may contain antibiotics and can contaminate the soil (Ding et al. 2011). In some arid and semi-arid regions, countries use wastewater for irrigation due to water scarcity. If wastewater is not treated well and later used for irrigation, antibiotics can be transmitted into soil and crops (Pan and Chu 2017a).

According to Jimenez et al. (2018), around 40-90% of veterinary antibiotics are excreted as parental compounds or metabolites into the environment. Most of the resistant bacteria is coming from the agriculture sector, where antibiotic residues in manure could reach from few ug/Kg to g/Kg of manure (Popowska et al. 2012).

It is important to study the uptake of veterinary antibiotics in plants through irrigation and through soil and manure in open field. The purpose of this thesis is to measure:

1) The uptake of gentamicin and oxytetracycline by radish, cucumber and lettuce using antibiotics treated irrigation water and soil amended with manure in a pot experiment
2) The uptake of gentamicin and oxytetracycline by radish, cucumber, and lettuce in the open field with and without addition of manure.

CHAPTER II

LITERATURE REVIEW

A. Antibiotics

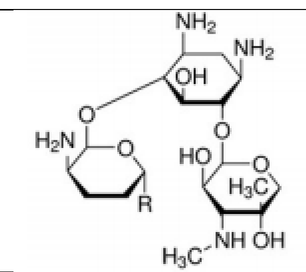
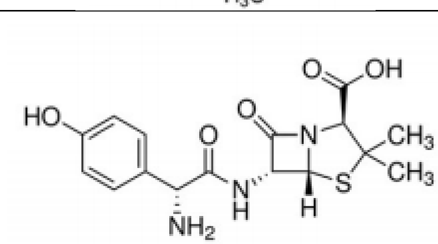
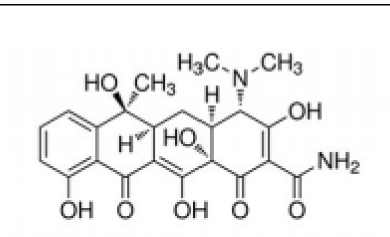
1. What are antibiotics

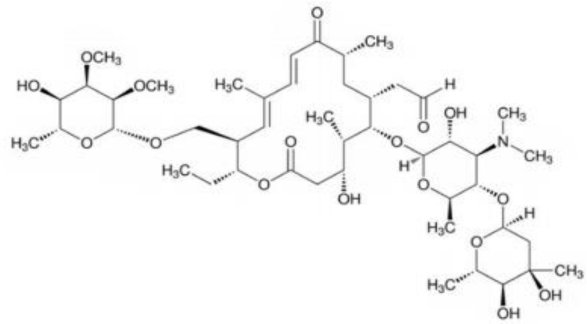
Antibiotics are substances produced by certain microorganisms that inhibit or kill other microorganisms. Generally, there are three major sources of antibiotics: synthetic, semisynthetic, and naturally produced microorganisms. Examples of antibiotics produced naturally are Tetracyclines from the *Streptomyces species*, gentamicin from the *Micromonospora purpurea* and penicillin from the *Penicillium* species. Examples of synthetic antibiotics are sulfonamides, fluoroquinolones, and trimethoprim (Coates et al. 2011). As cited in Zhou and Yao (2020), antibiotics are used by humans and livestock to prevent and treat diseases. According to Etebu and Arikekpar (2016), antibiotics are divided into two groups, bacteriostatic and bactericidal. Bacteriostatic antibiotics inhibits the growth of bacteria while bactericidal antibiotics kills it.

2. Classification of antibiotics

Antibiotics are classified according to their molecular structure, spectrum of activity and function (Sabundayo, and Calderon 2007). Antibiotics that are within the same class can have the same pattern of efficacy and side effects. According to Zhi et al. (2019), some classes of antibiotics include Tetracyclines, Aminoglycosides, Beta- Lactams and Macrolides. Examples of Aminoglycosides are gentamicin, streptomycin and kanamycin, where they have two or more amino sugars joined by glycoside linkage to a hexose nucleus of the compounds. Aminoglycosides are basic and have high solubility in water (Kerlin, 2017). Beta-Lactams have 5 to 6 members sulfur containing ring fused to a lactam ring (Decuyper et al. 2018). Macrolides contains monocyclic lactone with few saccharides combined to hydroxyl group (Miller et al. 2016). Tetracyclines are made up of 4 fused rings, with the addition of some ionized groups such as amino or keton. Tetracyclines have been found widely in soils because they have high affinity to soil organic matter and have the ability to connect in the cation exchange capacity of soil particles (Li et al. 2016). Molecular structure of some antibiotics is found in Table 1.

Table 1. Structural formula for different types of antibiotics

Class	Example	Molecular structure
Aminoglycoside	streptomycin	
Beta-lactams	Amoxicillin	
Tetracycline	Tetracycline	

Macrolide	Tylosin	

Source: Cycoń et al. (2019)

Antibiotics can have narrow or broad spectrum. Narrow spectrum acts against small range of microorganisms unlike broad spectrum. As cited by El Gemayel et al. (2018), narrow spectrum antibiotics work against either gram positive or gram negative while broad spectrum act against both gram positive and negative. Broad spectrum antibiotics are tetracyclines and aminoglycosides and narrow spectrum antibiotics are beta-lactams.

According to Ullah and Ali (2017), antibiotics can be divided according to different functions such as preventing the growth of cell membrane, nucleic acid synthesis and cell wall. Most of Aminoglycosides, macrolides and tetracyclines inhibit protein synthesis of microorganisms. Fluoroquinolones inhibit nucleic acids and beta-lactams work on cell wall synthesis (Brown et al. 2017)

B. Antibiotic usage and consumption

China is the biggest producer and consumer of antibiotics in the world, where it produced 248,000 tons and used around 162,000 tons in 2013. China also leads the highest consumer of veterinary antibiotics usage, which primarily demonstrated to swine and chicken with 62% and 23% respectively (Zhang et al., 2015)

Tetracyclines are the mostly used antibiotics worldwide and are capable of being excreted in an active form in feces and urine of animals and humans (Xu et al., 2020). Most wastewater treatment methods don’t remove tetracyclines, so different methods for the degradation of tetracyclines should be acquired (Xu et al., 2020).

Tetracyclines antibiotics include tetracycline, oxytetracycline, chlortetracycline, and doxycycline. (Suzuki and Hoa, 2012). The global consumption of antibiotics was estimated to increase more than 50% from 2010 to 2030 (Van Boeckel et al., 2015). Moreover, it is estimated that the use of antibiotics for edible animals will increase up to 70% in 2030 (Alhaji and Isola, 2018). China consumed 38,500 tons in 2012, where 23,176 tons were tetracyclines, penicillin, sulfonamides (Lin et al., 2015). Tetracyclines consumption reached 33.4% from the total veterinary antibiotics in 2014. The second most consumed antibiotics goes for β-lactams. It is expected that the consumption of antibiotics in food-producing animals will reach up to 105,596 tons in 2030 (Van Boeckel et al., 2015). Also, China is highest consumer of veterinary antibiotics used in livestock industry in 2010 where it reached around 15,000 tons (Jimenez, et al., 2018). China will double its antibiotic consumption in the livestock industry in 2030, where it will reach around 35,000 tons (Jimenez, et al., 2018). However, the leading consumers in the high-income countries include USA, Italy, and France (Klein et al., 2018).

Some of the antibiotics approved by FDA (2016) for the use in food-producing animals are found in the Table 2.

Table 2. Antimicrobial drug classes approved by FDA and their sales in 2015.

Class	Examples	Annual total sales for respective antibiotic class (Kg)
Macrolides	Tylosin Erythromycin	627,770
Aminoglycosides	Gentamicin Neomycin Spectinomycin	344,120
Penicillin	Amoxicillin Ampicillin Penicillin	936,669
Sulfonamides	Sulfamerazine Sulfamethazine	380,186
Tetracyclines	Chlortetracycline Oxytetracycline Tetracycline	6,880,465

Source: Antimicrobials Sold or Distributed for Use in Food-Producing Animals. Food and Drug Administration Department of Health and Human Services December, 2016

Table 3. Route of administration of different classes of antibiotics in food producing animals (examples Cattle and swine) and nonfood (examples Dogs and cat) in 2015

Route of administration	Class	Annual total (Kg)
Feed	Sulfonamides Tetracyclines	98,831 6,033,388
Water	Aminoglycosides Penicillin Tetracyclines	223,139 793,018 761,346
Other routes	Tetracyclines Other drugs	85,732 372,561

Source: Antimicrobials Sold or Distributed for Use in Food-Producing Animals. Food and Drug Administration Department of Health and Human Services, (2016)

C. Impact of Antibiotics on crops

1. Uptake of antibiotics in water culture and in soil amended with manure in pot experiments.

A Hydroponics experiment was done at AUB in 2018 by Lara El Gemayel. The results showed that lettuce leaves accumulated 70 ng/g, 320 ng/g, and 9 ng/g of enrofloxacin, tylosin, and oxytetracyline, respectively, in hydroponics water solution containing 10 mg/Kg of antibiotic solution in water. However, for radish bulbs, accumulation of antibiotics was 72 ng/g, 410 ng/g, and 38 ng/g for enrofloxacin, tylosin and oxytetracycline, respectively. Concentrations of 5 and 10 mg/Kg of antibiotics has decreased the growth of lettuce leaves, radish leaves and radish roots (Gemayel et al., 2020).

A pot experiment was done in the greenhouse at AUB in 2015 by Sandra Youssef. Results showed that lettuce leaves and radish roots accumulated 31.9 ng/g and 10.4 ng/g, respectively, in soils containing 10 mg/Kg gentamicin. With the same treatment, oxytetracycline accumulated 3.89 ng/g, 6.89 ng/g in radish roots and lettuce leaves, respectively (Youssef et al., 2020). Another pot experiment was conducted by Rani Bassil at AUB in 2012. The experiment was done to check the accumulation of gentamicin and streptomycin in radish, carrot, and lettuce. The three crops absorbed relatively higher amounts of gentamicin than streptomycin (Bassil et al., 2013).

2. Accumulation of antibiotics in crops from irrigation water

Wastewater reuse can help with the issue of water scarcity that developing countries are facing; however, in most cases, the water is not being screened and some contaminants of antibiotics are not being removed. As cited in Gudda et al. (2020), countries are using the reclaimed water from irrigation due to increase in water stress and increase in population. These countries include Australia, Columbia, USA, and China. Beside the benefits of using the wastewater for irrigation, it can spread the antibiotics in our soil and increase their accumulation in crops.

High percentages of antibiotics given to animals are excreted into feces and urine. After the excretion of antibiotics in manure and wastewater, they are used to irrigate and fertilize lands, which will later contaminate the soil and transfer the antibiotics to the crops that we eat (Du and Liu 2011).

A study was conducted in Lahore, Pakistan where pharmaceutical wastewater was used to irrigate wheat, spinach and carrots. They found that wheat, carrots and spinach accumulated between zero and 1 ng/g tissue of Ciprofloxacin, ofloxacin, levofloxacin, oxytetracycline, and doxycycline (Hussain et al. 2016). An experiment was also conducted in Spain, where lettuce, tomato, cauliflower, and broad beans were irrigated from river water (Tadić et al. 2021). Results showed that cauliflower inflorescences accumulated 3.61 ng/g of keto trimethoprim. However, concentration of enrofloxacin was 0-1, 1.5, and 2 ng/g in lettuce leaves, broad beans, and tomato fruits, respectively. Ofloxacin had a concentration of 1.7 ng/g in lettuce leaves. Broad beans can accumulate around 2.7 ng/g of decarboxyl ofloxacin. (Tadić et al. 2021). Another experiment was done in Ghana to evaluate the concentrations of tetracycline and amoxicillin in lettuce and carrots irrigated with known concentrations of these two antibiotics between 0.1 and 15 mg/L in a pot experiment. After that, antibiotics were extracted from lettuce leaves and carrot tubers via accelerated solvent extraction and analyzed on a liquid chromatograph-tandem mass spectrometer. Tetracycline was detected between 4.4 and 28.3 ng/g in lettuce and 12 to 36.8 ng/g in carrots. However, amoxicillin was detected between 13.7 to 45.2 ng/g in carrots and lettuce samples which indicated that amoxicillin was significantly higher than tetracycline (Azanu, et al., 2016).

Irrigating with treated wastewater can lead to accumulation of antibiotics in crops. For example, In Minnesota, USA, sulfamethoxazole was recorded in lettuce with ranges between 100 to 1200 ng/ng (Christou et al, 2017a). Moreover, trimethoprim and florfenicol were recorded in carrots and lettuce, respectively, with ranges between 2.8 to 13 ng/g in carrots and 6 to 120 ng/ng in lettuce (Boxall et al. 2012). In Pearl River delta, China, tetracycline was recorded in cabbage and spinach with concentrations of 6 and 7.4 ng/g, respectively (Pan et al, 2014). Also, it was cited in Gudda et al. (2020) that ciprofloxacin was recorded in cabbage with concentration of around 6.7 ng/g in King Abdullah Canal, Jordan.

3. Accumulation of antibiotics in crops from soil and manure in open fields

Antibiotics are found naturally in the soil. For example, oxytetracycline was first discovered in a soil sample that had soil actinomycete *Streptomyces rimosus* near Pfizer laboratories (Finlay et al. in 1950). It was named Terranycin and later OTC. Moreover, gentamicin could be found naturally in soil. It is produced naturally by the bacterium *Micromonospora purpurea* (Weintein et al. 1963). The bacterium belongs to the family Micromonosporaceae. They are aerobic and can form branched mycelium, where they can occur as saprotrophic forms in soil and water.

Antibiotics can also be found in the soil by contamination with different sources of treatments such as sludge, manure or reclaimed wastewater (Qiao et al. 2018). Antibiotics, such as tetracyclines, can have a high adsorption capability in manure, with a high half-life of up to 180 days (Pan and Chu, 2017b). Applying manure as a long-term treatment in agriculture fields will increase the concentrations of antibiotics in soil (DU and Liu, 2012). According to former research, it is found that soils are the major reservoirs of antibiotics (Kuppusamy et al. 2018). According to Miller et al. (2016), crops can accumulate high concentrations of antibiotics in soils amended with manure.

A study was conducted in Yangtze River Delta, Eastern China, where peanuts were planted in the open field. The soil was amended with pig manure on a yearly basis for 15 years. The pig manure was added each year at a rate between 10 to 80 tons/ha. It showed that peanut roots could accumulate oxytetracycline, chlortetracycline, tetracycline and doxycycline at concentrations 22.73, 8.33, 6.83, and 4.08 ng/g of tissue. Peanut kernels (edible part) can accumulate 0.91 ng/g of oxytetracycline, 15.42 ng/g of chlortetracycline and 20.06 ng/g of tetracycline. Moreover, the manure that was added to the soil had a concentration of 881.74 ng/g of oxytetracycline 24,853.02 ng/g of chlortetracycline, 9819.54 ng/g of tetracycline (Zhao et al. 2019).

D. Impact of antibiotics in the environment

1. Antibiotic contamination in rivers, surface water, and wastewater

It has been reported that enrofloxacin was detected at a concentration of 4.24 and 1.09 ug/L in river water and effluent in China (Wei et al. 2012). Other antibiotics like Ciprofloxacin, and sulfadiazine were also detected at a concentration of 5.93 and 0.108

ug/L in river water in China and Vietnam, respectively. As for Oxytetracycline, it was found at a concentration of 2.2 ug/L in river water in China (Watkinson et al. 2009). Some antibiotic concentrations were found in wastewater effluents such as Sulfamethoxazole in Spain, Korea, USA, Croatia, and Canada at concentrations of 0.05, 0.057, 0.342, 323.135 and 0.16 ug/L, respectively. As cited by Gudda et al. (2020), antibiotics like ofloxacin have been also detected in Spain, and China in wastewater effluents at concentrations of 0.816 and 3.520 ug/L, respectively; also irrigating from municipal wastewater effluents of treatment plants can expose crops to antibiotics. Antimicrobial resistant genes are emerging in the soil, water and air. Human activities are the main source of this spread (Zuccato et al., 2010).

2. *Contamination of antibiotics and their properties in soil and manure*

Antibiotics could be subjected to different abiotic and biotic factors that will let them to degrade or transform in the soil. (Duan et al., 2017). Also, desorption and adsorption in soil may occur (Martínez-Hernández et al., 2016). One of the abiotic degradations that the antibiotics may face is the hydrolysis. Some antibiotics are vulnerable to hydrolysis degradation such as beta-lactams while others are less susceptible such as sulfonamides and macrolides (Mitchell et al., 2015)

When manure and slurry are distributed along the surface of agricultural lands, photo degradation will contribute to degradation of certain antibiotics such as tetracycline and quinolones (Thiele-Bruhn and Peters, 2007).

the molecular structure of antibiotics plays a role in the degradation process in the soil (Pan and Chu, 2016). As cited by Cycoń et al. (2019), antibiotics differ in their rate of degradation in soils, where their half-life could reach from 1 to 3,466 days. According to Braschi et al. (2013), amoxicillin could have a half-life in less than 24 hours.

A study was done by Liu et al. (2014), where 10 mg/Kg of chlortetracycline was degraded in the soil , where DT50, the time required for the concentration to decline to half of the initial value, was less than 1. Nevertheless, outdoor experiments showed long term persistence of azithromycin, ofloxacin, and tetracycline in soils with a half-life reaching more than 408, 866, and 576 days, respectively (Walters et al., 2010).

Adsorption of antibiotics in the soil depends on the Kd coefficient, which is responsible for the transport behavior of organic molecules in the soil or amount of chemical substance adsorbed onto soil per amount of water. Substances that have high Kd values are more adsorbed into the soil particles and are less mobile as stated by Youssef et al. (2017). The adsorption ratio of tetracycline is between 400 to 1,147 L/Kg in clay loam soil; moreover, chlortetracycline had the highest adsorption ability in clay loam, with Kd of around 1,280 to 2,386 L/Kg. This will cause these two antibiotics to reach a degradation rate of 180 days in mud and manure. (Pan and Chu 2017c). Physiochemical properties of antibiotics such as the chemical formula, molecular weight, water solubility and Kd ratio (adsorption ratio) are found in Table 4. Concentrations of different antibiotics in manure and sludge are found in Tables 5 and 6.

Table 4. Physiochemical properties of some antibiotics

Antibiotic class	Example	Chemical formula	Molecular weight	Water Solubility (mg/L)	Kd (L/Kg)
Aminoglycosides	Streptomycin	$C_{21}H_{39}N_7O_{12}$	581.6	12,800	8 - 290
Tetracycline	Tetracycline	$C_{22}H_{24}N_2O_8$	444.43	231	417 - 1,026
Tetracycline	Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.4	1,000	417 - 1,026
β-Lactams	Amoxicillin	$C_{16}H_{19}N_3O_5S$	423.5	1,030	0.21 - 3.83
Macrolides	Tylosin	$C_{46}H_{77}NO_{17}$	916.1	5,000	5.4 - 172,480

Source: (Cyon et al., 2019)

Table 5. Maximum reported concentrations of antibiotics in manure ug/Kg

Class	Antibiotic	Concentration	Reference
Fluiroquinolones	Ciprofloxacin	45,000	Zhao et al, 2010
Tetracyclines	Oxytetracycline	354,000	Chen et al, 2012
Macrolides	Tylosin	7,000-8,000	Dolliver et al 2008

Source: (Cyon et al., 2019)

Table 6. Maximum reported concentrations of antibiotics in sewage sludge ug/Kg dw

class	Antibiotic	Concentration	Reference
Fluoroquinolones	Ciprofloxacin	426	Lillenberg el al, 2010
Macrolides	Azithromycin	1.3-158	Gobel et al, 2005
Sulfonamides	Sulfamethazine	0-20	Lillenberg el al, 2010
Tetracyclines	Tetracycline	8,326	Cheng et al., 2014

Source: (Cyon et al., 2019)

3. Antimicrobial resistant bacteria in soil and water

The increase of antibiotic resistant bacteria cause threat to animals' and humans' Health. According to Magnusson and Landin (2021), "it is estimated that in the coming decades, there will be several million deaths annually and a significant drop in livestock production attributable to infections by bacteria resistant to antibiotics". This problem is originating from the use of antibiotics haphazardly. Antibiotics should be given only when needed. Farmers and livestock animals are more prone to getting infected by antibiotic resistant bacteria (Magnusson and Lan din, 2021).

It was reported than some pathogens have resistant genes to antibiotics. For example, *Escherichia coli* has resistant genes in drinking water in India. These genes are responsible for different diseases such as diarrhea, septicemia, urinary tract infections, neonatal abdominal pain, fever, and pneumonia. Moreover, Salmonella sp. has been reported to express resistant genes in drinking water in Uganda. Antibiotic resistant bacteria can be transmitted in the recreational use of water, whether through marine or fresh water. The natural aquatic habitat like surface water, rivers, and sea are receiving water from sewage sludge, manure and wastewater treatments, and they all contain pathogens that are resistant to antibiotics (Manyi-Loh 2018 et al. 2018).

There is a link between antimicrobial resistances (AMR) to the exposure of antibiotics in the environment. The microbiome in the human body, which consists of multicellular organisms, bacteria, and viruses, can be disturbed by traces of antibiotics. The microbiome in the gut helps fight harmful pathogens by the production of certain vitamins and nutritional supply. Some antibiotic resistant bacteria have been found in many wastewater effluents. Also, heterotrophic bacteria have been found to be resistant to ampicillin and tetracycline in Northwest Beijing, China in domestic wastewater. The human exposure to antibiotics and their effect on humans starts from the sources of antibiotics like hospitals, agro activities, industries, and municipal wastewater and other sources, which will then be mixed in the sewage treatment plants, and later be used for the irrigation of crops. Using such water for irrigation will contaminate the soil and crops by up taking antibiotics and some resistant genes. Humans who consume vegetables that had been irrigated with antibiotics contaminated water will affect their gut microbiome.. Thus, humans will be at risk of exposition of resistant bacteria and genes. (Gudda, et al., 2020)

E. Situation in Lebanon

1. Antibiotics use in Lebanon

A survey was conducted by Mirna Choueiri in Bekaa and Mount Lebanon to check what antibiotics the farmers are using for cows in their farms. The survey was conducted on 17 farms, 12 of them in Bekaa and others in Mount Lebanon. It has shown that all farmers were using streptomycin and gentamicin. Fifty nine percent of the farmers were using penicillin and oxytetracycline for milk production. The farms were divided into 3 groups: small, medium (100-500 cows) and large-scale farms (more than 1000 cows). The tested antibiotics were gentamicin and streptomycin and were taken from manure. Mean concentrations of gentamicin and streptomycin from Bekaa were 139.16 ng/ml and 60.39 ng/ml, respectively. However, concentrations of gentamicin and streptomycin in Mount Lebanon were 80.74 ng/ml and 52.36 ng/ml, respectively. According to Choueiri et al. (2008), farmers were adhering to the recommended doses while administering the antibiotics.

A study was conducted by Jammoul et al. (2019) on 80 random chicken samples from a slaughterhouse in Keserwan area, Mount Lebanon. The objective of the study was to analyze the number of residues of four different chemical classes (sulfonamides, quinolones, tetracyclines, and beta lactams) of 30 antibiotics in chicken. All samples were from different farms in Jbeil, Keserwan, Batroun, and Akkar. Antibiotics were analyzed using the LC–MS/MS method. The highest concentrations of antibiotics detected in chicken were from the tetracyclines group. The mean detected in the 80 samples were 24.4, 22.6, 11.4 ug/Kg for tetracycline, oxytetracycline, and doxycycline, respectively. The second highest antibiotics goes to the family Beta-lactams. The mean detected in the 80 samples of Beta lactams was 8.5 ug/Kg for penicillin G and 5.4 ug/Kg for Amoxicillin. Sulfonamides (Sulfacetamide, sulfamethazine, sulfadimethoxine...) and Quinolones such as (sarafloxacin, Enrofloxacin, ciprofloxacin, and ofloxacin) were detected in ranges between 0 to 0.4 ug/Kg. Results has shown that the highest concentration of detection between Quinolones is ciprofloxacin (6.2 ug/Kg) and the second was ofloxacin (2.28 ug/Kg). The trend of antibiotic usage from which the samples were collected can tell us that tetracyclines are the most used antibiotics in poultry production. The tetracyclines detected in poultry were below the MRL values (200 ug/Kg). Moreover, MRL values of gentamicin in muscle, liver, fat and milk of cattle are: 100, 2000, 100, 200 ug/kg. The MRL values of oxytetracycline in muscle and milk of cattle are: 200ug/kg and 100 ug/L according FAO and WHO (2018).

A survey was made in Lebanon in 2008 to conduct the most used antibiotics in dairy cows. Twenty-six random farms were visited and more than 50% of them were small scale farms (less than 1000 cattle). The survey was conducted in Bekaa and Mount Lebanon. It has shown that farmers used streptomycin, Gentamicin, penicillin, Kanamycin and oxytetracycline (Abi Khalil et al. 2008).

A study was done by Mokh et al. (2017) in Lebanon to determine 63 pharmaceutical compounds in rivers and wells in different areas all over Lebanon. The method of detection was liquid chromatography coupled to tandem mass spectrometry (LC-ESI-MS/MS). Erythromycin and caffeine were the only compounds found in some rivers. For example, Anhydro-erythromycin was found in Beirut River at a concentration of 157 ng/L and 98 ng/l in Ibrahim River. However, tetracycline was not detected among the compounds.

F. Brief description of antibiotics used in the experimental study

1. Gentamicin

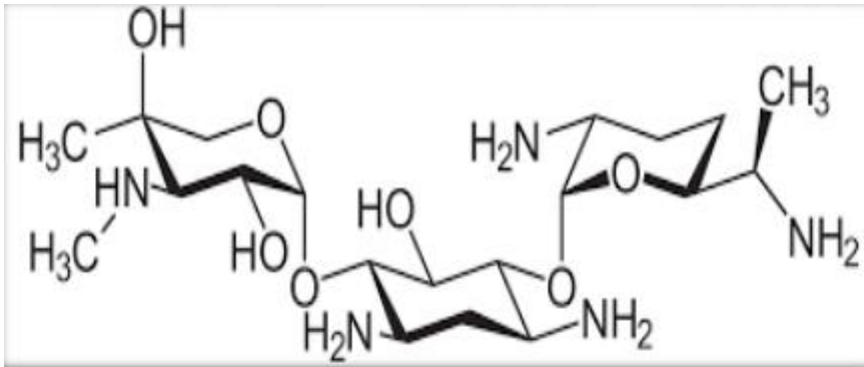


Figure 1. Gentamicin structure. Source: (Youssef et al., 2017)

The chemical structure of gentamicin (C12H43N5O7) with molecular weight of 477.595 g/mol Gentamicin works mostly on gram negative bacteria. It inhibits the translocation of the peptidyl-tRNA from the A-site to the P-site and causes misreading of mRNA, which causes the bacterium to stop synthesizing protein that increases its growth. As cited by Youssef et al. (2017), “Gentamicin is mainly used for the treatment of respiratory, gastrointestinal and urogenital infections”. Also it works against wide ranges of bacteria and most of them belong to gram-negative such as *Pseudomonas, Proteus, Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes,* and *Serratia* and some gram-positive bacteria like *Staphylococcus*.

2. Oxytetracycline

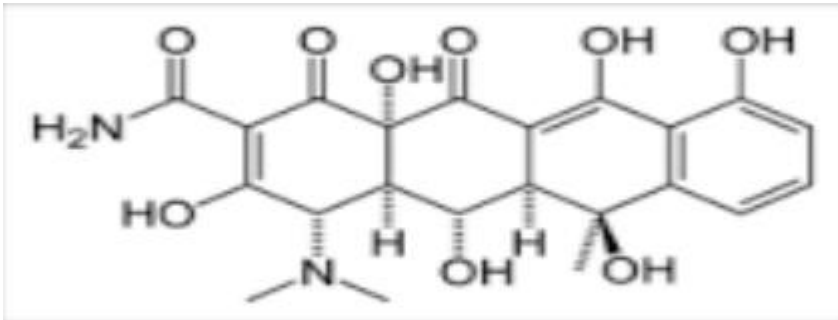


Figure 2. Oxytetracycline structure. Source: Xu et al., 2020

Oxytetracycline has the molecular formula C22H24N2O and molecular weight of 460.44 g/mol. It belongs to Tetracycline class, which is a broad-spectrum class that works on gram positive (Cocci, Actinomycetes) and gram negative (*Escherichia coli, Salmonella*, and *Pseudomonas*). According to Prescott and Dowling (2013), oxytetracycline is used to treat systemic infections, such as respiratory, gastrointestinal and skin bacterial infections, in livestock animals.. The mode of action of oxytetarcycline is the same as that of gentamicin.

CHAPTER III

MATERIALS AND METHODS

This chapter explains the materials and methods used in field experiment, pot experiment, Antibiotic analysis of water samples from Bekaa, column experiment, ELISA procedure and statistical analysis.

This chapter explains the materials and methods used in this study. The study was composed of 5 parts:

1. A field experiment at AREC (Agricultural Research and educational center)
2. A pot experiment at the greenhouse at AUB
3. Collection of water samples from Bekaa area
4. Column experiment at the greenhouse at AUB
5. ELISA procedure to detect antibiotics in plants

This chapter will also cover the procedure of soil analysis for the soil of the field and the pots. Moreover, the ELISA procedure and the statistical analysis will be illustrated.

A. Field experiment

This experiment was carried at AREC in plot D54 (Latitude 33°55'26.68"N Longitude 36° 4'22.54"E) during the summer (July) of 2019. The aim of the experiment was to determine whether radish, cucumber, and lettuce plants can accumulate gentamicin and oxytetracycline in their tissue or not. The treatments used were: soil with no addition of antibiotic, soil and manure with no addition of antibiotic, soil with the addition of antibiotic, and soil and manure with the addition of antibiotic. The rate of antibiotic used was 30 mg/Kg manure. The antibiotics were mixed with cow manure and added to the soil prior to planting. The cow manure used was collected from AREC and was well fermented. The field was prepared at beginning of July and the plants were transplanted into the field on 18th of July and harvested in September 5, 2019.

1. *Soil analysis*

A soil sample was collected prior to the start of the experiment from plot D54 at AREC and analyzed for soil texture, soil pH and EC, available phosphorous (P), available sodium (Na) and potassium (K), free calcium carbonate (CaCO3), and available micronutrients (Iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn)). The analyses were conducted according to the Methods of Analysis in Arid and Semi-Arid regions (Bashour and Sayegh, 2007). The sample was sieved using a 2 mm sieve. Gravels were removed and large clods were crushed using mortar and pestle. The sample was stored in a clean plastic container.

2. *Field design*

The field was arranged using a modified randomized complete block design with 3 replicates for each antibiotic treatment. The field was divided into a total of 18 plots, where each plot had an area of 6 m^2. The 18 plots were divided as follow: Nine plots amended with manure and 9 plots without manure. Three letters were given to the plots amended with manure, C, D and F (three treatments with 3 replicates), as shown in Figure 3. As for the plots without manure, the letters A, B, and E were given. Each letter is located on the left side of each plot as shown in Figure 3. The length of the field is 19 meters long and the width is 16 m wide, having a total area 304 m^2. A sub-main irrigation pipe was constructed at the bottom of the field, as shown in the Figure 3. The pipe was 1 inch in diameter and 6 tape lines were installed to the sub-main. The pressure used was 0.8 bars, since the maximum capacity for the tapes was 1 bar. The plants were irrigated equally, around 40 minutes per day from the D54 pool. The end of each tape was tied well so as no water can leak. Each plot was divided into 3 equal parts, where each part had a plant either lettuce, radish or cucumber. The plots amended with manure were on the left side as illustrated in Figure 4 while the plots without manure were on the right side as illustrated in Figure 5. Two meters spacing was done to separate the plots from each other vertically and 0.5 m horizontally.

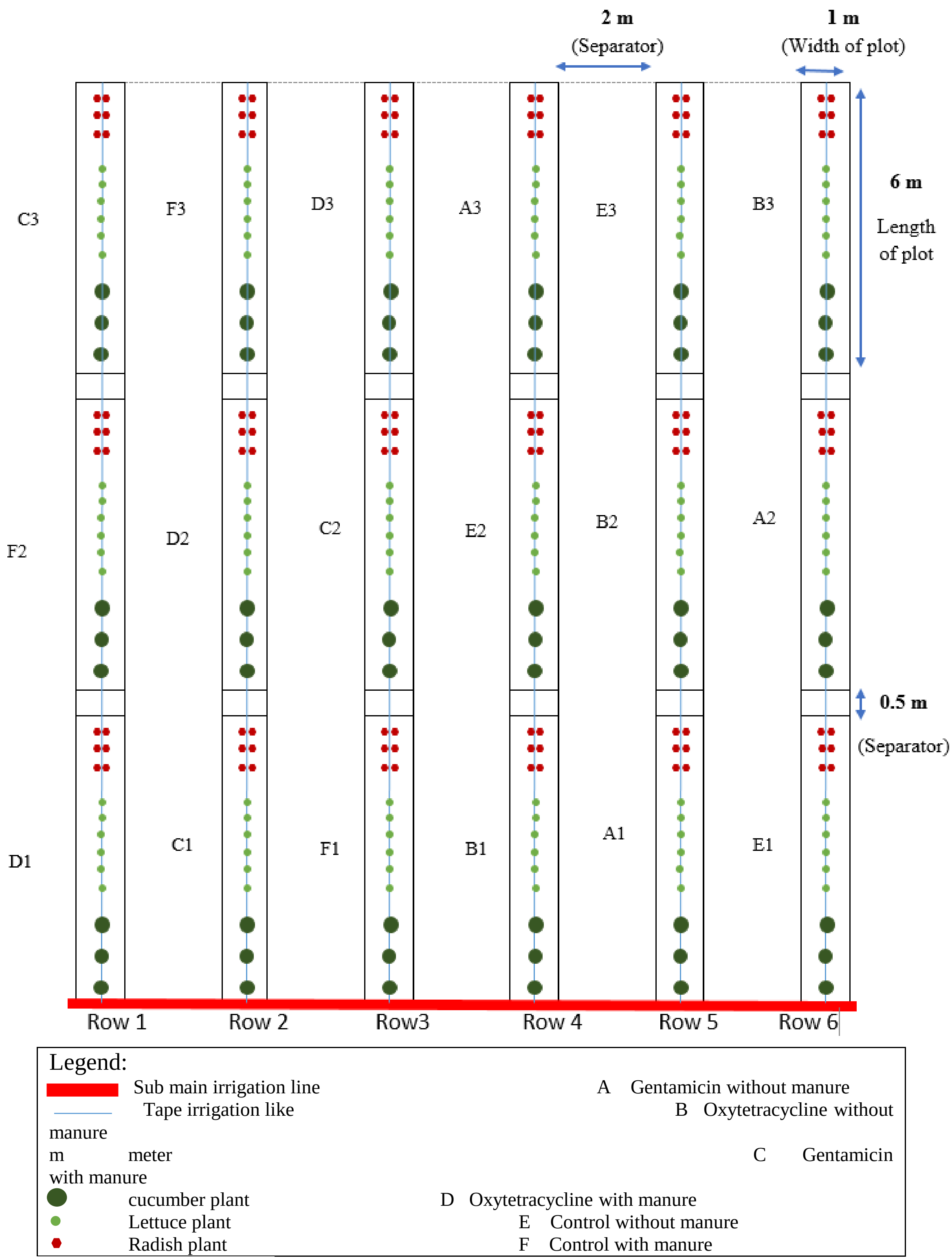


Figure 3. Field design

Figure 4. Plots treated with antibiotics and amended with manure

Figure 5. Plots treated with antibiotics free of manure.

3. Land preparation:

The land was prepared using primary and secondary tillage. A reversible moldboard plow was used in the first tillage and then rotary tiller and tandem disk harrow were used for the second tillage. In the plots amended with manure, the top soil was mixed with manure manually using a shovel. a granular fertilizer (15-15-15) was added at a rate of 50 Kg/ du. Around 0.3 Kg of fertilizer was used per plot. As for the plots treated with manure, the fertilizer was incorporated in the manure and mixed with the top soil.

4. Antibiotic rates

The rate of antibiotics used was 30 mg/Kg of antibiotic in dried manure. The amount of dried manure applied to each plot was 30 Kg making the total quantity of antibiotics applied per plot, is 900 mg/30 Kg manure. The antibiotics were bought from vet store in Bekaa. All antibiotics were added to water and mixed with manure before applying it to the soil.

5. Manure mix

The manure was brought from the manure stockpile near the cow pen inside AREC. The manure was carried by a bulldozer to a pickup and then transferred to the field. After that, big clods of manure and rocks were removed from the pile using a sieve as shown in Figure 6. The 30 Kg were weighed in big bags and distributed to the plots as shown in Figure 7. In the plots treated with manure, antibiotics were mixed with 20 liters of water. The water was then added to the manure distributed all over the treated plots. However, for the plots with no manure added, the antibiotics were mixed with 20 liters of water. the water was then spread along the tape line over the plot. The number of plants grown per plot were: 6 radish, 6 lettuce, and 3 cucumber.

Figure 6. Large manure clods being removed using a sieve

Figure 7. Bags of manure placed in each plot

Figure 8. Manure pile

6. Plant description

Freshly consumed crops were used in the field experiments: cucumber, lettuce, and radish. Cucumber and lettuce seedlings were bought from ROBINSONAGRI; however, radish was sowed as seeds in the field. In each plot (6 m^2), six lettuce seedlings, three cucumber seedlings and around 20 radish seeds were sown along the tape line. After the radish germinated, thinning was made to 6 radish plants per plot (Figure 9). The edible parts from each crop were later analyzed for antibiotic uptake.

Figure 9. Crop order in each plot (cucumber, lettuce then radish)

7. Plant tissue analysis

The crops were harvested on September 6, 2019 and were brought directly to the lab at AUB and stored in the fridge at 4°C for analysis. The extraction of antibiotics from cucumber fruit, lettuce leaves and radish bulbs were done two days after harvesting.

The extraction procedure used in this experiment was taken from the procedure used by Lara El-Gemayel in her thesis (2018). The method of tissue analysis is as follow:

- Wash the plant completely
- Blot the tissue using clean paper towels

- Finely chop the radish bulbs, lettuce leaves and cucumber fruits, using a knife
- Weigh some of the chopped material (representative sample) and place them in a 50 mL falcon tube (number the tubes indicating the plant material and antibiotic) – (6 g radish, bulbs, 6 g cucumber fruits, 6 g lettuce leaves)
- Add their respective extraction buffer (distilled water for gentamicin, Mcllvain buffer oxytetracycline, following the ratios: 6 grams in 18 ml distilled water or Mcllvain buffer)

- Blend the fresh mixture directly using a blender until all the plant material is fully cut and become a suspension (uniform grinding and mixing are crucial to get representative samples and accurate analytical results)
- Place each falcon tube on a vortex for at least 2 minutes to homogenize the sample suspension
    - Filter each mixture into its respective volumetric flask using (F40 Whatman) filter papers
- Pour the filtrates into a new falcon tube and place them in the refrigerator at a temperature of -4°C (each tube was labeled according to each treatment and plant with a date)

B. Pot experiment

The objectives of this experiment were to test whether radish, lettuce and cucumber plants accumulate antibiotics (gentamicin and oxytetracycline) via irrigation water and whether adding manure to the soil affects the accumulation of antibiotics in their edible parts. The experiment was carried out at the greenhouse at AUB in June 2020. The soil used was free of antibiotics. Manure was brought from AREC and was shredded into small pieces and dried before being mixed with the soil. Clean plastic bottles (20 L) were used for the preparation of irrigation water containing antibiotics. The greenhouse temperature was set at 24°C during the experiment.

1. Soil analysis

The soil used was analyzed for soil texture, soil pH and EC, available phosphorous (P), available sodium (Na) and potassium (K), calcium carbonate ($CaCO_3$), and available micronutrients (Iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn)). The analyses were conducted according to the Methods of Analysis in Arid and Semi-Arid regions (Bashour and Sayegh, 2007). The sample was sieved using a 2 mm-sieve and was stored in a clean plastic container for analysis.

2. Experimental scheme

The design of the pot experiment in the greenhouse was a 2x2 factorial with three replicates. The Factors are manure (with or without) and irrigation water (0 or 20 mg/L gentamicin or oxytetracycline). The crops used were similar to the ones used in the field experiment, cucumber, radish and lettuce. The total number of pots in the experiment were

72 (3 crops x 2 soil medias x 2 antibiotics x 2 levels each x 3 replicates), 24 pots for each crop.

3. Pot Preparation

The soil mix procedure was the same one used in Sandra Youssef's previous work at AUB. The soil was sieved via 10 mm sieve. The soil was then weighed in plastic bags before being placed in each pot. The soil was either mixed with manure (5 Kg soil + 0.25 Kg manure) or placed on its own (5 Kg). Then, one gram of 20-20-20 fertilizer was added in each bag. Finally, each bag was mixed very well, and the mixture was then poured gently in each pot and labeled properly. One seedling was planted in each pot for lettuce and cucumber. However, radish was sown as seeds and thinned after 5 days to keep a total of two seedlings per pot. (Figures 11 and 12).

Figure 10. Shredded manure exposed to heat in Greenhouse in May 2020

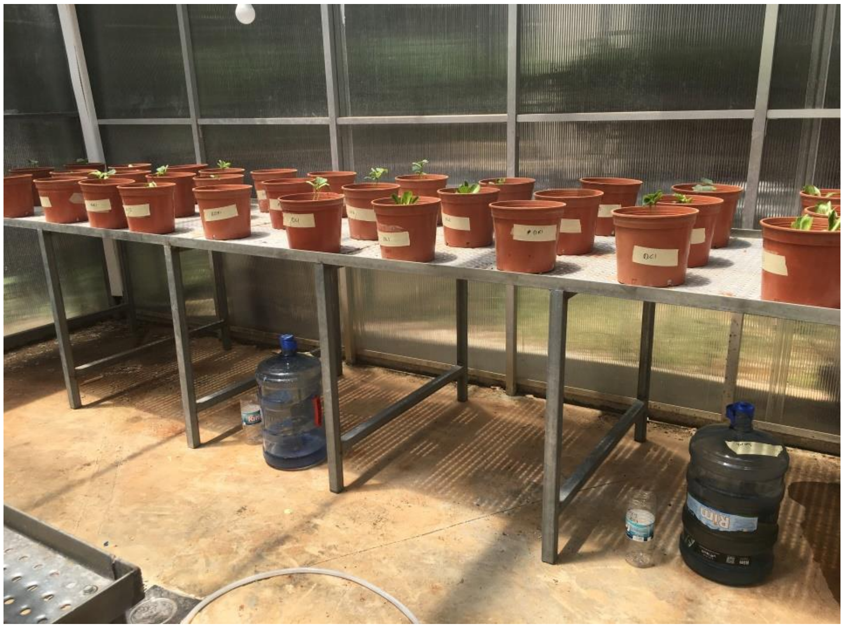


Figure 11. First week of the experiment

Figure 12. After 30 days of the experiment

4. Treatment distribution

The treatments for each crop are found in the Table 7.

Table 7. Treatments subjected to each crop

Number	Manure treatment	Type of Antibiotic Treatment in Irrigation Water (mg/L)
1	Soil + manure	0 gentamicin
2	Soil + manure	20 Gentamicin
3	Soil + manure	0 oxytetracycline
4	Soil + manure	20 oxytetracycline
5	Soil	0 gentamicin
6	Soil	20 Gentamicin
7	Soil	0 oxytetracycline
8	Soil	20 oxytetracycline

5. *Antibiotic concentration in water tanks*

Three clean plastic bottles (20 L) were filled with distilled water. Two of the tanks were subjected to antibiotics (gentamicin and oxytetracycline) and the third one was kept for the control, with distilled water only. The rate of antibiotics used in the irrigation water was 20 mg/L. Each tank was refilled with distilled water and the same concentration of antibiotics. The plants were irrigated on a daily basis with the ascribed treatments. Each tank was filled on a weekly basis.

6. *Tissue analysis*

All plants were harvested between July 28 and August 8, 2020.

The method of tissue analysis was similar to the one used in field experiment. The procedure includes:

- Weigh the crop and wash it well
- Dry the crops with paper towels
- Collect the edible parts, radish bulb, lettuce leaves and cucumber fruit
- Place 6 grams of tissue in the ELISA extraction bag. Add 18 ml of deionized water ratio (1:3)
- Grind the contents of the bag well by using an ELISA grinder to obtain a homogenized mixture
- Pour the suspension in a 50 ml falcon tube.
- Write the type of treatment and date on each falcon tube
- Use a vortex for two minutes to homogenize the extract

- Place the falcon tubes in the refrigerator at -4°C until the time of analysis.

C. Water samples from Bekaa

Four water samples were collected in July 2020 from AREC and the Litani River to determine their contamination with gentamicin and oxytetracycline. The samples were collected by submerging clean plastic bottles (2 L) inside the surface water (river or pool) and then stored at 4°C. The samples from the Litani River were collected from different locations. One sample was collected from main River near Joub Jannine and the other sample from Tal-Amara. The coordinates of the locations of the samples are found in Table 8. The samples from AREC were collected from the D54 pool and the earth pool. These pools are used for irrigating shrubs and crops at AREC. the D54 pool is filled by the Tell well at AREC while the Earth pools filled by different wells at AREC. The ELISA method was used in the determination of oxytetracycline and gentamicin in water samples.

Table 8. Coordinates of samples location

Location of water samples	Coordinates
Litani river	Latitude 33°38'19.0"N Longitude 35°46'47.5"E
Tal-Amara	Latitude 33°51'47.29"N Longitude 35°59'20.11"E
AREC-D54 pool	Latitude 33°55'25.92"N Longitude 36° 4'21.51"E
AREC-Earth pool	Latitude 33°55'31.55"N Longitude 36° 4'41.77"E

Figure 13. Litani River near Joub Jannine

Figure 14. Earth pool at AREC

Figure 15. D54 pool at AREC

D. Gentamicin and oxytetracycline leaching experiment

The aim of this experiment was to determine whether oxytetracycline and gentamicin leach in the soil using the column experiment. Four (20 cm) PVC cylinders were built, attaching 2 segmented rings (10 cm height and 8 cm in diameter each) together using a tape, as shown in Figure 16. Then, the columns were covered with a cheese cloth, from the bottom side. Two different soil types (sandy and clayey) were used in this experiment to determine the effect of the soil texture on the leaching of the antibiotics. Therefore, for each antibiotic treatment, there were two columns, each column having a different soil texture. The top 5 cm were kept empty in each column as shown in Figure 17. Moreover, a concentration of 20 mg/L per antibiotic (gentamicin and oxytetracycline) were mixed in water and added to each column. The leached water was then taken and stored in the fridge prior to testing using the ELISA method.

Figure 16. Empty PVC Columns

Figure 17. Columns filled with 2 soil types

E. ELISA procedure

Analysis for antibiotics was conducted using the enzyme-linked immunosorbent assay (ELISA) method. The kits were manufactured by PerkinElmer, Austin, TX USA.

1. Procedure for detection of oxytetracycline and gentamicin

According to the Maxsignal Gentamicin Test Kit (product no. 1027-01A) and to the Maxsignal oxytetracycline ELISA Test Kit (product no. 1081-01D), the procedure to be used is as follows:

"The method is based on a competitive, two-step colorimetric enzyme-linked immunosorbent assay (ELISA). The target analyte has been coated to the plate wells. During analysis, a sample is added along with a primary antibody specific to the target analyte. If the target analyte is present in the sample, it will compete for the antibody, thereby preventing the antibody from binding to the analyte coated to the plate well. A secondary antibody, tagged with a peroxidase enzyme, targets the primary antibody that is complexed to the analyte coated on the plate wells. The resulting color intensity, after addition of TMB substrate, has an inverse relationship to the target analyte concentration in the sample". The standards given with the kits are represented in Table 9. The reagents used by both kits are represented in the table 10.

Table 9. Standards used by both kits

Gentamicin standards (ng/ml)	**Oxytetracycline standards (ng/ml)**
0	0
0.05	0.15
0.1	0.375
0.3	0.75
0.9	1.5
2.7	4.5

*All samples and standards were removed from the fridge and kept at room temperature for 2 hours before use.

Table 10. Reagents given by both kits

Oxytetracycline reagents	Gentamicin reagents
Oxytetracycline-Coated Plate 1 x 96-well plate	Gentamicin-Coated Plate 1 x 96-well plate
Oxytetracycline Antibody #1 12 mL	Gentamicin Antibody #1 12 mL
100X HRP-Conjugated Antibody #2 300 µL	100X HRP-Conjugated Antibody #2 250 µL
20X Wash Solution 28 mL	20X Wash Solution 28 mL
Antibody #2 Diluent 20 mL	Antibody #2 Diluent 20 mL
TMB Substrate 12 mL	TMB Substrate 12 mL
Stop Solution 14 mL	Stop Solution 14 mL
5X Oxytet Extraction Buffer 2 x 25 mL	10X Sample Extraction Buffer 25 mL
10X TET Sample Diluent 28 mL	-
TET Balance Buffer Concentrate 5 mL	-
Standard Diluent 28 mL	-

2. ELISA Procedure for oxytetracycline proposed by the supplier is as follows:

- Add 75 µL of each standard in duplicate to different wells using a new pipette tip for each standard addition. Add standards from low to high concentration.
- Add 75 µL of each sample in duplicate to remaining wells using a new pipette tip for each sample addition.
- Add 100 µL of Oxytetracycline Antibody #1 to each well.
- Cover the plate. Incubate the plate for 55 minutes at controlled room temperature
- After incubation, wash the plate 3 times
- After the 3rd wash, invert the plate and forcibly tap it against paper towels until no 1X Wash Solution remains. Continue to the next step immediately to avoid drying of the plate
- Add 150 µL of freshly prepared 1X HRP-Conjugated Antibody #2 to each well

- Cover the plate. Incubate the plate for 25 minutes at controlled room temperature (20–25°C).
- After incubation, wash the plate 3 times
- After the 3rd wash, invert the plate and forcibly tap it against paper towels until no 1X Wash Solution remains. Continue to the next step immediately to avoid drying of the plate.
- Add 100 µL of TMB Substrate to each well.
- Cover the plate. Incubate the plate for 15 minutes at controlled room temperature (20–25°C) in the dark
- After incubation, add 100 µL of Stop Solution to each well to stop the substrate reaction
- Use a lint-free wipe to clean the bottom of the wells before continuing to the next step
- Obtain the absorbance values using a plate reader set at 450 nm primary filter (OD450).

3. ELISA Procedure for gentamicin proposed by the supplier is as follows

- Add 50 µL of each standard in duplicate to different wells using a new pipette tip for each standard addition. Add standards from low to high concentration.
- Add 50 µL of each sample in duplicate to remaining wells using a new pipette tip for each sample addition.
- Add 100 µL of Gentamicin Antibody #1 to each well.

- Cover the plate. Incubate the plate for 30 minutes at controlled room temperature (20–25°C).
- After incubation, wash the plate 3 times.
- After the 3rd wash, invert the plate and forcibly tap it against paper towels until no 1X Wash Solution remains. Continue to the next step immediately to avoid drying of the plate.
- Add 150 µL of freshly prepared 1X HRP-Conjugated Antibody #2 to each well.
- Cover the plate. Incubate the plate for 30 minutes at controlled room temperature (20–25°C).
- After incubation, wash the plate 3 times.
- After the 3rd wash, invert the plate and forcibly tap it against paper towels until no 1X Wash Solution remains. Continue to the next step immediately to avoid drying of the plate.
- Add 100 µL of TMB Substrate to each well. Avoid contamination by not touching the interior of the wells with the pipette tip. Discard the substrate if any coloration is observed before addition to each well.
- Cover the plate. Incubate the plate for 15 minutes at controlled room temperature (20–25°C) in the dark.
- After incubation, add 100 µL of Stop Solution to each well to stop the substrate reaction. Avoid contamination by not touching the interior of the wells with the pipette tip.

- Use a lint-free wipe to clean the bottom of the wells before continuing to the next step.
- Obtain the absorbance values using a plate reader set at 450 nm primary filter (OD450).

F. Statistical analysis:

For the field experiment, the mean concentration of antibiotics for all treatments (Soil + antibiotics) were compared using two-way ANOVA (analysis of variance) and the post hoc analysis was used to compare the significance between all groups using Bonferroni's range test in SPSS version 22. Manure and antibiotics application were the two factors and the output antibiotics concentration in each crop was the dependent variable. Differences were considered significant when P-value<0.05. The same method of analysis was used in the greenhouse experiment to determine the significant difference in the accumulation of antibiotics in the crop (radish, cucumber, or lettuce) with respect to both factors (with or without manure and irrigation water treated with antibiotics (0 or 20 mg/lit antibiotic)). The average means of the accumulated antibiotics per crop were considered significant when P-value<0.05.

CHAPTER IV

RESULTS AND DISCUSSION

In this chapter, the results of the soil analysis for the field experiment and pot experiment will be presented and discussed. The results of the field, pot, and column experiments will be analyzed and discussed.

A. Field experiment

In this section, the analysis of the soil of the field experiment will be presented and discussed. Also, the accumulation of gentamicin and oxytetracycline in radish, lettuce and cucumber grown in the open field at AREC will be presented.

1. Physical and chemical properties of soil

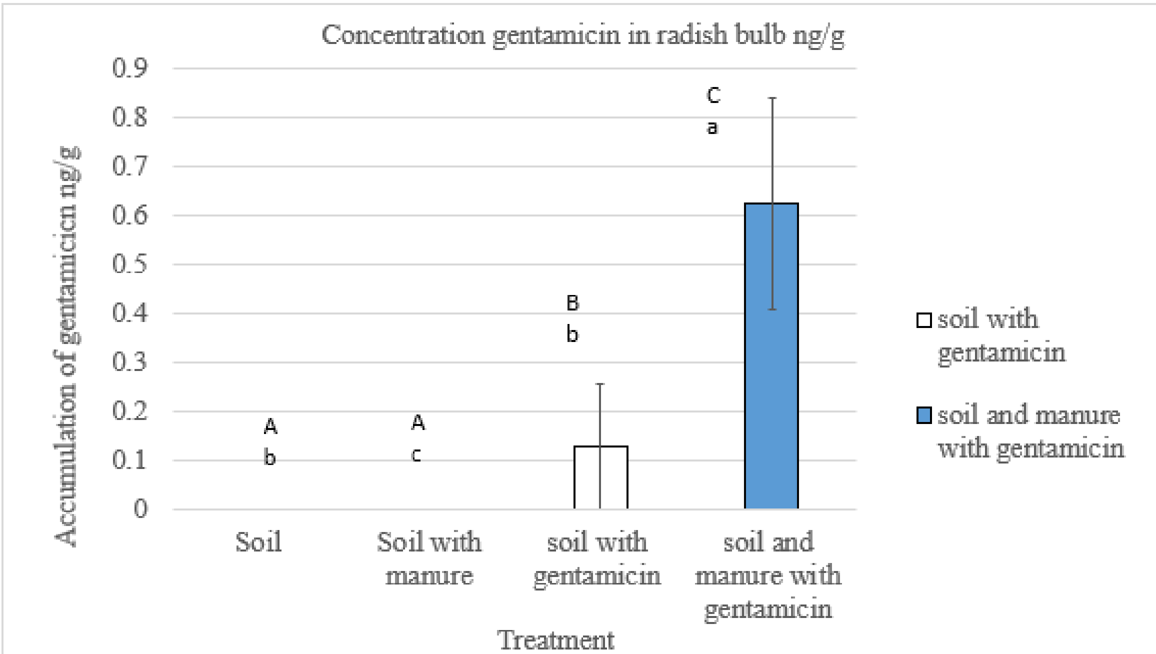
A soil sample from D54 plot at AREC was sampled before starting the experiment. The sample was then taken to the lab at AUB for analysis. It has shown that the soil is non-saline (EC less than 4 mS/cm), calcareous, and slightly basic. Available elements, such as K and P were high. Microelements extracted with DTPA such as Fe, Cu, and Mn were considered high, and Zn was in medium range. (Bashour and Sayegh, 2007) (Table 11).

Table 11. Soil physical and chemical properties of field experiment

Parameter	Result
Texture	Clay
pH	7.4
EC	584 uS/cm
%CaCO3	25
Available P	30 mg/kg
Available K	668 mg/kg
Available Fe	9 mg/kg
Available Zn	2.1 mg/kg
Available Cu	2.16 mg/kg
Available Mn	10 mg/kg

2. Gentamicin antibiotic

a- <u>Radish</u>



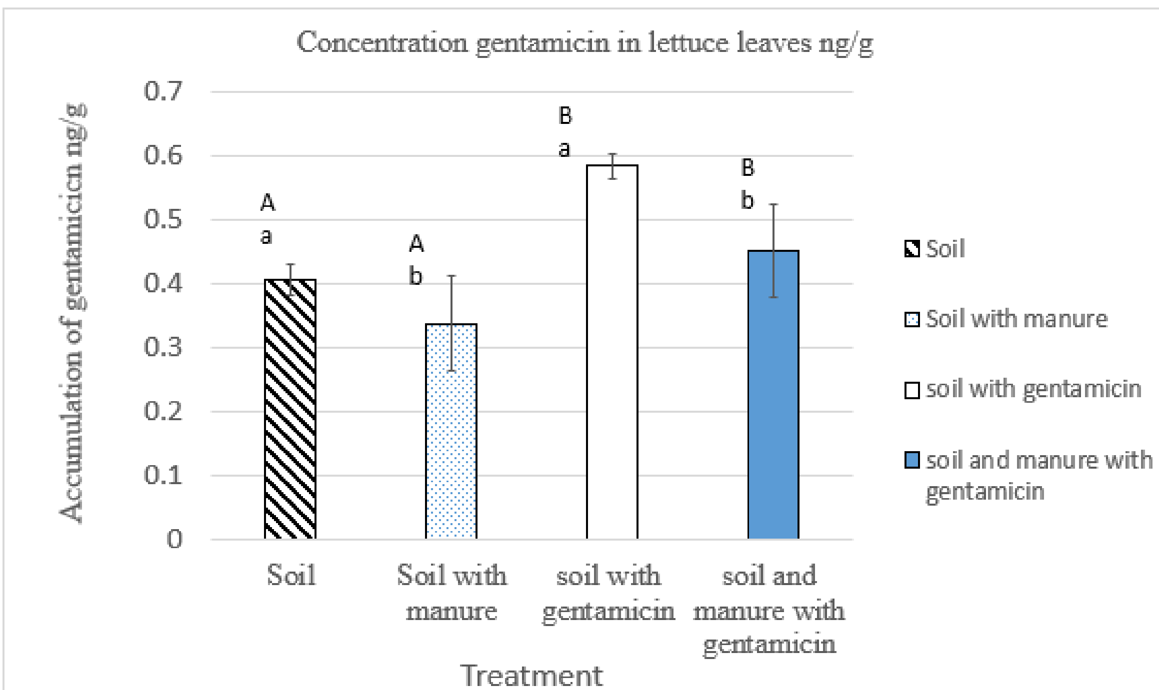
*small letters correspond to the significant difference between no antibiotic treatments (soil or soil and manure) and with antibiotics treatments (soil or soil and manure). Capital letters correspond to the significant difference within the same treatment of antibiotic (with and without manure)

Figure 18. Concentration of gentamicin in radish.

As shown in Figure 18, the addition of gentamicin in soil without manure didn't have a significant difference compared to the control (soil); however, the addition of gentamicin to manure showed a significant difference compared to (soil and manure with no antibiotic) and (soil with antibiotic) treatments . This shows that the manure increases the accumulation of gentamicin in the radish bulb, where it reached 0.625 ng/g tissue; it is higher than that without manure, 0.129 ng/g. As cited by Basil et al. (2013), manure boosts the uptake of gentamicin in radish.

A pot experiment conducted by Sandra Youssef in the greenhouse at AUB in 2016, where gentamicin was added in manure and mixed with soil at different rates of the antibiotic, showed that the addition of gentamicin and manure in the pots increased the accumulation in radish roots. This tells us that radish can accumulate gentamicin from soils in open fields and from those in the pots. However, the accumulation in pots was higher than that in the field.

b- <u>Lettuce and Cucumber</u>



*small letters correspond to the significant difference between no antibiotic treatments (soil or soil and manure) and with antibiotics treatments (soil or soil and manure). Capital letters correspond to the significant difference within the same treatment of antibiotic (with and without manure)

Figure 19. Concentration of Gentamicin in Lettuce leaves.

There was no significant difference between all the treatments of gentamicin in lettuce leaves. All values were below 1 ng/g tissue. However, results showed that the highest gentamicin accumulation in lettuce leaves was in the plots treated with gentamicin without manure, the 0.58 ng/g tissue. The concentration of gentamicin in the lettuce leaves of the plots treated with gentamicin and manure was 0.45 ng/g tissue.

A previous pot experiment at AUB has shown that applying gentamicin to manure in the soil can increase its accumulation in lettuce leaves. Adding gentamicin to the soil without mixing it with the manure, shows no significant difference between treatments (gentamicin in soil with no manure) compared to the control (0 mg/Kg gentamicin in soil with no manure) (Youssef et al. 2020). This shows that the lettuce plants in the open field accumulate gentamicin less than in those in the pots. This could be due to the fast degradation of gentamicin during the summer, and high temperatures. Also, antibiotics in the field are much more diluted than when added in pots; this is because the amount of soil in the field is higher than in pots.

The cucumber fruits did not accumulate gentamicin. The gentamicin concentrations were very low, less than 0.1 ng/g of tissue, and there was no significant difference between all the treatments.

3. Oxytetracycline antibiotic

Accumulation of oxytetracycline didn’t show any significant difference between all treatments in each crop. The oxytetracycline couldn’t be detected in most of the replicates. Yet, a few replicates showed traces of oxytetracycline, less than 0.1 ng/g. This may be due to different factors, either the concentration of oxytetracycline in manure wasn’t enough or the plant didn’t absorb the oxytetracycline in the field. However, previous research in China has shown that oxytetracycline can be accumulated in some crops in the open field like peanuts, but the field was subjected to manure each year for 8 years (Zhao et al. 2019).

The pot experiment that was done by Youssef et al. 2020, showed that oxytetracycline is accumulated in the roots, where the accumulation showed a significant difference in the uptake by lettuce and radish roots. However, the concentration in radish and lettuce leaves was low and didn't show any significant difference. Similar to the field experiment, oxytetracycline couldn't be detected in all edible crops (radish bulbs, lettuce leaves and cucumber fruits). This agrees with Migliore et al.'s results (2010) where they measured the accumulation of oxytetracycline on Zea mays in the field and in pots. The soil in the field was subjected to pig slurry with a concentration of 15 mg/L of oxytetracycline. Field plants were fertilized with a total 1.8 L per plant of pig slurry. At the end of the vegetative cycle, oxytetracycline was tested in the roots, stalks, leaves and cobs. The results showed that oxytetracycline couldn't be accumulated in all the parts of corn in the field. However, the same crop was planted in pots at a concentration of 1000 ng/g oxytetracycline. The results showed that the roots reached a concentration of 54.5 ng/g and the aerial portion of the crop reached a concentration of 2.81 ng/g. This shows that plants can avoid the uptake of oxytetracycline in the field more than in pots. Oxytetracycline is highly adsorbed to the soil and its organic matter. Tetracyclines can adsorb strongly to clay soils and sediments under different environmental conditions (Allaire et al., 2006). The reason why oxytetracycline is difficult to be accumulated in the field is because it has a charge. Oxytetracycline will have a zero charge at pH of 5 and interacts with organic matter mainly through hydrophobic partitioning. When pH is lower than 5, oxytetracycline becomes positively charged and when pH is above 5, it becomes negatively charged. After being charged, it will be highly adsorbed to soil minerals, mainly through cation exchange sites (Kulshrestha et al., 2004). However, Gentamicin doesn't have a charge and has a lower half life than oxytetracycline, which makes gentamicin much easier to be accumulated in crops and doesn't stay in soil as much as oxytetracycline. Gentamicin is highly soluble compared to oxytetracycline.

Although oxytetracycline couldn't be detected in the field experiment, it stays in the soil for long periods of time. The half-life of oxytetracycline can reach 300 days as cited by (Tasho & Cho 2016). The half-life of many antibiotics is found to increase at low temperatures and in the dark, suggesting their persistence in deeper soil layers and deep waters for a longer time interval (Hektoen et al., 1995).

Highly contaminated soils with antibiotics will change the microbial community in the soil. Long-term exposure, genetic variation and transfer of antibiotic-resistant genes (ARGs) result in the evolution of resistant pathogens and bacteria (Boxall et al., 2003). A recent study by Awad et al. (2015) has shown that the widespread use of veterinary antibiotics by CAFOs (Concentrated Animal Feeding Operation in Korea) has the potential to generate ARGs as emerging contaminants in solid environment matrices. As cited by Tasho & Cho (2016), resistance can ultimately eliminate our chances of effectively treating diseases. Moreover, these resistant microbes can be transported from the "farm to our home" via unregulated consumption of agricultural produce.

Moreover, Soil samples were collected from the same field experiment from all treatments and the number of total bacteria was counted on a weekly basis in FAFS lab. The soil in the lab was treated with minimum inhibitory concentration (MIC) of gentamicin oxytetracycline. The results showed that the treatments of (soil with antibiotics) and (soil with manure and antibiotics) had the highest total bacterial counts after 2 days incubation period.

B. Green house experiment

In this section, the soil of the pot experiment conducted at the greenhouse at AUB will be presented and discussed. Also, the accumulation of gentamicin and oxytetracycline by in radish, lettuce and cucumber grown in the pots will be presented.

1. Physical and chemical properties of soil

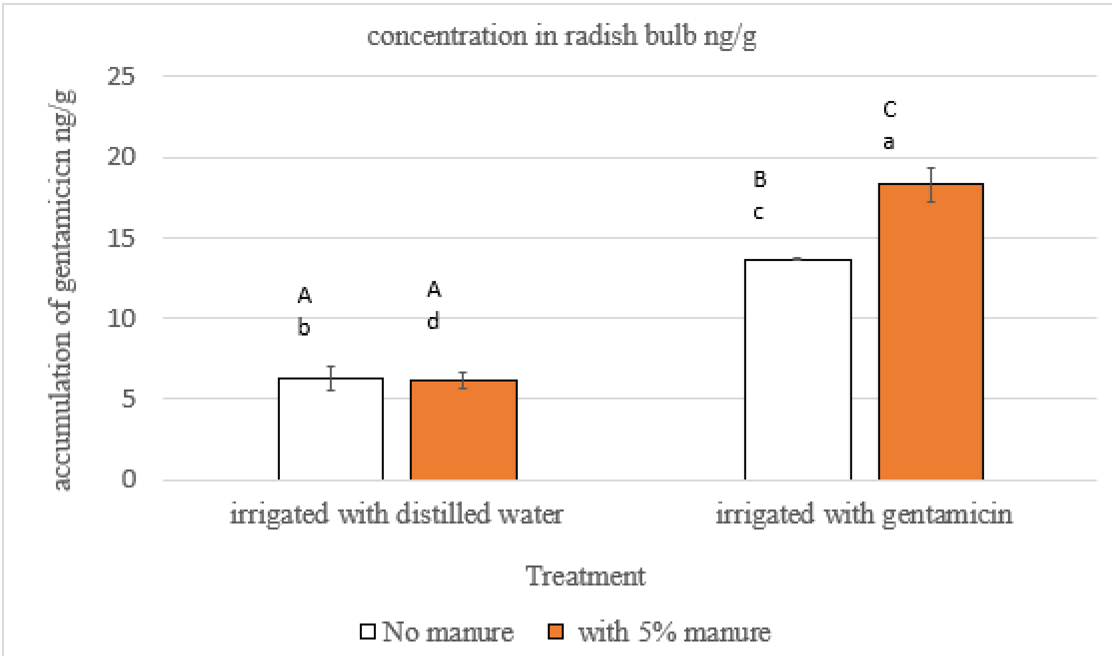
The physical and chemical properties of the soil used in the greenhouse experiment were analyzed. The analysis showed that the soil is sandy loam, slightly basic, non-saline and low in calcium carbonate (not calcareous). Available P and K were considered low and all the exchangeable microelements were high (Fe, Zn, Cu, and Mn) (Table 12).

Table 12. Soil physical and chemical properties of greenhouse experiment

Parameter	Result
Texture	Sandy loam
pH	7.7
EC	291 uS/cm
CaCO3 %	10%
Available K	100 mg/kg
Available P	7 mg/kg
Available Fe	40 mg/kg
Available Zn	8 mg/kg
Available Cu	6 mg/kg
Available Mn	25 mg/kg

2. Gentamicin accumulation in crops by irrigation water and effect of manure

a- <u>Accumulation of gentamicin Radish</u>

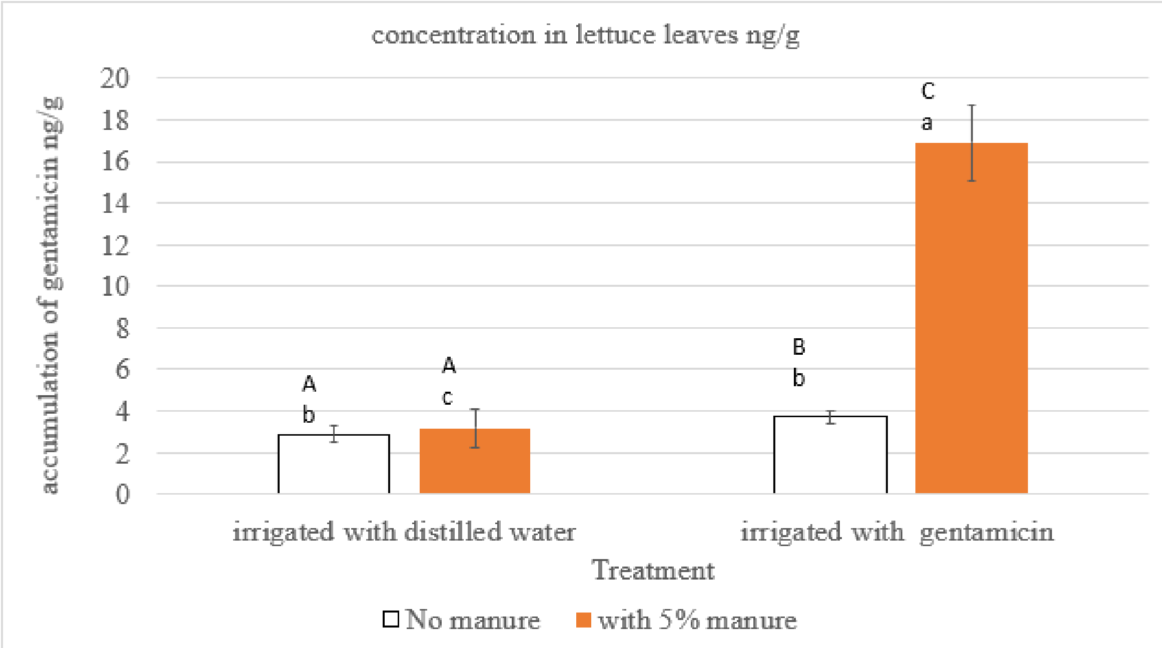


*Small letters correspond to the significance between irrigation treatment (irrigation distilled water and irrigation gentamicin in soil and soil with 5 % manure) and different capital letters correspond to significance between the same irrigation treatment (with or without 5 % manure).

Figure 20. Concentration of gentamicin in radish bulb

The accumulation of gentamicin in radish bulb is shown in Figure 20. As illustrated, the highest concentration of gentamicin was in the treatment that included manure and was irrigated with gentamicin (20 mg/lit) contaminated water having a concentration of 18.36 ng/g in tissue. There was a significant difference between the treatment of (irrigation gentamicin without manure) to (irrigation with distilled water without manure). This agrees with Sandra Youssef's results regarding the radish roots, were the concentration of gentamicin in the roots increased when gentamicin was incorporated with manure (Youssef et al. 2020).

b- Accumulation of gentamicin in lettuce

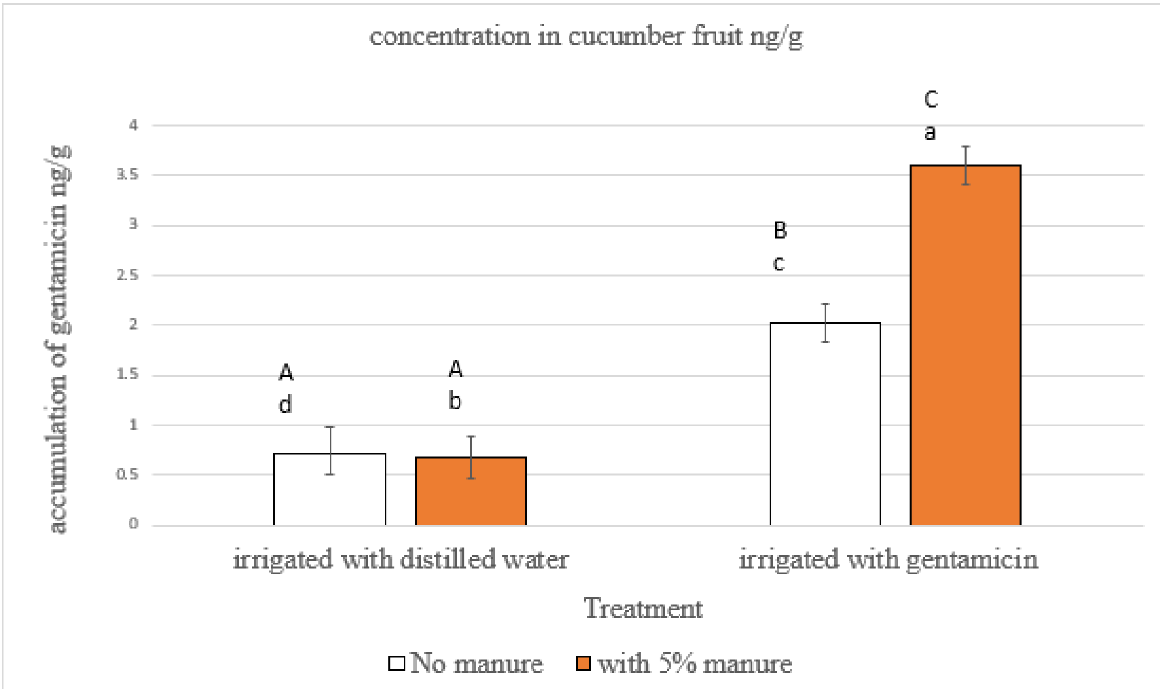


*small letters correspond to the significance between irrigation treatment (irrigation distilled water and irrigation gentamicin in soil and soil with 5 % manure) and different capital letters correspond to significance between the same irrigation treatment (with or without 5 % manure).

Figure 21. Concentration of gentamicin in lettuce leaves

Figure 21 shows that the highest concentration of gentamicin was in the treatment of soil and manure irrigated with gentamicin contaminated water (16.97 ng/g tissue). This treatment showed a significant difference when compared to the treatment of soil without manure irrigated with gentamicin contaminated water. This agrees with Sandra Youssef's study (2020), where soil amended with 5 % manure (5 and 10 mg/Kg of gentamicin in manure) showed a significant difference than the one that didn't include manure. So, irrigating a soil that has 5 % manure with gentamicin or amending the gentamicin in manure will have the same effect on lettuce leaves with respect to control in pots.

c- Accumulation of gentamicin in cucumber fruit



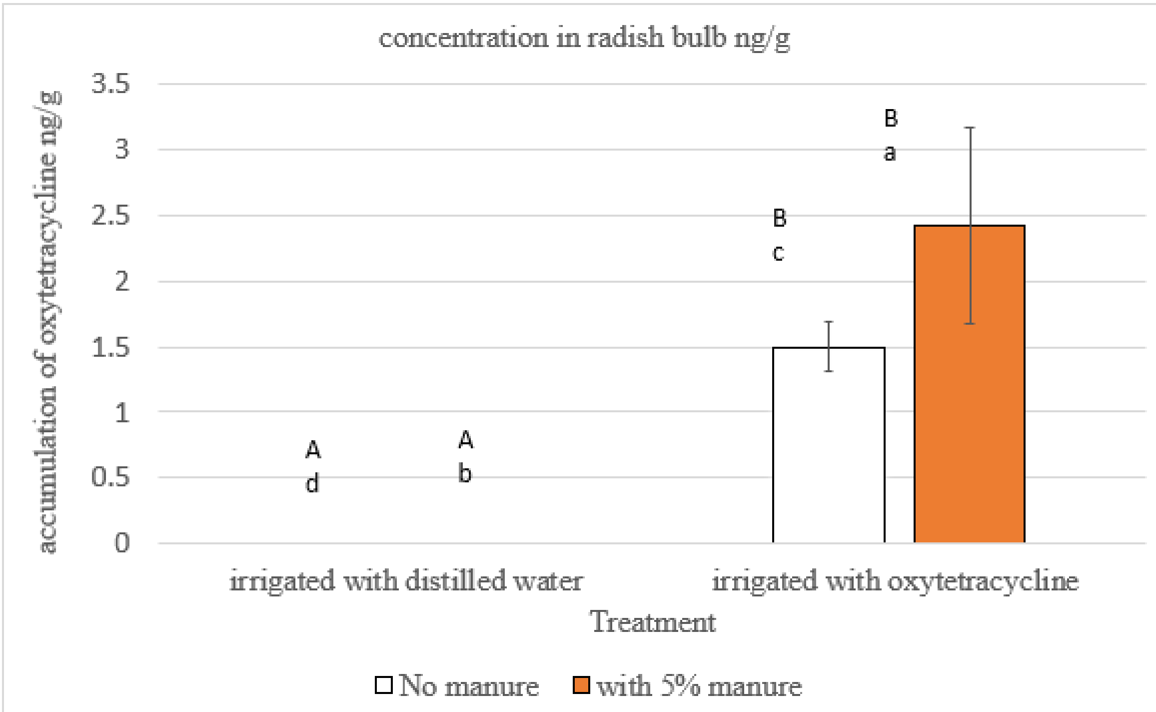
*small letters correspond to the significance between irrigation treatment (irrigation distilled water and irrigation gentamicin in soil and soil with 5 % manure) and different capital letters correspond to significance between the same irrigation treatment (with or without 5 % manure).

Figure 22. Concentration of gentamicin in cucumber fruit

As shown in Figure 22, the highest concentration of gentamicin in cucumber fruit was found in the treatment of the soil with manure irrigated with gentamicin contaminated water (3.6 ng/g tissue). However, the treatment of the soil without manure and irrigated with gentamicin contaminated water showed a lower accumulation concentration (2.03 ng/g tissue). This shows that manure interacted gentamicin in the irrigation water since it gave higher accumulation in cucumber fruit with significant difference. Also irrigating with gentamicin alone without manure showed a significant difference compared to that of the control (irrigating distilled water without manure). This shows that cucumber fruit can accumulate gentamicin from contaminated irrigation water.

3. Oxytetracycline accumulation in crops by irrigation water and effect of manure

a- <u>Radish</u>

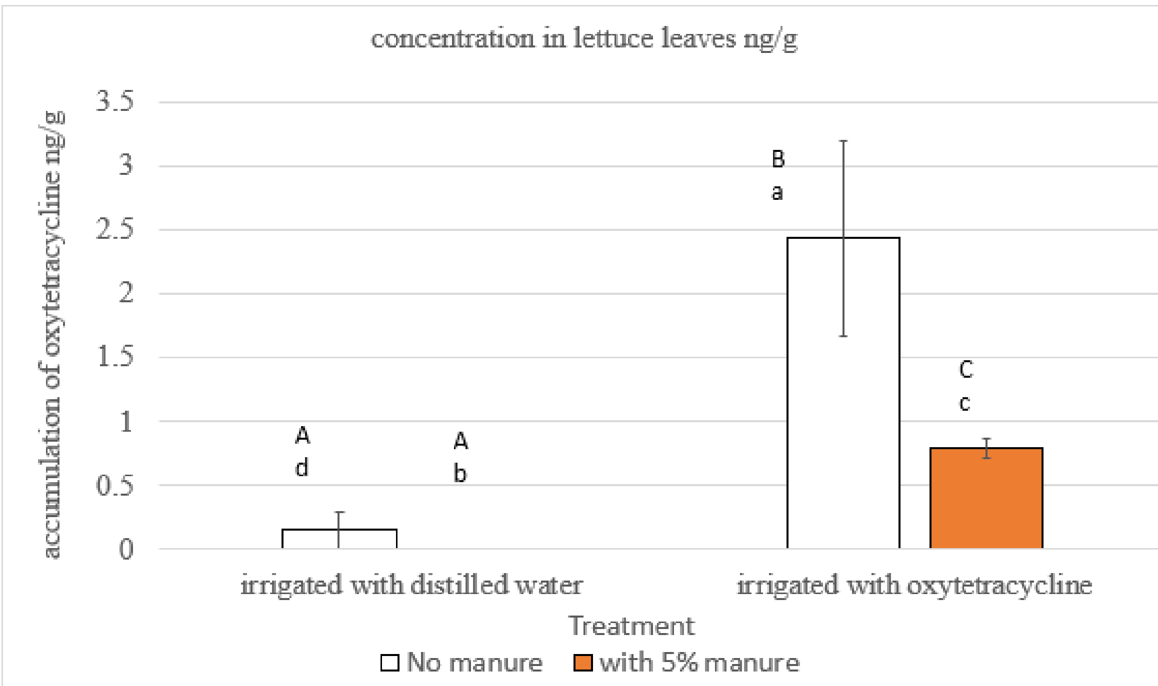


*small letters correspond to the significance between irrigation treatment (irrigation distilled water and irrigation gentamicin in soil and soil with 5 % manure) and capital letters correspond to significance between the same irrigation treatment (with or without 5 % manure).

Figure 23. Concentration of oxytetracycline in radish bulbs

As shown in Figure 23, in the oxytetracycline treatment without manure, accumulation of antibiotic in radish was significant with respect to the treatment irrigated with distilled water without manure. Also, the addition of 5 % manure to the oxytetracycline irrigation treatments, gave a significant increase with respect to the one irrigated with distilled water with 5% manure. However, there wasn't significant increase within the same treatment of oxytetracycline (with and without 5 % manure). This confirms that manure didn't interact with the oxytetracycline in the treatments. A similar experiment was conducted in Ghana by Azanu et al. (2016), where different concentrations of tetracycline and amoxicillin were irrigated to carrots in pots. The ones irrigated with tetracycline and amoxicillin gave significant difference with the control ones (no antibiotic in irrigation water). Tetracycline is in the same classification of oxytetracycline, and carrots are tubers similar to the radish bulb, both edible parts under the soil.

b- Lettuce

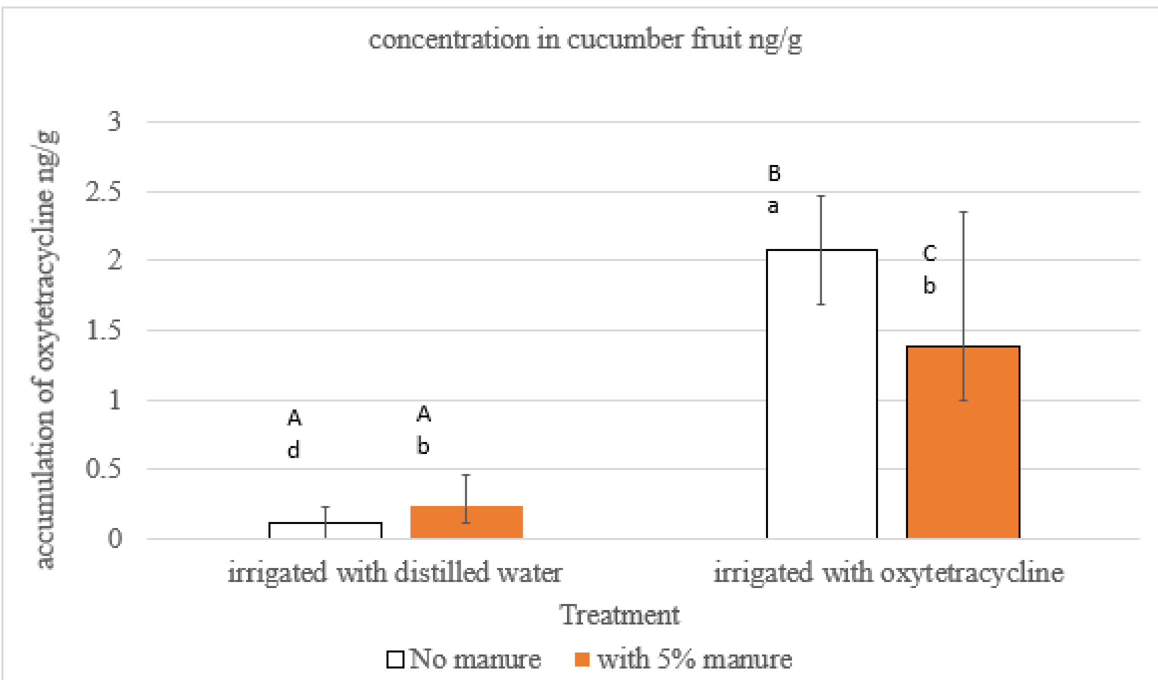


*small letters correspond to the significance between irrigation treatment (irrigation distilled water and irrigation gentamicin in soil and soil with 5 % manure) and capital letters correspond to significance between the same irrigation treatment (with or without 5 % manure).

Figure 24. Concentration of oxytetracycline in lettuce leaves

Accumulation of oxytetracycline in lettuce leaves are shown in Figure 24. It has shown that irrigating with oxytetracycline without the addition of manure can lead to the accumulation of oxytetracycline in lettuce leaves, reaching 2.43 ng/g tissue, and it was significantly higher than the control (irrigating with distilled water without addition of manure). However, the addition of manure withing the same oxytetracycline treatment gave lower results than without manure. This agrees with previous research by Sandra Youssef in 2016 at AUB, where incorporating oxytetracycline in manure didn't give any significant difference with other treatments where no manure was added. The manure decreased the accumulation of oxytetracycline in lettuce leaves. This is due to the high adsorption ratio of oxytetracycline in the soil and the antibiotic is able to stick to the cation exchange sites of organic matter, which makes it harder for the plants to uptake it.

c- Cucumber



*small letters correspond to the significance between irrigation treatment (irrigation distilled water and irrigation gentamicin in soil and soil with 5 % manure) and capital letters correspond to significance between the same irrigation treatment (with or without 5 % manure).

Figure 25. Concentration of oxytetracycline in cucumber fruit

As shown in Figure 25. Irrigating with oxytetracycline without addition of manure has shown an accumulation in the cucumber fruit, where it reached 2.08 ng/g tissue. It was significantly higher from the control treatment (irrigation with distilled water without manure). In the treatment of (irrigation with oxytetracycline) with the addition of 5% manure has shown accumulation, where it reached 1.39 ng/g but the accumulation wasn't significant with one irrigated with distilled water with 5% manure.

Compared to the field experiment, the accumulation of gentamicin and oxytetracycline in edible crops were higher in the irrigation experiment. Moreover, the pot experiment done by Youssef er al. 2020, showed that oxytetracycline is only accumulated in the root part of lettuce and radish. However, in the irrigation experiment oxytetracycline could be accumulated in all edible parts (cucumber fruit. Radish bulb and lettuce leaves). This shows that oxytetracycline is more available to the plants when placed in irrigation water rather than placed in the soil media in pots. Oxytetracycline in water is not attached to solid particles unlike when incorporated in soil. Also, the antibiotic in the soil is highly adsorbed to the clay particles and organic matter; this could be one of the reasons why the accumulations of oxytetracycline decreased when manure was added in irrigation in lettuce and cucumber. However, the manure didn't decrease accumulation of oxytetracycline in radish bulb. This agrees with El Gemayel et al. (2020), where radish and lettuce were grown in a water culture media contaminated with 10 mg/L of oxytetracycline. The accumulation of oxytetracycline was significant in lettuce leaves and roots, as well as in radish leaves and bulbs.

C. Concentration of gentamicin and oxytetracycline in water samples in bekaa

Water samples from Bekaa showed low concentrations of gentamicin and oxytetracycline, barely detectable. The sample taken from the Litani River near Joub Jannine had a concentration of 0.16 ng/ml and the sample taken near Tal-Amara had a concentration of 0.24 ng/ml. Moreover, water samples from AREC had also low concentrations of gentamicin and oxytetracycline. Sample from D54 pool had a concentration of 0.24 ng/ml gentamicin; however, the sample from earth pool had concentration of 0.19 ng/ml gentamicin. The concentration of gentamicin in the water samples were considered very low compared to the concentrations in the pot experiment, which was 20 mg/L gentamicin. The chance of accumulation of gentamicin and oxytetracycline in lettuce leaves, radish bulbs, and cucumber fruits by irrigation is very low.

D. Concentration of gentamicin and oxytetracycline in leached water from column experiment

The leached water was collected from the columns that were irrigated with 20 mg/lit of oxytetracycline. Both soil textures (sand and clay) gave low concentrations of oxytetracycline, 1.45 and 0.93 ng/ml from the sand and clay columns, respectively. Moreover, gentamicin gave similar results, were its concentrations in sand and clay were 1.27 and 2.15 ng/ml, respectively. Therefore, this experiment tells us that more 99% of the 20 mg/lit of both antibiotics stayed in the soil of the column. This agrees with the literature that states Tetracyclines and Beta-lactams have the tendency to get adsorbed in the soil and only a small percentage gets leached to ground. Unlike other antibiotics like the Sulfonamides class, where they have a high tendency of being leached to the underground water. (Boy-Roura et al. 2018).

CHAPTER V

SUMMARY, CONCLUSION, AND RECOMMENDATIONS

The extensive use of antibiotics in animal food has raised a big question concerning the antibiotic resistant bacteria in the environment, as well as the appearance of antibiotics in food supplies and water. The main reason behind the spread of antibiotics in food and water supplies is because of the use of manure, a major antibiotics contaminant, that is holding antibiotics in agriculture lands, and then accumulating them in the consumed plants. It was important to study the accumulation of oxytetracycline and gentamicin in cucumber, radish, and lettuce in the field and in the pots. The results of irrigation experiment have shown:

- Gentamicin and oxytetracycline can be accumulated in radish bulbs, cucumber fruits, and lettuce leaves.
- The addition of 5 % manure in pots can increase the accumulation of gentamicin in lettuce leaves, radish bulbs, and cucumber fruits.
- The addition of 5 % manure in pots didn't increase the accumulation of oxytetracycline in all edible parts.

The results of the field experiment showed that radish bulbs accumulate gentamicin more when manure was added to the soil. However, for the lettuce leaves and cucumber fruits, the manure didn't increase the accumulation of gentamicin. Oxytetracycline wasn't detected in all edible plants in the field.

More research should be done on the antimicrobial resistant bacteria that are found in the soil. It was shown that the bacteria are acquiring resistance when adding antibiotics to the soil. If humans are affected by these antibiotic resistant bacteria, then there is a chance humans will not be cured of diseases that require the use of antibiotics. It's important to control the haphazard use of antibiotics in livestock production and use the antibiotics for treating sick animals and not as preventive agent. Also, expand of animal husbandry, hygiene, and health management on farms and improve vaccination programs is important.

APPENDIX

Table 1. Accumulation of gentamicin in radish bulb in field experiment

Treatment gentamicin mg/Kg manure	Soil	Soil with manure
	Accumulation gentamicin ng/g tissue ± SE	
0	0 Ab	0 Ac
30	0.129±0.042 Bb	0.625±0.216 Ca
P-Value	Less than 0.05	

*Different capital letters refer to significant within each row and different small letters refer to significant within each column

Table 2. Accumulation of gentamicin in lettuce leaves in field experiment

Treatment gentamicin mg/Kg manure	Soil	Soil with manure
	Accumulation gentamicin ng/g tissue ± SE	
0	0.406±0.023 Aa	0.338±0.065 Ab
30	0.585±0.073 Ba	0.453±0.02 Bb
P-Value	Less than 0.05	

*Different capital letters refer to significant within each row and different small letters refer to significant within each column

Table 3. Accumulation of gentamicin in radish bulb in pot experiment

Irrigation treatment gentamicin mg/L	Soil	Soil with manure
	Accumulation gentamicin ng/g tissue ± SE	
0	6.29±0.71 Ab	6.21±0.5 Ad
20	13.68±0.08 Bc	18.36±1.07 Ca
P-Value	Less than 0.05	

*Different capital letters refer to significant within each row and different small letters refer to significant within each column

Table 4. Accumulation of gentamicin in lettuce leaves in pot experiment

Irrigation treatment gentamicin mg/L	Soil	Soil with manure
	Accumulation gentamicin ng/g tissue ± SE	
0	2.97±0.44 Ab	3.24±0.93 Ac
20	3.62±0.28 Bb	16.97±1.8 Ca
P-Value	Less than 0.05	

*Different capital letters refer to significant within each row and different small letters refer to significant within each column

Table 5. Accumulation of gentamicin in cucumber fruits in pot experiment

Irrigation treatment gentamicin mg/L	Soil	Soil with manure
	Accumulation gentamicin ng/g tissue ± SE	
0	0.72±0.26 Ad	0.68±0.21 Ab
20	2.03±0.19 Bc	3.60±0.18 Ca
P-Value	Less than 0.05	

*Different capital letters refer to significant within each row and different small letters refer to significant within each column

Table 6. Accumulation of oxytetracycline in radish bulbs in pot experiment

Irrigation treatment oxytetracycline mg/L	Soil	Soil with manure
	Accumulation oxytetracycline ng/g tissue ± SE	
0	0 Ad	0 Ab
20	1.5±0.19 Bc	2.42±0.76 Ba
P-Value	Less than 0.05	

*Different capital letters refer to significant within each row and different small letters refer to significant within each column

Table 7. Accumulation of oxytetracycline in lettuce leaves in pot experiment

Irrigation treatment oxytetracycline mg/L	Soil	Soil with manure
	Accumulation oxytetracycline ng/g tissue ± SE	
0	0.15±0.15 Ad	0 Ab
20	2.43±0.76 Ba	0.79±0.08 Cc
P-Value	Less than 0.05	

*Different capital letters refer to significant within each row and different small letters refer to significant within each column

Table 8. Accumulation of oxytetracycline in cucumber fruits in pot experiment

Irrigation treatment oxytetracycline mg/L	Soil	Soil with manure
	Accumulation oxytetracycline ng/g tissue ± SE	
0	0.11±0.11 Ad	0.23±0.23 Ab
20	2.08±0.39 Ba	1.39±0.95 Cb
P-Value	Less than 0.05	

*Different capital letters refer to significant within each row and different small letters refer to significant within each column

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