AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF RESIDUAL ANTIBIOTICS IN SOIL AND WATER ON PLANT GROWTH

by
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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Agriculture
of the Faculty of Agricultural and Food Sciences
at the American University of Beirut

Beirut, Lebanon
April 2018

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ACKNOWLEDGMENTS

First and foremost, I would like to thank the American University of Beirut for giving me the opportunity to further my studies in my desired field. My advisor Dr. Isam Bashour who gave me constant encouragement, guidance and motivation to complete my thesis topic completely and impeccably. I would also like to thank the committee members Dr. Youssef Abou Jawdah, Dr. Mohamad Farran and Dr. Nadim Farajallah for their contribution, collaboration and support to perfect my thesis topic in the best ways possible. Great thanks also go to Ms. Hana Sobh and Dr. Houssam Shaib who were constantly available for assistance.

Where would I be without my family? I am the person I am today thanks to my family who always fought to give me the best life and finest education, and for always believing in me. Deep and warm appreciations go to them for their constant guidance, inspiration and prayers. Their love and persistent confidence in me have helped me in pursuing my dreams and visions.

Thank you to Sandra Youssef with whom I conducted the pot experiment and who walked me through its procedure. Also, many thanks go to all my friends especially: Chafik Abdallah, Nour Masri, and Ann-Marie Agajanian. I am very grateful for their never-ending willingness to help, motivate, and advise me.

At last, I would like to thank everybody else who was important to the successful fulfillment of this thesis, as well as conveying my apologies that I could not mention them personally one by one.

AN ABSTRACT OF THE THESIS OF

Lara Jacques El-Gemayel for Master of Science
Major: Plant Sciences

Title: Effect of residual antibiotics in soil and water on plant growth

Antibiotics such as oxytetracycline, enrofloxacin, tylosin and gentamicin are extensively used in Lebanon as a mean to prevent and treat illnesses in animals, promote growth and increase feed efficiency. They are mainly excreted via animal defecates used as fertilization mean on agricultural lands and found in the environment as their parent compound or metabolite. Researchers have identified that most of these antibiotics are absorbed and accumulated by plants via roots and leaves; however, studies are still lacking on antibiotics effect on plant growth and persistence in soil and water. The aim of this study is to evaluate the uptake and accumulation sites of antibiotics by plants grown in soil and nutrient solution and their effect on plant growth, as well as study the antibiotic persistence in soil. Antibiotic analysis was done using the Enzyme Linked Immunosorbent Assay (ELISA). A pot experiment was conducted in the greenhouse of the American University of Beirut where lettuce and cucumbers were grown in two growing media (soil without and soil with 5% manure), administered with gentamicin and enrofloxacin at 4 different antibiotic levels (0, 5, 10 and 20 mg/kg). In nutrient solution, lettuce and radish were grown in 3 different levels of enrofloxacin, oxytetracycline and tylosin at 0, 5 and 10 mg/kg. Lastly, the persistence of enrofloxacin, oxytetracycline and tylosin was investigated in a pot experiment at 5 mg/kg and extracted with water. In the pot experiment, results demonstrated that gentamicin and enrofloxacin mainly accumulated in cucumber leaves and manured soil increased the uptake of antibiotics. In nutrient solution, enrofloxacin, oxytetracycline and tylosin were absorbed by lettuce and radish plants; in radish bulbs (edible part) it accumulated at an average of 67.7 ng/g, 30.98 ng/g and 407.45 ng/g respectively. In lettuce, enrofloxacin was translocated all over the crop, tylosin accumulated the most in leaves (343.83 ng/g) and oxytetracycline the most in lettuce roots (20.43 ng/g). Enrofloxacin and oxytetracycline reduced lettuce and radish weight by around 70%, whereas tylosin had no significant effect on plant growth. Enrofloxacin and oxytetracycline persisted in the soil and showed a half-life of ~24 days, whereas tylosin was completely degraded after 22 days. Further research should be done on the persistence of antibiotics in soil and their effect and fate in the environment.

Keywords: Lebanon, antibiotics, enrofloxacin, gentamicin, tylosin, oxytetracycline, pot experiment, hydroponics, persistence

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ABBREVIATIONS

$(NH_4)_6Mo_7O_{24}.4H_2O$	Ammonium Molybdate Tetrahydrate
°C	Degree Celsius
µL	Microliter
ADI	Acceptable Daily Intake
APBWT	Average Plant Bulb Weight
APLWT	Average Plant Leaf Weight
APRWT	Average Plant Root Weight
APWT	Average Plant Weight
AREC	Agriculture Research Education Center
AUB	American University of Beirut
B	Boron
Ca	Calcium
$Ca(NO_3)_2.4H_2O$	Calcium Nitrate Tetrahydrate
CE	Common Era
Cl	Chlorine
cm	Centimeter
Cu	Copper
$CuSO_4.5H_2O$	Copper Sulfate Pentahydrate
DTPA	Diethylenetriaminepentaacetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
EMA	European Medicines Agency
F	Fluorine
Fe	Iron

Fe-EDDHSA	Iron – ethylenediaminedi (2-hydroxy-5-sulfophenylacetic) acid
g	Gram
g/L	Gram Per Liter
g/mol	Gram Per Mole
GLM	General Linear Models
H_3BO_3	Boric Acid
ha	Hectare
i.e.	*Id est.*
K	Potassium
KCl	Potassium Chloride
k_d	Distribution Coefficient
kg	Kilogram
kg/ha/year	Kilogram Per Hectare Per Year
KNO_3	Potassium Nitrate
L/kg	Liter Per Kilogram
M	Molar
Mg	Magnesium
mg	Milligram
mg/kg	Milligram Per Kilogram
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate
mL	Milliliter
mL/L	Milliliter per Liter
mm	Millimeter
mM	Millimolar

Mn	Manganese
$MnSO_4.H_2O$	Manganese Sulfate Monohydrate
Mo	Molybdenum
MRL	Maximum Residue Limit
MSc.	Master in Science
N	Normal
ng/g	Nanogram Per Gram
ng/mL	Nanogram Per Milliliter
$NH_4H_2PO_4$	Ammonium Dihydrogen Phosphate
$NH_4OAC\text{-}K^+Na$	Ammonium Acetate – Potassium Sodium
P	Phosphate
PBS	Phosphate Buffered Saline
ppm	Parts Per Million
RPM	Round Per Minute
S	Sulfur
SAS	Statistical Analysis System
TE	Trace Element
USA	United States of America
USDA	United States Department of Agriculture
Zn	Zinc
$ZnSO_4.7H_2O$	Zinc Sulfate Heptahydrate

CHAPTER 1

INTRODUCTION

Research has divulged that contrary to people believes, antibiotics are not confined to the modern “antibiotic era”. In some human skeletal remains dating back to 350-550 CE from ancient Sudanese Nubia, traces of tetracycline were found (Aminov, 2010). Undoubtedly, the availability of tetracycline in these ancient peoples’ bones is only justifiable after exposure to tetracycline-containing materials in the diet of these primal people. Paul Ehrlich and Alexander Fleming are usually the two names connected to the beginning of the modern “antibiotic era”. Ehrlich reasoned that chemical compounds that could be synthesized would “be able to exert their full action exclusively on the parasite harbored within the organism” (Aminov, 2010). This led him in 1909 to find a drug against an endemic disease that was almost incurable at the time: syphilis. On September 3, 1928, through serendipitous events, Fleming discovers penicillin; however, it’s not until 1945 that it was produced in large quantities and commercialized (Aminov, 2010).

To fight illness prevention, disease treatment and growth promotion, antibiotics are widely used in human medicine, animal and fish farming (Pan & Chu, 2017a). In recent decades, their usage as feed additives in livestock production and agriculture has raised several questions about the residual effects of these antibiotics in food and water supplies, as well as their limits and sources. As antibiotics are constantly added into the environment, they are said to be pseudopersistent (Van Boeckel et al., 2015). Antibiotics enter the agro ecosystems through different ways from which wastewater irrigation and soil application with biosolids or animal manures (Daghrir & Drogui,

2013). Scientists are startled about the residual effects of these antibiotics as the antibiotics used for humans and animals belong to the same general classes or they share the same mode of action (Phillips et al., 2004). Many studies are being conducted to investigate if plants are capable of spreading antibiotics from the soil into the food chain (Pan & Chu, 2017a). As humans consume those crops, then a major problem would be their consequences on human health.

Antibiotic residues present different persistence and transportation modalities and values in agricultural soil, including sorption, degradation and leaching. Therefore, when biosolids, animal manure or wastewaters are utilized in the soil-plant system, it is possible that the antibiotics accumulate in the irrigated crops, thus absorbed by plants. When crops are grown in soil contaminated with antibiotics, root uptake is suspected to be a major route of exposure for the crop. Earlier studies have shown that antibiotics and other pharmaceuticals can accumulate in different plant tissues (Bassil, Bashour, Sleiman, & Abou-Jawdeh, 2013; Youssef, 2016). Nonetheless, the effect of environmental antibiotics on human health and terrestrial ecosystems are still unclear. Also, studies on phytotoxicity of antibiotics and antibiotics uptake mechanism by plants remain limited. In the following pages, an overview of the sources of entry of antibiotics to the environment, their uptake by plants and the result of residual effect of antibiotics in soil and water on plant growth will be outlined.

The objectives of this research were to:

1. Evaluate the uptake of enrofloxacin, tylosin, oxytetracycline and gentamicin in hydroponic and soil cultures by lettuce, cucumber and radish. Four different antibiotics used in Lebanon in human and animal medicine as well as for animal fattening

2. Find major accumulation sites of these antibiotics in lettuce, cucumber and radish crops grown in soil and hydroponic media
3. Evaluate the effect of enrofloxacin, tylosin and oxytetracycline in water culture on lettuce and radish growth
4. Investigate the persistence of enrofloxacin, tylosin and oxytetracycline in soil

This study was conducted at the Department of Agricultural Sciences at the American University of Beirut (AUB). Controlled greenhouse pot experiments and hydroponic systems were used to plant lettuce, cucumber and radish. The systems were spiked with known levels of different antibiotics. The measurements of antibiotic concentrations in roots, shoots and fruits of lettuce, cucumber and radish was done by the ELISA technique.

CHAPTER 2

LITERATURE REVIEW

A. Antibiotics

1. Definition

Antibiotics are antimicrobial organic substances that are produced from natural microorganisms such as fungi or bacteria or through industrial synthesis: synthetic or semi-synthetic chemical compounds (Khan et al., 2008; X. Wang, Ryu, Houtkooper, & Auwerx, 2015). They are compounds that are recognized to fight infections triggered by bacteria in both: animals and humans. The general term "antibiotic" symbolizes any organic molecule class that kills or inhibits microbes via specific interactions with microbial targets. The source of the class or compound is not taken into consideration (Davies & Davies, 2010; Michael et al., 2013). Examples of man-made antibiotics with no natural origins are trimethoprim, fluoroquinolones and sulfonamides (Coates, Halls, & Hu, 2011). Undeniably, most naturally occurring antibiotics have been chemically improved to provide developed properties of the drug. Instances of the former are: tetracyclines, streptogramins, glycopeptides, beta-Lactams, aminoglycosides, macrolides and lincosamides.

Antibiotics can be classified as narrow or broad spectrum, bacteriostatic or bactericidal, or based on their modes of action. Bactericidal are antibiotics that kill bacteria by interfering with either the development of the bacterium's cell content or cell wall such as beta-Lactams, fluoroquinolones and aminoglycosides. Bacteriostatic on the other hand are the ones that keep the bacteria in its stationary phase of growth.

Sulfonamides, macrolides and tetracycline groups of antibiotics are bacteriostatic. Surely, few antibiotics could be both bacteriostatic and bactericidal: this depends on the dosage, the state of the invading bacteria and the period of exposure (Pankey & Sabath, 2004). More concretely, fluoroquinolones and aminoglycosides killing characteristics are concentration dependent. As the drug concentration rises, their rate of killing rises as well. Medical authorities advise that bactericidal and bacteriostatic drugs ought not to be mixed or utilized simultaneously as their properties will cancel one another.

2. Classification and types of antibiotics

a. Based on their spectrum of activity

Antibiotics or antibacterial agents can be classified based on their target specification. They could either be broad or narrow spectrum. The narrow spectrum antibacterials are the ones which can act upon a narrow range of microorganisms (Clatworthy, Pierson, & Hung, 2007). In other words, they either act specifically against Gram-negative or Gram-positive bacteria and the broad-spectrum ones work against a broad range of pathogenic bacteria, involving both Gram-negative and Gram-positive bacteria. For medical treatments, the narrow spectrum antibacterials are favored over the broad-spectrum antibacterials and are considered as perfect antibacterials. This is because in the body, narrow spectrum antibiotics do not destroy as many of the natural microorganisms as the broad-spectrum antibiotics. Therefore, its ability to cause superinfection is lower. Additionally, as narrow spectrum antibiotics only deal with specific bacteria, then this reduces the chance of resistance occurrence (Ullah & Ali, 2017).

Examples of broad-spectrum antibacterials are: quinolones, aminoglycosides including gentamicin, chloramphenicol, tetracycline and oxytetracycline. Examples of narrow spectrum antibacterials are: beta-Lactamase (1st generation: penicillin G, penamecillin), sulfonamides and glycopeptide (Ullah & Ali, 2017). In addition to their spectrum of activity, antibiotics can also be classified based on their mechanism of action.

b. Based on their mechanism of action

The mode of action is one of the most essential factors connected to each antibacterial compound. Different antibiotics may have diverse modes of action and that is due to their structure's nature and to the extent of their affinity to some target sites inside bacterial cells. Antibiotics can also be divided based on their target sites in the bacterium (Kohanski, Dwyer, & Collins, 2010). The major antibiotic functions are responsible of inhibiting bacterial growth and cell membrane function, cell wall synthesis, protein and nucleic acid synthesis, etc. Antibacterials thus can be divided into four groups: inhibitors of membrane function, inhibitors of cell wall synthesis, inhibitors of nucleic acid synthesis and inhibitors of protein synthesis (Ullah & Ali, 2017). Table 1 lists few of the principal antibiotics with different mechanisms of action and spectrum of activity.

Table 1. List of few antibiotics with distinctive modes of action and spectrum of activity

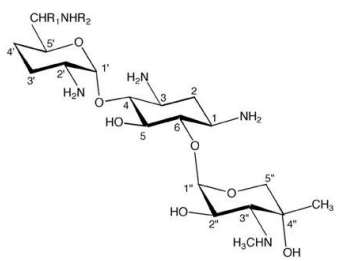
Antibiotic Class	Spectrum of activity	Mechanism of action
Aminoglycosides	Broad spectrum	Protein synthesis inhibitors
Macrolides	Broad spectrum	
Tetracyclines	Broad spectrum	
Fluoroquinolones	Broad spectrum	Nucleic acid inhibitors
Beta-Lactams	Broad spectrum	Cell wall synthesis inhibition

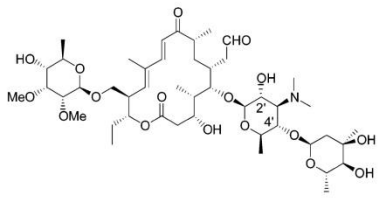
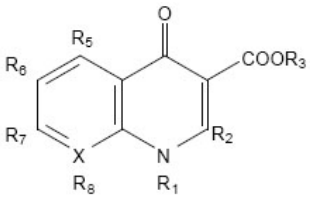
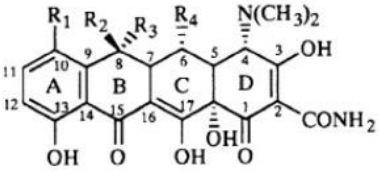
Source: (K. Brown, Uwiera, Kalmokoff, Brooks, & Inglis, 2017)

c. Based on their chemical formula/structure

The chemical structure of antibiotics is the sole property which unmistakably separates one antibiotic from another. Diverse skeleton comprising antibiotics exhibit different behaviors. For this reason, it is fundamental to categorize antibacterials based on their chemical structure. Besides, classifying antibiotics is essential as similar structural components detain analogous patterns of effectiveness, toxicity and additional related properties (Ullah & Ali, 2017). Moreover, it is the chemical structure of the antibiotics that determines all its chemical, physical, pharmacological, clinical and lastly microbiological properties (Béahdy, 1974). Table 2 below elaborates on the characteristics of the antibiotics included in this study that differentiate one antibiotic class from another.

Table 2. List of antibiotics classes with their respective characteristics

Antibiotic Class	Examples	Main Skeleton	Characteristics
Aminoglycoside	Gentamicin, streptomycin	1 	Two aminosugars joined by glycosidic bond to an aminocyclitol
Macrolides	Tylosin	2	Consist of a

			macrocyclic lactone ring, typically 14-, 15- or 16-member to which one or more deoxy sugars could be linked
Quinolones and fluoroquinolones	Enrofloxacin, ciprofloxacin (2nd generation)	[3] 	Quinolones are quinine derived structural units. An added fluorine at position 6 is named fluoroquinolone
Tetracyclines	Oxytetracycline	[4] 	Four rings hydrocarbon containing compounds

Source: Modified from (van der Marel, 2013)
(Yoshizawa, Fourmy, & Puglisi, 1998)[1] (Phan et al., 2004)[2] (Smith, Pennefather, Kaye, & Hart, 2001)[3] (Sarmah, Meyer, & Boxall, 2006)[4]

B. Utilization of antibiotics

In the past decade, the use of antibiotics in agriculture and animal industry has been booming. With the increasing world population, estimated to reach 9.6 billion by 2050, several challenges are faced to provide individuals with sufficient amounts of food (K. Brown et al., 2017). For this reason, veterinary drugs (mainly hormonal growth promoters and antibacterial drugs) have been resorted to in the fields of aquaculture, agro-industry and animal husbandry as a mean to improve weight gain rate and feed efficiency (growth promotion) or treat and prevent illnesses (therapeutic and prophylactic use) in food producing animals (Beyene, 2016; NAAS, 2010). Experts divulge that following subtle differences, the antibiotics used to prevent and treat

bacterial diseases in food producing animals maintain similar principles to the antibiotics consumed in human medicine (Gustafson & Bowen, 1997). In 2011, FDA (2013) stated that in the USA, humans consumed ~3.3 million kg of antibiotics and animals consumed ~13.6 million kg of antibiotics. This indicates that around 80% of antibiotics are used for agricultural purposes (X. Wang et al., 2015). This notable difference in usage is mainly due to the large number of livestock and poultry flocks raised by farmers, thus obliging the grower to administer all of his healthy herds with antibiotics as soon as he senses clinical signs of a disease in only a portion of his flock: otherwise great economical losses would occur. The latter have henceforth become a fundamental part of animal production systems and their misuse or overuse have drastic effects such as promoting the development of antibiotic resistant bacteria (X. Wang et al., 2015).

1. Human use of antibiotics in the world

During World War II, penicillin was produced in large quantities where its need was essential to treat troops and war injuries. Antimicrobials were previously invented and consumed for the purpose of controlling infectious diseases which were the primary cause of human mortality and morbidity (Aminov, 2010). After the war, Moore et al. (1946) reported the use of streptomycin as a growth promoter in poultry and with time, antibiotics are nowadays more utilized for veterinary and livestock production purposes than for human medicine. Since the 1970s the discovery of new antibiotic classes dropped and resistance started growing (Aminov, 2010).

X. Wang et al. (2015) stated that in developing countries, from 2000 to 2010, human consumption of antibiotics increased by 36%. In 2013, China was stated to

manufacture and consume the highest amount of antibiotics among all countries with an estimate of 162 million kg (X. Wang et al., 2015). Some of the most employed antibiotic classes are: macrolides, quinolones, cephalosporins, penicillins, and tetracyclines (Pan & Chu, 2017a; Van Boeckel et al., 2014). From 2000 to 2010, cephalosporins and penicillins use increased by 41% and their total consumption accounted for around 60%. As for tetracyclines, it's highly resorted to in China as well as it ranked second among antibiotics in production and usage on a global level in 2013. In 2014 it accounted for around 33.4% of veterinary antibiotics consumption. It represents 90% of all the antibiotics used in the UK as well as more than 50% in Korea (Kim et al., 2011; Pan & Chu, 2017a). It has been reported that in Europe, human antibiotic intake progressively increased between 2000 and 2015. In 2015, tetracyclines, sulfonamides, macrolides, β-lactams and quinolones represented 95% of the total sales.

2. Antibiotics use in livestock production

As mentioned previously, veterinary antibiotics are primarily administered to food producing animals to promote growth and development as well as prevent and treat diseases. In 2013, the use of antibiotics in food-producing animals in the US augmented to ~14.8 million kg. Comparing it to the 2009 data, it shows an increase of 17% (X. Wang et al., 2015). In 2030, it is projected that the international antibiotic intake in food producing animals will rise to 105,596 tons (Pan & Chu, 2017a; Van Boeckel et al., 2015).

a. Use of antibiotics in livestock production

Moore et al. (1946) and Jukes et al. (1950) reported that in the late 1940's, growth promoting properties of antibiotics in farm animals such as swine and poultry were first discovered (Dibner & Richards, 2005). Farm animals have been fed sub-therapeutic doses of antimicrobials for two primary reasons: to treat and prevent infections as well as promote animal growth and feed efficiency. A list of the most frequently administered antibiotics in livestock and the total amount of veterinary antibiotics given per livestock head are enumerated in tables 3 & 4 respectively. The prophylactic use of antibiotics is administered to healthy animals through water and feed in small doses over a long period of time, whereas their therapeutic use is resorted to prevent the infected animals from displaying any symptoms. As mentioned by NAAS (2010) the use of antibiotics as growth promoters is defined as the "administration of antibiotics to healthy livestock animals at concentrations below 200 mg/kg in feed for more than 14 days" (NAAS, 2010). Sometimes, growth promoting antimicrobials are administered at low concentrations between 2.5 and 125 mg/kg depending on the animal and drug type (K. Brown et al., 2017).

Nevertheless, the constant feeding of antibiotics through animal feeds over a long period of time results primarily in conditions favorable for the development of resistance in bacteria and secondarily it introduces low levels of antibiotics in water and soil through the animal feces and urine (Jayalakshmi, Paramasivam, Sasikala, Tamilam, & Sumithra, 2017). This leads to antibiotics resistance of both: soil bacteria as well as pathogenic bacteria (NAAS, 2010). In fact, Starr and Reynolds (1951) following an experimental feeding of streptomycin in turkeys reported one of the first resistances in food animals (Dibner & Richards, 2005).

Table 3. List of the most frequently administered antibiotics in livestock

Antibiotic Class	Antibiotic	Use in animals
Tetracycline	Oxytetracycline	Growth promoter in cattle, veterinary medicine
Tetracycline	Chlortetracycline	Growth promoter in cattle, veterinary medicine
Beta-Lactam	Penicillin	Disease treatment and prevention, growth enhancement
Sulfonamide	Sulfamethazine	Disease treatment
Fluoroquinolone	Enrofloxacin	Treatment of respiratory and alimentary tract infections in pigs and poultry
Aminoglycoside	Neomycin	Treatment and control of bacterial enteritis
Aminoglycoside	Gentamicin	Disease prevention, treatment of infections, growth and feed efficiency
Macrolide	Tylosin	Disease treatment, growth enhancement in some cases

Source: (Sarmah et al., 2006; Tasho & Cho, 2016)

Table 4. Total quantity of veterinary antibiotics used in different countries and the quantity used per livestock head

Country	Head (x1000)			Amount used (tons)				Amount used (g/head)		
	Cattle	Pig	Poultry	Cattle	Pig	Poultry	Total	Cattle	Pig	Poultry
Australia	4 500	700	80 700	-	-	-	932	-	-	-
Denmark	1 107	2578 5	121 735	11	93	0.4	104.4	9.9	3.6	0.003
Korea	1 819	8962	109 628	112	831	335	1 278	62	93	3.1
UK	10 378	4851	159 323	7	281	20	308	0.7	58	0.12
USA	29 000	92600	780 000	1 675	4694	4 779	11 148	58	51	6.1

Source: (Kim et al., 2011)

b. Mode(s) of action of antibiotics on animals

The complexity in the mammalian gastrointestinal tract is what causes a challenge in establishing the mode(s) of action of antimicrobial growth promoters. This

includes connections between bacterial, host and environmental factors (K. Brown et al., 2017). Two primary hypotheses have been formulated by K. Brown et al. (2017): (i) bacteria-centric and (ii) host-centric. Primarily, the bacteria-centric hypothesis consists of antimicrobial agents who may work as growth promoters by decreasing the pathogen load and/or by modifying the composition of the microbiota to diminish competition for nutrients. On the other hand, the host-centric hypothesis consists of direct immunomodulatory agents that permit the shifting of resources to metabolic functions. K Kumar, Gupta, Baidoo, Chander, and Rosen (2005) also clarified that growth depressing microbial metabolites reduction and clinical infections inhibitions are some antibiotics' mechanisms through which animal growth enhancement occurs.

3. Antibiotic use in plants

In the USA, around 22,680,000 kg of antibiotics are produced yearly; less than 0.5% of these are used as antibiotics on plants (McManus, Stockwell, Sundin, & Jones, 2002). Plant-grade antibiotics are normally prepared as powders and contain 17 to 20% of the active ingredient (McManus et al., 2002). Subsequently, these powder antibiotics are suspended or dissolved to a concentration of 50 to 300 ppm in water and then sprayed onto the targeted parts of the plant (Bhalsod, 2016). As these antibiotics are relatively costly, they are mainly administered on high value vegetables, fruits and ornamental plants where the antibiotic value will be recuperated (McManus et al., 2002).

Orchards are a considerable economical asset for farmers, hence the possible losses caused by pathogen infection authorizes the growers to spray antibiotics. In plantations, bacterial diseases occur less frequently than viral or fungal diseases.

Therefore, antibiotics in crops are solely resorted to in specific circumstances (Vidaver, 2002). One of the way to treat diseases and bacterial infections on ornamental plants and crops was the use of antibiotics (Moats, 1986). For example Moats (1986) reported that crowngall bacterium was treated with penicillin and tomato canker was treated with tetracycline. Also, fire blight which is caused by *Erwinia amylovora* and bacterial spot on peaches caused by *Xanthomonas campestris* are regularly treated with antibiotics (Stockwell & Duffy, 2012)**.** Gentamicin is prepared as gentamicin sulfate and is used in Latin America as a mean to control bacterial diseases such as *Pseudomonoas, Xanthomonas, Erwinia* and *Ralstonia* on vegetable crops (McManus et al., 2002). As of 2002, oxytetracycline and streptomycin are the two most frequently used antibiotics in plants (Bhalsod, 2016)**.**

Compared to the residues of antibiotics in soil and water and although their numbers are high, the use of antibiotics for planting purposes has been given mild attention. Annually in the US, millions of kilograms of antibiotics are used: only 0.1% of them are used for agricultural crops (Vidaver, 2002). In 2009, around 16 000 kg of antibiotics which is equivalent to 0.12% of total veterinary antibiotics were used in orchards. It was reported by the USDA (Vidaver, 2002) that in 1999, 40% of the total number of pear orchard areas obtained 5 400 kg of oxytetracycline, 20% of the total apple orchards received around 52 000 kg of streptomycin and about 1 300 kg of oxytetracycline on 5% of the apple orchard areas. Bhalsod (2016) suggested that antibiotic residues identified on crop surfaces are neglected as they are non-persistent and quickly deactivate by sunlight.

As animals have encountered bacterial resistance against antibiotics, the same has occurred on crops. In her article, Moats (1986) stated that resistance against

streptomycin on peppers and tomatoes has arisen. The resistance of streptomycin can be limited by resorting to efficient and thorough management strategies. An alternative way to combat resistance of fire blight to streptomycin was to reduce its usage in orchards, incorporate new antibiotics and include integrated pest management schemes (Stockwell & Duffy, 2012); such as pruning and eliminating infected branches.

4. Sources of antibiotics in the environment and plants

As mentioned earlier, bacterial therapy and animal growth are globally dealt with via antibiotics. For instance, in animal manure in China, a wide difference between concentrations of tetracyclines were spotted: 29 µg/kg and 43 500 µg/kg (X. Hu, Zhou, & Luo, 2010; Pan & Chu, 2017a). This is justifiable by the fact that a great percentage of antibiotics pass through animals' bodies while being poorly absorbed (Sarmah et al., 2006). Generalizing that to the human scale, people as well excrete undigested antibiotics or discard unused antibiotics into the toilets. Additionally, industries release wastewater, hospitals inappropriately throw medical wastes, and animals defecate parent and metabolite antibiotics. These antibiotic residues ultimately contaminate the soil, ground or surface water by runoff or leaching. Consequently, antibiotic residues primarily enter the environment from manufacturing wastewater and via feces, urine and manure from animals and humans after they have consumed the medication (Daghrir & Drogui, 2013).

Those feces containing antibiotic residues are applied as animal manure: a mean of land fertilization. Therefore antibiotics enter the environment via the spread of animal manure as fertilizers onto farmland or as biosolids after livestock wastewater treatment (Pan & Chu, 2017a). Undoubtedly, through anaerobic fermentation or

composting, some of the antibiotics are destroyed. Nevertheless, it's the repeated applications of manure that result in the buildup of residual antibiotics in farmlands, soils and adjacent areas. Leaching and runoffs will even accumulate them in surface and ground water (Hirsch, Ternes, Haberer, & Kratz, 1999). Based on the physicochemical properties of the antibiotic, it can either be held by the soil or absorbed by plants. Recent studies have indicated that irrigation water or manure contaminated with antibiotics, increased their uptake by plants from the soil (Awad et al., 2014) .

Since sewage water often contain high concentrations of antibiotics and conventional treatments are not sufficient for cleaning, Carvalho, Basto, Almeida, and Brix (2014) reported that additional treatments were added to the system to improve it**.** With these additions only 60% of most drug treatments were eliminated and 24 to 36% of the tetracycline was removed after the addition of two wastewater treatment plants in China.

Furthermore, the illegal discharges of wastewater by antibiotic manufacturers to their near environment, leads to additional contamination of waterways, groundwater, soil and local communities. It has been recurrent to find antibiotic residues in rivers, soils and sediments (Michael et al., 2013; Pan & Chu, 2017a). For example, Fick et al. (2009) detected 14 mg/L of quinolones in sewages as well as high concentrations of seven other pharmaceuticals.

C. Availability of antibiotics in the environment

1. Antibiotic concentration in different media

Antibiotics are commonly detected at different concentration levels in different environmental matrices: wastewater, soil, animal manure and plant tissues. Climate

change and regional drought, population growth and pollution have all led to water shortages. As a mean to fight that, wastewater has been resorted to in order to irrigate urban landscaping, agricultural land, and replenish ground or surface water (Pan & Chu, 2017a). On a global level, a minimum of 20 million ha of arable lands is irrigated with treated wastewater (Jimenez & Asano, 2008) and according to Pan and Chu (2017a), 3.3 million ha of arable land in China, have been contaminated because of wastewater irrigation.

Worldwide, several classes of antibiotics have been identified in wastewater (Yidong et al., 2017). Tetracycline showed to be present in Korea at the highest concentration in wastewater: 255 μg/L (Pan & Chu, 2017a). Moreover, it has been stated that in Shandong province, a certain pharmaceutical manufacturer threw away polluted water holding over 50 μg/L of antibiotics. Compared to the concentrations in clean water: it is 104 times higher (Pan & Chu, 2017a). In lake water in India, the quinolones concentration was 105 to 106 times higher than the approved levels in effluents in China and surface water in the USA (Pan & Chu, 2017a).

As mentioned previously, the primary sources of antibiotics to agricultural soil are biosolids or manure and wastewater. Livestock manure containing antibiotic residues is being applied on agricultural lands as a mean of fertilization to improve soil quality, consequently absorbed by and accumulating in plants and affecting the soil flora. Accurately, due to animal's poor gut absorption, 90% of pharmaceuticals consumed and utilized by animals are evacuated as their parent compound. They may enter the groundwater intact or mineralized by soil organisms (Lillenberg, Litvin, Nei, Roasto, & Sepp, 2010). That is explained by the fact that excretory organs remove polar compounds (e.g. tylosin and tetracycline) more effectively than compounds with high

lipid solubility characteristics (Kuldip Kumar, Gupta, Chander, & Singh, 2005). Often, lipid soluble antibiotics are not removed till they are metabolized to polar compounds.

Lillenberg et al. (2010) along with several other studies, suggested different fates of antibiotic residues in soil. The substance may either be easily changed and degraded into water and carbon dioxide or, if the matter is lipophilic then it may require some time to degrade, or, it could be metabolized into a substance which is more hydrophilic. In this case, it does not decompose at all, thus affecting the environment.

In the environment, antibiotic persistence and fate depends on numerous factors like: biodegradation, binding to soil, chelation or chemical complexation, photolysis and hydrolysis. In 2005 Kuldip Kumar et al. (2005) distinguished antibiotics may be inactivated in manure or soil when chelation or chemical complexation of drugs with inorganic or organic compounds or ions occur.

After analyzing 30 pig manure samples, the highest concentration of tetracyclines reached up to 23 mg/kg while that of quinolones in chicken manure from China was a high as 1 420 mg/kg (Pan & Chu, 2017a). Also, Choueiri (2008) in Lebanon stated that sulfonamides which are synthetic antibiotics are employed against gram-positive and gram-negative bacteria as well as after excretion; they detain a 90% recovery rate in cow manure. Sulfamethazine is a member of the sulfonamide antibiotic group. Accinelli, Hashim, Epifani, Schneider, and Vicari (2006) reported that in Germany, after seven months of manure fertilization, the concentration of sulfamethazine was 15 µg/kg of soil.

Many wastewater treatment plants do not completely remove antibiotics; therefore, antibiotics survive the treatment and become sorbed to biosolids which are then applied as fertilizers on agricultural lands (Ding, Zhang, Gu, Xagoraraki, & Li,

2011; Gottschall et al., 2012). In a study reported by Pan and Chu (2017a), seventeen antibiotics were identified in biosolids and animal manure and eighteen in soil. The most abundant one was tetracycline found at a rate of 2.68 μg/g in soil and 184 μg/g in manure (Pan & Chu, 2017a). Ciprofloxacin was also found abundantly in biosolids: 3.26 μg/g (Pan & Chu, 2017a). Table 5 enumerates a list of the most used veterinary antibiotics concentrations in different media as reported by several researchers.

Table 5. Concentrations of veterinary antibiotics in different media

Antibiotic	Country	Medium	Concentration	Reference
Oxytetracycline	Denmark	Soil	2.5 – 50 μg/L	(Kong et al., 2007)
Oxytetracycline	Denmark	Pig manure	33 to 22000 mg/g	(Kong et al., 2007)
Tetracycline	China	Swine manure	0.3 – 56.8 mg/kg	(Y.-x. Li et al., 2013)
Tylosin	China	Swine manure	0.2 – 1.9 mg/kg	(Y.-x. Li et al., 2013)
Enrofloxacin	Turkey	Poultry manure	0.01 – 0.08 mg/kg	(Karcı & Balcıoğlu, 2009)
Chlortetracycline	Canada	Poultry manure	23 mg/kg	(Warman & Thomas, 1981)
Oxytetracycline	Italy	Fresh cattle manure	872 mg/kg	(De Liguoro, Cibin, Capolongo, Halling-Sørensen, & Montesissa, 2003)
Tetracycline	China	Dairy cow manure	0.2 – 10.4 mg/kg	(Y.-x. Li et al., 2013)
Ciprofloxacin	-	Surface water	9 ng/L	(Christian et al., 2003)
	-		Up to 30 ng/L	Kolpin et al 2002
	-		Up to 26.2 ng/L	(Calamari, Zuccato, Castiglioni, Bagnati, & Fanelli, 2003)
Chlortetracycline	-	Surface water	Up to 690 ng/L	(Kolpin et al., 2002)
Oxytetracycline	-	Surface water	Up to 340 ng/L	(Kolpin et al., 2002)
Tylosin	-	Surface water	Up to 280 ng/L	(Kolpin et al., 2002)
Tylosin	-	Surface water	Up to 2.8 ng/L	(Calamari et al., 2003)

Enrofloxacin	Italy	Soil	51 μg/L	(Riaz et al., 2018)
Ciprofloxacin	Turkey	Soil	0.204 mg/kg	(Riaz et al., 2018)
Enrofloxacin	Austria	Poultry manure	8.3 mg/kg	(Riaz et al., 2018)
Chlortetracycline	-	Poultry manure	57 – 11 900 μg/kg	(Nieder, Benbi, & Reichl, 2018)
Chlortetracycline	-	Cattle manure	11 – 208 μg/kg	(Nieder et al., 2018)
Tylosin	-	Poultry manure	3700 μg/kg	(Nieder et al., 2018)
Sulfamethazine	-	Pig manure	9 990 μg/kg	(Nieder et al., 2018)
Ciprofloxacin	-	Biosolid	3.26 μg/g	(Pan & Chu, 2017a)
Tetracycline	Korea	Wastewater	255 μg/L	(Pan & Chu, 2017a)

Source: Collected from different sources

2. Antibiotic levels in manure

Nowadays, some countries frequently use and incorporate veterinary antibiotics into animal feeds in order to improve feed efficiency and growth rate (Sarmah et al., 2006). In China, in 2013, the overall usage of antibiotics was around 162,000 tons, from which 52% were employed for animal consumption (Q. Yang, Zhang, Guo, & Tian, 2016). Significantly, a high percentage of veterinary antibiotics are mostly defecated via feces and urine. Following repeated manure applications, those antibiotics eliminated through excreta might accumulate and persist in soil. In the feces, 30 to 90% of them are metabolites or parent compounds (Q. Yang et al., 2016). In vegetable cultivation in China, animal manure application on land at a level of 15 000 to 150 000 kg/ha/year leads antibiotics to infiltrate the soil (Q. Yang et al., 2016).

Animal manure encloses significant quantities of antibiotic resistant bacteria, antibiotic residues and antibiotics resistance gene (Udikovic-Kolic, Wichmann, Broderick, & Handelsman, 2014). Numerous reports have evaluated the persistence, transfer and alteration of veterinary antibiotics residues in soil, and discovered that the application of manure as a mean of fertilization might cause the antibiotic

concentrations to increase significantly (Y. Xu, Yu, Ma, & Zhou, 2015). Accurately, Baguer, Jensen, and Krogh (2000) stated that manure contaminated with antibiotics and added on agricultural land appears to be the dominant path through which antibiotics are released into the environment. In fact, it is the chief cause of microbial resistance. Subsequently, Song and Guo (2014) declared that there is a yearly release of 3 000 to 27 000 tons of drugs through livestock manure into the environment due to the global heavy usage of veterinary antibiotics in confined animal feeding procedures.

Thanks to innovative analytical techniques, numerous researchers projected the amount of antibiotics in manure. For instance, sediments from different countries have been studied and antibiotics like tylosin, sulfadimidine, tetracycline and sulfathiazole have been identified in cattle manure, swine slurry, fish farm and poultry litter at a broad concentration extending from traces to 200 mg/kg (K Kumar et al., 2005). Hamscher, Sczesny, Höper, and Nau (2002) confirmed in his study that the concentration of chlortetracycline and tetracycline in manure were 0.1 and 4.0 mg/kg respectively. Furthermore, Song and Guo (2014) testified that more than 50 main antibiotics have been identified in swine, poultry, horse and cattle manure at concentrations ranging from 0.01 to 765 mg/kg of dry manure mass.

3. Uptake and accumulation site of antibiotics in plants

Ever since they have been discovered, antibiotics have been a chief factor in treating infectious diseases. Nevertheless, their extensive usage as feed additives in animal husbandry has raised concerns on the manifestation of antibiotics in water and food supplies. It has been estimated by Vidaver (2002) that annually in the USA, 53 000 ha of vegetables and fruit plants are sprayed with antibiotics (Kuldip Kumar et al.,

2005). Several studies tackled the uptake and accumulation of veterinary antibiotics by different crops. A repeated launching of veterinary antibiotics into the soil via a continual application of manure or a constant irrigation with water contaminated with antibiotics could ultimately add the antibiotic's concentration high enough to go as a potent hazard into the terrestrial environment. Most of the studies regarding the uptake of veterinary antibiotics by plants resort to two approaches: growing plants in a soil covered with manure or in a medium high in antibiotics (soil or water).

Root crops are widely consumed worldwide. It has been established through several studies that from the growth media, plants are able to uptake pharmaceutical compounds thru their roots (Kong et al., 2007). A study sponsored by the USDA in 2007 revealed that some vegetables take up antibiotics when cultivated in a soil amended with livestock manure (Tasho & Cho, 2016). The vegetables were planted in a greenhouse, on soil fertilized with liquid hog manure comprising sulfamethazine (Dolliver, Kumar, & Gupta, 2007). This antibiotic was found in the leaves of crops. In another study directed by X. Hu et al. (2010) examined the occurrence and movement of antibiotics taken by organic vegetables fertilized with manure; eleven antibiotics were stored in the crops. Additionally, in his experiment, Sabourin et al. (2012) studied the uptake of antibiotics by crops grown in soils amended with municipal biosolids; they distinguished three different antibiotics in carrot, sweet corn, tomato and potato. The antibiotics found were trimethoprim, quinolones and sulfonamides and they were detected in the comestible parts of the crops at concentrations varying from 0.01 to 14 ng/g of dry weight (Grote et al., 2007). Also, Michelini, Reichel, Werner, Ghisi, and Thiele-Bruhn (2012) conducted a study where maize and willow were grown in a greenhouse for 40 days in potting soils contaminated with 10 mg/kg of sulfadiazine.

The chemical was not detected in the above ground tissues but rather in the roots of maize and willow at a concentration of 26.6 and 333 mg/kg of dry weight respectively.

Contrarily, X. Hu et al. (2010) discovered the veterinary antibiotics to be in the following ascending order: leaves, stem, roots. Nevertheless, the former reported a greater hypogeal antibiotic level in an undeveloped test plant, which was sampled two weeks prior to harvest. Lillenberg et al. (2010) stated that antibiotics accumulate in plants at a greater amount when the vegetative period is longer; it was lowest in cucumbers and highest in lettuces. Also, L. Liu et al. (2013) demonstrated that under an antibiotic concentration of 1,000 µg/L, the plant *P. autralis* detained oxytetracycline HCl, sulfamethazine and ciprofloxacin HCl concentrations of 6 901, 2 047 and 12 834 ng/g dry weight respectively. A summary of the results is that all plants contained antibiotics in the following ascending sequence: ciprofloxacin HCl, oxytetracycline HCl, then sulfamethazine.

In an experimentation held in Lebanon, Basil et al. (2013) testified that gentamicin was relatively absorbed at a greater amount than streptomycin by radish, lettuce and carrot. It was also stated that in plant tissue, the concentration of antibiotics increased when the antibiotic level in the manure increased (1 > 0.5 mg/kg). L. Liu et al. (2013) experiment also revealed a positive correlation between the buildups of antibiotics with their concentrations in the media. Nevertheless, this conclusion is not in accordance with the results of Youssef (2016) where it was demonstrated that a growing media administered with a greater antibiotic concentration, does not always result in a greater accumulation level in plant tissues.

In many aquatic media like groundwater, surface water and municipal sewage, antibiotic residues are extensively prevalent and documented. Azanu et al. (2016)

studied the uptake of tetracycline and amoxicillin by lettuce and carrots irrigated with contaminated water of known antibiotic concentrations. The latter discovered that tetracycline was identified in all plant samples, at concentrations varying in lettuce from 4.4 to 28.3 ng/g and in carrot's fresh weight: 12.0 to 36.8 ng/g.

D. Uptake of antibiotics by plants

1. Phytotoxicity

Most studies on the phytotoxic effect of antibiotics have been examined in vitro, hence not under soil conditions. Pan and Chu (2017a) stated that the phytotoxicity of antibiotics varies among plant species and antibiotic compounds. There are three most scrutinized phytotoxic endpoints: germination, growth and development. Note that antibiotics are recognized to detain a biphasic effect on plant growth which is depicted by hormesis with a high dose of inhibition and a low dose of stimulation (Q. Li, 2006) . More concretely, tetracyclines reduced the production of *Phaseolus vulgaris* (pinto beans) whereas in *Triticum aestivum* (wheat) and *Zeamays* (corn) it encouraged nutrient uptake (Batchelder, 1982). This is justified by the fact that in maize plants, tetracyclines caused a noticeable increase in the activities of the stress protein glutathione S-transferases and peroxidases; however, the latter did not occur in pinto beans (Pan & Chu, 2017a). In consonance with previous findings, antibiotics had a greater phytotoxic effect on root/shoot elongation than on seed germination (Pan & Chu, 2016b; M. Wang & Zhou, 2005). An, Zhou, Sun, and Zhang (2009) justified the latter by stating that it might be difficult for the antibiotics to penetrate the seed coat, hence the growth of the embryonic roots is not affected as the antibiotics cannot be absorbed. On one hand, root elongation was a more sensitive endpoint compared to seed germination and shoot

elongation (An et al., 2009; Sresty & Rao, 1999). On the other hand, Jin, Chen, Sun, Zhou, and Liu (2009) discovered that the inhibition of sulfonamides on root elongation was significantly lower than on shoot elongation (Pan & Chu, 2017a). Additionally, Hillis, Fletcher, Solomon, and Sibley (2011) determined that the sensitivity of carrots to tetracyclines is greater than that of lettuce and alfalfa.

One of the utmost significant factors leading to phytotoxicity is hydrophobicity (Pan & Chu, 2016b). Nevertheless, not only do the experimental parameters impact the outcomes of the toxicity tests by order of magnitude (i.e. antibiotic concentration and time duration), but also, most outcomes provided by in vitro phytotoxicity tests are improbable to befall in environmental soil. Over and above, the possible environmental consequences of the metabolites of antibiotics haven't been studied considerably yet. Undeniably, it is indispensable to study the prolonged phytotoxicity effects of antibiotics in a representative soil environment with the application of animal manure or wastewater contaminated with antibiotics (Pan & Chu, 2017a). Table 6 illustrates some phytotoxic effects perceived on plants at some concentrations.

Table 6. Phytotoxic effect of some antibiotics on plants

Antibiotic	Plant species	Toxicity Effect
Amoxicillin	Carrot, Lettuce	10 000 μg/L was seen to be toxic for root growth
Chlortetracycline	Pinto beans	Plant growth was affected when grown in sandy loam but no effect was seen in clay loam (concentration 10 mg/L)
Chlortetracycline Enrofloxacin	Carrot, Lettuce	1 000 μg/L was seen to be toxic for root growth
	Alfalfa	100 μg/L was seen to be toxic for root growth
	Cucumis sativus, Lactuca sativa, Phaseolus vulgaris, Raphanus sativus	High concentration (5 000 μg/L) was seen to have toxic effect on post germinative development of plant
Levofloxacin	Carrot	10 000 μg/L was seen to be toxic for

		root and shoot growth
Tetracycline	Carrot	100 µg/L was seen to be toxic for root whereas 1 000 µg/L was observed to be toxic for shoot growth
Tylosin	Carrot Lettuce Alfalfa	10 000 µg/L was seen to be toxic for root growth 10 µg/L was seen to be toxic for root growth
Metronidazole	Soybean	With increase in drug concentration (0 – 4 g/kg of soil) plant growth decreases

Source: (Kalaji et al., 2017)

2. Effects of antibiotics on plant growth

Studies have shown that antibiotics detain either enhancement or harmful effects on the growth and the performance of plants (L. Liu et al., 2013; Migliore, Rotini, Cerioli, Cozzolino, & Fiori, 2010): they can modify biomass production, branching patterns, number of leaves, internode and shoot length, root to shoot ration, dry and fresh weight, C to N and K to Ca ratio, etc. (Michelini et al., 2012). It was stated that responses of plants could be dose-dependent (Minden, Deloy, Volkert, Leonhardt, & Pufal, 2017). For example, the toxic effects are perceived at high concentrations whereas increased growth is perceived at lower concentrations (Migliore et al., 2010). Also, Azanu et al. (2016) informs that antibiotics typically accumulate in roots . Hence, the former has an adverse impact on root elongation, root length, and number of lateral roots, which holds consequences on crop water uptake (Michelini et al., 2012; Piotrowicz-Cieślak, Adomas, Nałęcz-Jawecki, & Michalczyk, 2010). Accordingly, a study conducted hydroponically by Kong et al. (2007), revealed that at concentrations greater than 0.02 mM, oxytetracycline exerted an inhibitory effect on the growth of alfalfa; shoot growth was less sensitive than root growth. Additionally, some authors report a positive correlation between root elongation inhibition and

concentrations of pollutants (Hillis et al., 2011). In addition to affecting roots, antibiotics have been shown to have a significant effect on the germination frequencies of seeds.

Phytotoxicity of antibiotics differentiates between plant species and antibiotic compounds. In their research, Minden et al. (2017) studied the effects of sulfadiazine, penicillin and tetracycline on two grass species and two herb species and demonstrated that the antibiotics do not lead to a lower germination rate but rather initiate a delay in germination. At a level greater than 1 µg/L, all treatments led to a significant delay in germination (10 to 45 hours of delay); the greater the antibiotic concentration, the further the delay (Minden et al., 2017). Similarly, Eluk, Nagel, Zimmermann, Molina, and Althaus (2016) concluded that the germination of seeds was delayed due to the antibiotics (enrofloxacin and penicillin); phytotoxic effect were also observed on crop growth. On another hand, Eluk et al. (2016) showed that even when the Maximum Residue Limits (MRL) of antibiotic is followed, phytotoxic effects are still caused. Indeed, their results demonstrated that different crops (corn, soybean and sorghum) were affected by different antibiotic concentrations of kanamycin, enrofloxacin, penicillin, and tylosin.

Consequently, antibiotics greatly affect the growth and the performance of plants. Nevertheless, it is still vague how much these effects reach humans and introduce a hazard into their lives. Table 7 records the accumulation and effects of some antibiotics on crops.

Table 7. Accumulation and effects of different antibiotics on crops

Antibiotic	Crop	Concentration	Effect on plants	Reference
Chlortetracycline, Oxytetracycline, Tylosin	Lettuce, Carrot, Alfalfa	1 – 10000 μg/L	No effect on germination; decrease of shoot and root lengths at different concentrations	(Hillis et al., 2011)
Chlortetracycline	Corn, Green onion, Cabbage	0.02 μg/mL	Bioaccumulation	(K Kumar et al., 2005)
Gentamicin	Carrot	0, 0.5, 1 mg/kg	Bioaccumulation, somewhat reduced growth	(Bassil et al., 2013)
Tetracycline, oxytetracycline, chlortetracycline	Pea	0 – 8 mg/kg	Bioaccumulation, decreased root length and at 0.4 mg/kg and more decreased peroxidase activity	(Ziolkowska, Piotrowicz-Cieslak, Margas, Adomas, & Nalecz-Jawecki, 2015)
Tetracycline	Carrot	0 – 300 mg/L	Decrease in germination rates, inhibition of shoot and root elongation	(Pan & Chu, 2016b)
Gentamicin	Lettuce	31.9 – 56.7 ng/g	N/A	(Youssef, 2016)
Tylosin	Lettuce	2.38 – 18.27 ng/g	N/A	(Youssef, 2016)
Enrofloxacin	Radish	9.2 – 16.9 μg/mL	N/A	(Chowdhury, Langenkämper, & Grote, 2016)
Chlortetracycline, oxytetracycline	Pinto bean plants	160	Root dry weight reduced by 66 – 94%	(Sarmah et al., 2006)
Sulfamethazine	Corn	<1.06 mg/kg	Not mentioned	(Du & Liu, 2012)

Source: Modified (Minden et al., 2017)

3. Mechanism through which plants absorb antibiotics

The accumulation and translocation of antibiotics in crops is affected by different mechanisms and factors. Some of these factors are the nature and concentration of the antibiotic applied, crop species, quality of the water and soil properties (Pan & Chu, 2017c). Nevertheless, the uptake process appears to be the most affected by the antibiotics' physicochemical properties. To measure the ability of an antibiotic to move from root to shoot, the translocation factor is resorted to. When the translocation factor is less than 1 then this means that the translocation of the antibiotic from roots to leaves or fruits are restricted. When it is greater than 1, then the translocation is not restricted (Pan & Chu, 2017a). When plants absorb antibiotics, they are transferred to shoots, leaves and fruits through a passive diffusion via the phloem or xylem into the symplastic pathway (Pan & Chu, 2017c). Consequently these compounds are moved into roots thru the Casparian strip and are then carried to fruits by the phloem or to leaves by the xylem (Miller, Nason, Karthikeyan, & Pedersen, 2016). In plants, the xylem transports water, organic compounds and nutrients from root to shoot through the transpiration stream it provides. The stem holds the lower concentration as its purpose is considered as a conductive channel for antibiotics (L. Liu et al., 2013). Therefore, it is possible that an increase in the transpiration rate could speed the uptake of antibiotics from the soil into the plant (Pan & Chu, 2017a). Also, Pan and Chu (2017a) indicates that the translocation of chloramphenicol, tetracyclines, and lincomycin was higher in leafy vegetables. This signifies that the transportation process of these antibiotics mainly occurs through the xylem. After being absorbed, most of the tetracyclines henceforth exist in the cytosol and symplast of plants (pH ~7.2) under their neutral form. Subsequently, they are moved more freely and stored at

a higher concentration in leaves or fruits (Pan & Chu, 2017a). Additionally, the tetracyclines that are more water-soluble are translocated more easily in plants through water mass flow (Pan & Chu, 2017a). Kong et al. (2007) primarily suggested in their study that the transport of oxytetracycline into the roots of alfalfa is energy-dependent, which necessitates selective binding sites and secondarily that the uptake of oxytetracycline is aquaporin independent as water channels are not the mean through which oxytetracycline enters into the roots.

Nevertheless, the passage of organic compounds in the phloem and xylem is still unclear as it chiefly depends on the ability of the antibiotic to cross membranes. Consequently, as it has been hypothesized that the accumulation of antibiotics into plants are hazardous to human and animal health then further research experiments should tackle the uptake, translocation and accumulation mechanisms of different veterinary antibiotics into vegetables' roots and shoots.

E. Residual effect on humans and the environment

1. On humans

As several previous studies have been conducted regarding the consumption of antibiotic-contaminated edible crops by humans then the former can henceforth be evaluated. The available data for humans varies widely as they are provided by different sources, hence different experimental conditions. Some experiments were conducted in a hydroponic system and others in an open field or greenhouse. Pan and Chu (2017a) specified the annual exposure of human to different antibiotic classes and it fluctuated between 0.01 to 8 456 μg/g in different crops. Sulfonamides in celery leaves, detained a concentration of 0.01 μg/g and chloramphenicol in rice grains 8.456 g/kg.

The minimal therapeutic antibiotic dose that humans can tolerate and consume per day falls between 20 and 200 mg. The predicted values of annual potential human exposure to antibiotics in edible plants were less than the minimum therapeutic dose or below the recommended acceptable daily intake (ADI) values (Pan & Chu, 2017a). In other words, human exposure to antibiotic is likely to be low through annual consumption of edible crops grown in manure-amended or wastewater-irrigated soil. Nevertheless, some studies with antibiotics showed that in presence of heat or during cooking, loss of microbiological activity occurred.

Resorting to the scarce data available on antibiotic contaminated edible crops, the possible human exposure to antibiotics through daily or annual consumption of those crops is likely to be low. It is believed that the available rate of most antibiotics in edible plant tissue represented a *de minimis* risk to human health (Prosser & Sibley, 2015). Note that the antibiotic structures would be damaged and its level decreased if the crop is cooked before ingestion (Phillips et al., 2004). Consequently, the residual antibiotic concentrations would be much lower than those reported, hence human exposure could be negligible (Pan & Chu, 2017a).

Haphazard administration of drugs for therapeutic and non-therapeutic reasons in animal husbandry has been studied to persist in the foods produced by these animals, thus posing a health hazard to consumers. Truly, it was tackled that antibiotic residues that approached human in a direct or indirect manner resulted in an increase of resistance to the drug, alteration of human intestinal flora and bacterial count (T. Chen, Li, & Wei, 2014; Marshall & Levy, 2011). This subject is not studied in Lebanon and the region and with detailed investigation.

2. In soil (adsorption, degradation, leaching):

a. Adsorption of antibiotics

Several antibiotics are adsorbed and fixed onto soil particles depending on the physico-chemical properties of the antibiotic, type of soil, quality and content of soil organic matter, soil pH, soil minerals, main climatic conditions and other environmental aspects (Kuldip Kumar et al., 2005; Tasho & Cho, 2016). Veterinary antibiotics are relatively easy to adsorb to soil particles as they are organic compounds displaying a wide range of functional groups as well as they can be amphoteric, amphiphilic or ionic. It's the interaction of veterinary antibiotics with organic matter and clay minerals that causes their binding, sorption and fixation on the soil matrix. The different binding mechanisms involve van der wall interactions, anion exchange, cation bridging and electrostatic attraction (Jeon et al., 2014). Amphoteric and acidic antibiotics bind to soil via non-ionic interactions, whereas cationic antibiotics bind to soil via ionic interaction (NAAS, 2010). Under acidic conditions, the primary adsorption mechanism of tetracyclines is: cation exchange (Jia, Zhou, Wang, Zhu, & Chen, 2008). Due to the structure of tetracyclines which contains electron donor groups, strong complexes can be formed between tetracyclines and metal ions. Tetracyclines and divalent metal cations are hereafter prone to bind and adsorb onto soils, organic matter and minerals, hence their extractability is reduced, which in turn reduces their accessibility for plant uptake (Y. Zhang et al., 2016).

In the soil, the behavior and transport of organic contaminants are predicted by the sorption coefficient (K_d), which is also usually used to predict the solute sorption to soil. The adsorption affinity of antibiotics is measured and denoted by the sorption coefficient (K_d) which confirms the antibiotic sorption and mobility in the environment.

The K_d value ranges from 0 to greater than 200 L/kg (Hashmi, Strezov, & Varma, 2017). A K_d value greater than 200 L/kg has a high tendency to bind to soil particles, from 5 to 200 L/kg it is considered mediate and 0 to 5 L/kg low (Hashmi et al., 2017). The soil mobility determines the ability of antibiotics to pass through the soil into surface runoff and groundwater. Kulshrestha, Giese, and Aga (2004) indicated that the mobility of antibiotics increased due to the dissolved organic matter which reduced the sorption of the antibiotics to clay. Compounds that have a low K_d value are not strongly bound to the soil, thus are more mobile, whereas compounds with a high K_d value are strongly bound and are less mobile in the soil.

Due to their numerous structural classes, the physicochemical properties of antibiotics greatly differ from one another. Depending on the pH of the soil, some antibiotics are dissociated or highly water soluble whereas others are non-polar or hydrophobic (Kuldip Kumar et al., 2005). Sorption of ionizable chemicals to sediments and soil are influenced by ionic strength and pH (ter Laak, Gebbink, & Tolls, 2006). Alternation of the pH causes deprotonation and protonation of ionizable compounds, thus altering the physicochemical properties and consequently the sorption ability of a compound. Increasing pH leads to a lower sorption coefficient (ter Laak et al., 2006). Also, alteration of the interfacial potential or competition for ion exchange sites by ionic strength can impact the sorption of ionized antibiotics. Usually, clay loam soils sorb compounds stronger than loamy sand soils (ter Laak et al., 2006). The sorption capacity of tetracyclines (i.e. oxytetracycline) and fluoroquinolones (i.e. enrofloxacin) is much bigger than other pharmaceuticals, hence; they are prone to showing low mobility and accumulating in the soil surface. Macrolides (i.e. tylosin) too demonstrate low mobility and ease to adsorb onto soils rich in mineral contents (Fe, Mn and Al): Kay, Blackwell,

and Boxall (2004) showed that 13% of tylosin desorbed from the soil and Rabølle and Spliid (2000) showed that oxytetracycline desorption rate fluctuated between 0.5 to 2.3 % in soil. On another hand, sulfonamides indicated a low ability to adsorb onto soil, hence a stronger mobility in the soil (Accinelli, Koskinen, Becker, & Sadowsky, 2007). Therefore, due to different environmental factors and compound properties different antibiotics revealed different behaviors and distribution in soils.

b. Degradation and persistence of antibiotics in soil

Antibiotics in soil may be degraded or transformed because of biotic or abiotic reactions. Chemically mediated transformations like oxidation, reduction, hydrolysis and photochemical transformations are the major abiotic reactions. Humic substances, mediate photo-degradations, which primarily takes place on soil surfaces (Thiele-Brun & Peters, 2007). In the case of hydrolysis, the dissociation of water releases hydrogen and hydroxide ions which attack antibiotics, thus breaking an existing bond and forming a new one. That is either dependent or independent of the pH (Yaron, Calvet, & Prost, 1996). Additionally, biodegradation also plays an important role in the exclusion of antibiotics from the environment (J.-F. Yang et al., 2012).

Soil biodegradation mainly takes place by the activities of microorganisms. The latter relies on several factors such as pH, oxygen, temperature, water activity or moisture level, degree of adaptation, microbial population, accessibility of nutrients, cellular transport properties and chemical structure of the compound (Selvam & Wong, 2017). In an experiment conducted by Pan and Chu (2016a), the fraction of degraded quinolones and tetracyclines was 44-75% in sterilized soils and 82 to 100% in non-sterilized agricultural soil. This denotes an important role of microbial degradation.

Differences in experimental conditions and media provide a challenge to compare the degradation behaviors of different antibiotics. Studies have indicated that the half-lives of sulfonamides in non-sterilized soil varied from 5 to 30 days and in a sterilized soil from 59 to 265 days (Kümmerer & Henninger, 2003; Lin & Gan, 2011).

The degradation of antibiotics may also be affected by soil properties like the organic carbon content. It has been asserted by J. Xu, Wu, and Chang (2009) that a high organic carbon content in the soil could lower the bioavailability of antibiotics and consequently hinder their degradation rate. On another note, the addition of biosolids or animal manure intensifies the sorption capacities of antibiotics in soil and commonly stops their degradation (Kay et al., 2004). Aminov and Mackie (2007) informed that antibiotic residues are found in agricultural soils at a depth of 10 meters and more. Christian et al. (2003) studied the residues of the most utilized antibiotics in liquid manure and soil. Among their findings was that sulfonamides and macrolides are highly stable in both media, while fluoroquinolones and beta-lactams were barely distinguished and tetracyclines were not found because of their high sorption coefficient. However, fluoroquinolones are insensitive to hydrolysis and several antibiotics belonging to this class such as quinolones are characterized by a high chemical stability (S. Wang & Wang, 2015). In another study conducted by Schlüsener and Bester (2006) the half-lives of tylosin and macrolides (oleandomycin and erythromycin) was 8 days, 20 and 27 days over a period of 120 days respectively. When it comes to the class of beta-lactams (cephalosporins and penicillins), they are rarely detected in the environment as their unstable lactam ring leads to a fast degradation (Thiele-Bruhn, 2003). Tetracyclines strongly adsorb to soil particles due to their high sorption coefficient (K_d); they do not move easily. Nevertheless, the tetracyclines can

absorb light, thus they are prone to photodegradation and after 30 days, chlortetracycline degraded by 50% and the half-life of oxytetracycline ranges from 18 to 79 days (S. Wang & Wang, 2015).

In summary, the half-life of an antibiotic can range from few days up until 300 days in different media such as soil, marine sediments, sewage, etc. (Kuldip Kumar et al., 2005). Antibiotics may persist in deep soil layers and deep waters for a longer period as lack of light (darkness) and low temperatures lower the degradation rate, hence increase the half-life of several antibiotics (Hektoen, Berge, Hormazabal, & Yndestad, 1996).

Table 8. Values of veterinary antibiotics degradation in different media

Class	Antibiotic	Medium	Temperature (ºC)	Degraded (%)	Time (days)
Tetracyclines	Tetracycline	Pig manure	8	50 – 70	48
		Soil	25	50	31.5 – 86.6 ($t_{1/2}$)
	Oxytetracycline	Soil and slurry	N/A	50	18 – 79 ($t_{1/2}$)
		Soil and cattle manure	N/A	-	30
	Chlortetracycline	Sandy loam soil and cattle feces	4	12	30
			20	56	30
			30	56	30
Sulfonamides	Sulfamethazine	Soil	25	50	24 – 57.8 ($t_{1/2}$)
		Biosolids	N/A	50	173 ($t_{1/2}$)
	Sulfadiazine	Soil	25	50	2 – 265 ($t_{1/2}$)
Macrolides	Tylosin	Sandy loam soil and manure	4	60	30
			20	100	30
			30	100	30
		Sand and slurry, sandy	N/A	50	3.30 – 8.1

		loam and slurry			($t_{1/2}$)
		Liquid manure	23	50	2.4 ($t_{1/2}$)
		Manure (composting)	40	50	16 – 23 ($t_{1/2}$)
	Erythromycin	Sandy loam soil and cattle feces	4	-	30
			20	75	30
			30	100	30
		Soil	20	50	11 ($t_{1/2}$)
Quinolones	Norfloxacin	Soil + manure	25	50	24 – 153 ($t_{1/2}$)
	Ciprofloxacin	Biosolids	30	50	3.6 – 5.8 ($t_{1/2}$)

$t_{1/2}$ = half-life
Source: (Pan & Chu, 2017a)

c. Leaching of antibiotics

In 2010, the worldwide consumption of veterinary antibiotics by livestock was 63 000 tons minimum, and it was projected to rise in 2030 to 106 600 tons (Van Boeckel et al., 2015). Ötker and Akmehmet-Balcıoğlu (2005) informs in his article that in veterinary medicine, antibiotics have been extensively used as a growth promoter or for therapeutic reasons. Following administration, antibiotics are not fully digested. Around 75% are excreted through feces and 90% through urine (Halling-Sørensen, 2001). They are excreted either as the parent compound and/or as metabolites, which are subsequently added to the environment by spreading animal manure as a mean of fertilization onto agricultural lands, direct addition by grazing livestock, and the release of wastewater (Pan & Chu, 2017b). The direct consequence of adding animal manure on farm lands are surface runoff and leaching of veterinary antibiotics into deeper soil layers (Ji et al., 2012). Depending on their mobility in soils, these compounds represent

a great threat to close rivers, groundwater, streams and aquatic life, thus contaminating them. In surface water, soil, ground water, sediment and drinking water, more than 30 different veterinary antibiotics were detected (C. Chen et al., 2014).

Rabølle and Spliid (2000) informed that as the affinity of several antibiotics is high in soils, then antibiotic losses are more prone to occur through surface runoffs than through leaching from lands amended with manure contaminated with antibiotics. Rabølle and Spliid (2000) resorted to four antibiotics and tested their leaching ability in a soil column study. In saturated steady-state conditions, most of the antibiotics persisted in the top few centimeters of the soil columns. This has been interpreted by the fact that the soil surface compared to subsurface layers, detains a greater concentration of organic matter. Therefore, this encourages the adsorption of antibiotics, hence decreasing their leaching (Jones, Bruland, Agrawal, & Vasudevan, 2005). Consequently, the addition of animal manure in soil might decrease the leachability of some antibiotics (i.e. tetracycline-hydrochloride) (Engels & Winckler, 2004). Other factors such as pH, dissociation constants and sorption desorption processes, water solubility, and partitioning coefficients of antibiotics affect their leaching potential (Pan & Chu, 2017a).

The leaching ability of antibiotics in the environment depends on their soil physicochemical characteristics, weather conditions and organic waste application. Due to its physicochemical properties such as organic content and texture, sandy soils present a high veterinary antibiotics leachability (Pan & Chu, 2017b). A study by Kay et al. (2004) indicated that sulfonamides detain a high ability to be leached into groundwater. This could possibly be credited to its low soil partition coefficient. Compounds detaining a low K_d value, are more mobile in the soil as they are not

strongly adsorbed to its particles. Therefore, the group of antibiotics detaining these properties, can easily be moved and contaminate the surface as well as ground water. On another hand, antibiotics that are tightly bound to soils, can mainly be transported during run off losses of soil to surface waters (NAAS, 2010). H. Chen, Gao, Li, and Ma (2011) explained that antibiotics are likely to be leached down by percolation or lost though runoff when they are weakly bound to the soil (small K_d value), whereas when they are strongly bound to the soil (high K_d value), they move to other areas with soil particles by runoff water. Sorption experiments by Rabølle and Spliid (2000) have proved that olaquindox which has weak adsorbing capacities leached completely thru soil columns whereas depending on soil properties, tylosin which has stronger adsorbing capacities, persisted in several depths; oxytetracycline was not transported at all, hence not leached.

Table 9. Persistence and chemical properties of commonly administered veterinary antibiotics

Antibiotic	**Half-life (days)**	**K_d value (L/kg)**	**Water solubility (g/L)**	**Mobility**
Enrofloxacin	>50	496 – 61 000	5 - 200	Non-mobile
Gentamicin	N/A	417 – 1026	10 - 500	N/A
Oxytetracycline	10 – 50	420 – 1030	> 200	Non-mobile
Tylosin	< 10	8.3 – 128	5 – 200	Slightly mobile
Penicillin	10 – 50	N/A	5 – 200	Slightly mobile

Source: Modified: (Call, Matthews, Subbiah, & Liu, 2013; Hashmi et al., 2017; Pikkemaat, Yassin, Fels-Klerkx, & Berendsen, 2016)

F. Description of antibiotics used in the study

In this research, oxytetracycline, tylosin, enrofloxacin and gentamicin uptake and accumulation in soil and water by lettuce, cucumber and radish was studied, as well

as their persistence in soil. These four antibiotics are extensively used by Lebanese farmers.

1. Oxytetracycline

a. Chemical structure and formula

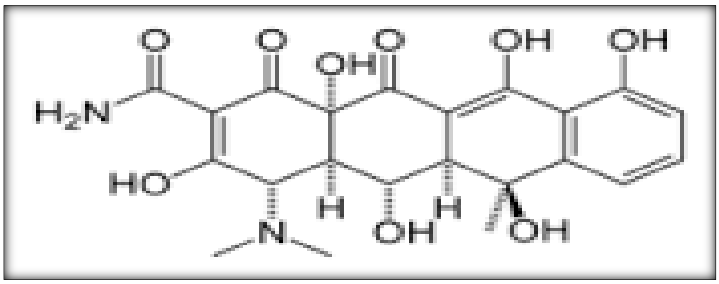


Figure 1. Oxytetracycline chemical structure
Source: (L. Yang et al., 2018)

Molecular formula: $C_{22}H_{24}N_2O_9$
Molecular weight: 460.44 g/mol

b. Overview

In the 1940s, the tetracyclines were discovered. Their derivatives are yellowish in color, crystalline, and amphoteric substances that form salts with both bases and acids in aqueous solution. Oxytetracycline and chlortetracycline were the first antibiotics of the tetracycline group to be labeled (Chopra & Roberts, 2001). These two were produced by *Streptomyces rimonus* and *Streptomyces aureofaciens*, respectively. They manifest bacteriostatic activity. Their mode of action is characterized by the inhibition of protein synthesis (Roberts, 2005). Protein synthesis is inhibited by

stopping the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) (Chopra & Roberts, 2001).

In 2015, data on the distributions and sales of veterinary drug product revealed that 6,880,365 kg of tetracycline were sold in the USA alone and 71% of them are used in food producing animals (Gokulan, Cerniglia, Thomas, Pineiro, & Khare, 2017). Their frequent use is due to their low cost, availability, low toxicity as well as their broad-spectrum activity. They exhibit activity against gram-negative (*Salmonella, Haemophilus, Pasteurella, Escherichia coli, Bordetella, Pseudomonas, Brucella,* etc.) and gram-positive (*Corynebacterium, Erysipelothrix, Cocci, Actinomycetes,* etc.) bacteria as well as atypical organisms like mycoplasmas, rickettsiae, chlamydiae and protozoan parasites.

In veterinary medicines, oxytetracyclines are extensively utilized to treat respiratory, gastrointestinal and skin bacterial infections (Prescott & Dowling, 2013). They are primarily used in pigs, beef cattle, goats, horses, sheep, dogs, poultry, cats, fishes and rabbits. Their purpose as veterinary medicine is to treat systemic infections, sepsis and infectious diseases of genito-urinary tract and locomotive organs (Prescott & Dowling, 2013). On another hand, oxytetracycline could also be utilized in livestock to fix breathing disorders. In this case, it is administered thru intramuscular injection or as powder.

In plant agriculture, oxytetracycline is found under two formulations: oxytetracycline hydrochloride or oxytetracycline-calcium complex. In the USA, it is registered to be used on peach and nectarine to control *Xanthomonas arbricola* which forms bacterial spots. Note that to alleviate the symptoms of lethal yellow diseases spawned by phytoplasmas, oxytetracyclines are rarely injected directly into the trunk of

elm and palm trees. In the environment, oxytetracycline persists for more than 100 days (van der Marel, 2013).

The oxytetracycline injection used in the experiment contains 300 mg/ml of oxytetracycline base as amphoteric. Regarding withdrawal times; oxytetracycline treatment should be ceased at least 21 days prior to slaughter for cattle and swine, 5 days for poultry whereas for milk it is 6 days.

2. Tylosin

a. Chemical structure and formula

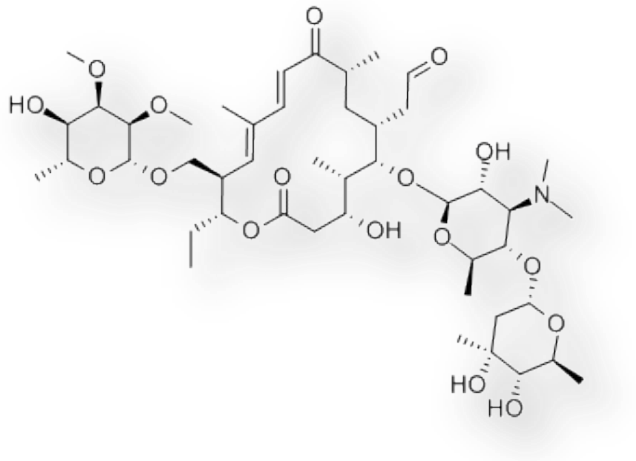


Figure 2. Tylosin chemical structure
Source: (Rabølle & Spliid, 2000)

Molecular formula: $C_{46}H_{77}NO_{17}$
Molecular weight: 916.1 g/mol

b. Overview

Tylosin is naturally obtained as a fermentation produce of *Streptomyces fradiae*. It has a broad spectrum of activity against mycoplasma, gram positive

pathogens and a narrow range on gram negative bacteria. It inhibits protein synthesis by binding to the bacterial ribosome 50S subunit (Kaneko, Dougherty, & Magee, 2007). Tylosin falls under the antibiotic class of macrolides, which have tendency to be unstable in acids and weak bases.

In veterinary medicine, it is used as feed additive as well as to control mastitis, respiratory diseases and dysentery in farm animals (Kaneko et al., 2007). It could be given in the milk replacer, orally to calves, at a dosage of 40 mg/kg body weight and to cattle through intramuscular injections at a dosage of 4 to 10 mg/kg body weight. In pigs, it could be given in the feed at a dosage of 3 to 7 mg/kg of body weight or via the drinking water at a dosage of 25 mg/kg of body weight or thru intramuscular injection at a dosage of 2 to 10 mg/kg for the control and prevention of enzootic pneumonia and swine dysentery. As for poultry, it is provided to them at a dosage of 35 mg/kg body weight through their drinking water. When given as a feed additive to, it could be integrated in pig food at concentrations of 5 to 40 mg/kg of feed (depending on the age). In the environment, it persists for less than 10 days (van der Marel, 2013).

3. Enrofloxacin

a. Chemical structure and formula

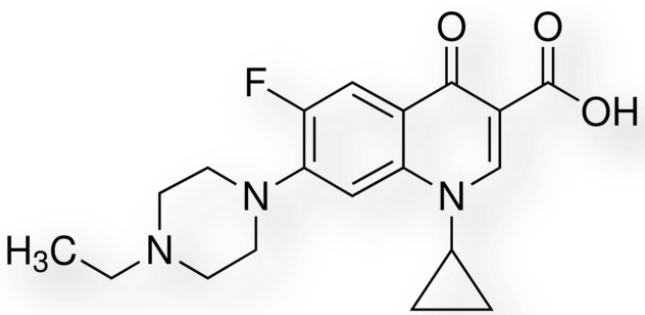


Figure 3. Enrofloxacin chemical structure
Source: (Trouchon & Lefebvre, 2016)

Molecular formula: $C_{19}H_{22}FN_3O_3$
Molecular weight: 359.39 g/mol

b. Overview

Enrofloxacin was first patented in 1984 (Trouchon & Lefebvre, 2016) and it belongs to a subfamily of quinolones: fluoroquinolone. Enrofloxacin is a broad spectrum antimicrobial, efficient on most gram negative and gram positive pathogens, but not anaerobic bacteria. It is utilized to treat systemic and local diseases. It has demonstrated a great effect on several bacterial diseases in poultry, rodents, cattle, and domestic carnivores.

In most species, enrofloxacin is metabolized by de-ethylation into its active primary metabolite: ciprofloxacin, which has antimicrobial effects. Also drug residues of both (enrofloxacin and ciprofloxacin) have been found in animal tissues and muscles (Yan, Tian, & Row, 2008; F. Yu et al., 2014). Additionally, it is stated that

ciprofloxacin appears to be a drug that is more potent than enrofloxacin (Pasquali & Manfreda, 2007). Therefore, the European Medicines Agency (EMA) have defined the enrofloxacin ADI value at 6.2 µg/kg of body weight (Yan et al., 2008).

In the environment, enrofloxacin and ciproflocacin are mainly released by direct discharge of aquaculture products and the defecation of livestock animals' feces and urine (Trouchon & Lefebvre, 2016) and their degradation half-life in the environment is more than 50 days (van der Marel, 2013). This leads to the contamination of surface water, sediment, soil and biota (Van Doorslaer, Dewulf, Van Langenhove, & Demeestere, 2014). The elimination parameters demonstrate a significant difference between different species. It seems to be the highest in pigs, with a half-life of 26 hours. Also, ciprofloxacin clearance for pigs and chickens is five times greater than the clearance of enrofloxacin. In the environments, different processes such as biodegradation, oxidation, and photolysis except hydrolysis cause the degradation of enrofloxacin and ciprofloxacin. Regardless of the latter, enrofloxacin and ciprofloxacin half-lives are very long: between 1,155 and 3,466 days for ciprofloxacin in soil (Trouchon & Lefebvre, 2016).

4. Gentamicin

a. Chemical structure and formula

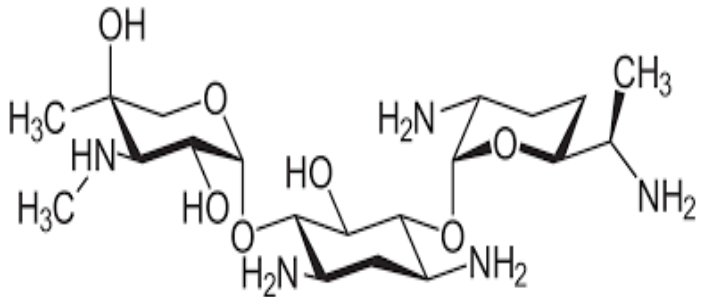


Figure 4. Gentamicin chemical structure
Source: (Yoshizawa et al., 1998)

Molecular formula: $C_{21}H_{43}N_5O_7$
Molecular weight: 477.59542 g/mol

b. Overview

Gentamicin falls under the aminoglycoside class of antibiotics. The fermentation of *Micromonospora purpurea* produces it. Its mode of action relies on inhibiting the bacterial capability to synthesize proteins and multiply by irrevocably binding to 30S ribosomal subunits. It is primarily utilized to treat urogenital, gastrointestinal and respiratory infections (pneumonia, cystitis, endometritis, bronchitis, salmonellosis, infected wounds, etc.) which are caused by organisms that are gentamicin-sensitive.

Gentamicin has a polar organic base and detain bactericidal activities that work against aerobic gram-negative bacteria (Gehring et al., 2005). When administered orally, gentamicin is broadly not absorbed. Nevertheless, when it is injected intramuscularly, it is well absorbed and it is excreted unchanged through the kidneys (S.

Brown & Riviere, 1991). When both types of bacteria are present: gram-positive and gram-negative, aminoglycosides are normally joined with other antibiotics.

In crop agriculture, gentamicin is formulated as gentamicin sulfate and occasionally, it is mixed with oxytetracycline. It is used in some Latin American countries to control several vegetables crops' bacterial diseases initiated by species of *Pseudomonas, Erwinia, Pectobacterium, Xanthomonas* and *Ralstonia* (McManus et al., 2002).

In humans, it is used every 8 hours, intramuscularly in order to provide an overall daily dose of 3 mg/kg of body weight per day (EMEA, 2002). When used as veterinary medicine, gentamicin is sold as a solution to inject in pigs, horses, and cattle and as an oral solution for poultry. In animals, it is administered intravenously, orally or intramuscularly. Note that gentamicin is promptly absorbed and it is defecated via urine unchanged. Undoubtedly, the dose administered varies depending on the animal species. For instance, the 10% solution gentamicin is provided to ruminants at 2 to 4 mg/kg body weight, to pigs at 5 mg/kg body weight and for day old chickens at 0.2 mL/L of distilled water (EMEA, 2002).

The gentamicin injection used in the experiment contains 100 mg/ml. Regarding withdrawal periods: gentamicin treatment in meat should be ceased 7 days after the last application (Tan, Jiang, Huang, & Hu, 2009) whereas for chicken, its withdrawal period is of 5 weeks (ANADA, 2014). Aminoglycoside residues persistence varies as it depends on different factors: formulation utilized, dosage interval, dose provided, as well as the physiological and health status of the animal (Gehring et al., 2005).

Table 10. The physicochemical characteristics of different antibiotics.

Antibiotic	Class	Formal Weight (g/mol)	Charge	Usage[1]	Release to environment
Enrofloxacin	Fluoroquinolone	359.39	Positive (acidic conditions) Negative (basic conditions)[2]	Therapeutic use in cattle	Ciprofloxacin[2]
Gentamicin	Aminoglycoside	477.595	0 (basic conditions) Positive (acidic conditions)[3]	Disease prevention in swine	Gentamicin[4]
Oxytetracycline	Tetracycline	460.434	Positively charged (acidic conditions) Negatively charged (basic conditions)[5]	Therapeutic use, disease prevention, feed efficiency and weight gain in chickens, swine, cattle and sheep Growth promotion in swine, chicken and cattle	Oxytetracycline[6]
Tylosin	Macrolide	916.1	Positive[7] (Polar)	Disease prevention in chicken, cattle and swine Growth promotion, weight gain, and	Tylosin B (desmycosin), Tylosin A-Aldol, Dihydro-desmycosin, Tylosin D (relomycin)[8]

	feed efficiency in swine

Source: (K. Brown et al., 2017) [1], (Ötker & Akmehmet-Balcıoğlu, 2005) [2] , (Miranda-Andrades et al., 2017) [3], (Fraser, 1991; Y. Liu et al., 2017) [4], (Bansal, 2013) [5], (Tian, Khalil, & Bayen, 2017) [6], (Q. Zhang et al., 2016) [7], (Wegst-Uhrich, Navarro, Zimmerman, & Aga, 2014) [8]

CHAPTER 3

MATERIALS AND METHODS

This chapter elaborates on the methods and materials resorted to in this study. It is divided into six different segments. First, soil analysis methods, second, the pot experiment, third, the persistence of antibiotics in soil, fourth, the hydroponic experiment, the fifth is the antibiotic analysis (ELISA procedure) and the sixth is the methods of statistical analysis.

A. Soil pot experiment

To examine the accumulation of antibiotics in crops grown in soil, and based on the procedure adapted by Youssef (2016), the pot preparations were set in the greenhouse area of the American University of Beirut (AUB) on the 17th of October 2016. The pot preparation follows an official experimental scheme, with defined replications, treatments and controls. The soil was provided by a supplier, whereas the antibiotic free manure was left to dry for more than 6 months after collection from the Agriculture Research and Education Center (AREC) in the Beqaa Valley. Noting that for the last 6 months and more, before collection, no antibiotics were administered to these cows. The section below elaborates on the crop description, antibiotics used, concentrations added, the soil mix (experimental scheme, pot preparation and antibiotic treatments) and at last the tissue analysis.

1. Crop description

To study the uptake and accumulation of residual antibiotics in crops from soil, lettuce and cucumber, two vegetables consumed fresh, were chosen to be tested on in this experiment. The lettuce was chosen as a leafy green and the cucumber as a fruit. Cucumbers as well as lettuces were brought as young seedlings from a nursery, in Sidon, Lebanon; it has a coastal Mediterranean climate, thus no stress was exerted on the plants during their transportation to Beirut. The young seedlings were transplanted as one lettuce and one cucumber per pot respectively. They were grown in a greenhouse at AUB and watered as needed. After harvesting lettuces on November 21, 2016 (35 days after transplant) and cucumbers on December 14 and 15 2016 (56 and 57 days after transplant), the antibiotics concentrations were measured through the ELISA assay method in the roots and leaves of both crops and in the fruits for cucumbers.

2. Antibiotics included in the study and their concentrations

Two antibiotics extensively utilized in livestock and poultry in Lebanon were used in this experiment. The antibiotics are gentamicin and enrofloxacin (Choueiri, 2008). They were purchased from a veterinarian store in Al-Bekaa, Lebanon. The chemical properties of each are illustrated in Table 11.

Each antibiotic was tested at four different levels (0, 5, 10 and 20 mg/kg) and every treatment was replicated three times. In both growing media (soil with 5% manure and soil without manure), enrofloxacin was tested on both cucumbers and lettuces whereas gentamicin was only tested on cucumbers, because it was experimented on lettuce earlier in another study.

Table 11. Chemical properties of the four different antibiotics used

Antibiotic	Family	Molecular formula	Molecular mass (g/mole)	Formal charge
Oxytetracycline	Polyketide	$C_{22}H_{24}N_2O_9$	460.434	+ve at pH<7 -ve at pH>7[1]
Gentamicin	Aminoglycoside	$C_{21}H_{43}N_5O_7$	477.596	0 at pH>7 +ve at pH<7[2]
Enrofloxacin	Fluoroquinolone	$C_{19}H_{22}FN_3O_3$	359.401	+ve at pH<7 -ve at pH>7[3]
Tylosin	Macrolide	$C_{46}H_{77}NO_{17}$	916.10	+ve[4]

Source: (Bansal, 2013)[1] (Miranda-Andrades et al., 2017)[2] (Ötker & Akmehmet-Balcıoğlu, 2005)[3] (Q. Zhang et al., 2016)[4]

3. Soil mix

This section elaborates on the experimental scheme, the method used in pot preparation and the different antibiotic treatments.

a. Experimental scheme

The design used in this experiment was 2 x 4 factorial arrangement of treatments with interaction in a complete randomized design with defined replicates. The factors are: two soil media (soil without manure and soil with 5% manure) and 4 levels of antibiotics (0, 5, 10 and 20 mg/kg)

Crops used: 2 crops (lettuce and cucumber)

Antibiotics used: 2 antibiotics (gentamicin and enrofloxacin)

Replicates: 3 replicates per treatment (3 pots)

The total number of pots in this experiment is: Total number of pots for enrofloxacin + Total number of pots for gentamicin = 48 + 24 = 72 pots

b. Pot preparation

- Two growing media:
    i. Soil without manure: after sieving the soil in a 10 mm sieve, 5.25 kg were added into the pots
    ii. Soil with 5% manure: 5 kg of soil and 0.25 kg of manure were mixed together and added into the pot
- Total number of pots for enrofloxacin:

  2 crops x 4 levels x 2 growing media x 3 replicates x 1 antibiotic = 48 pots
- Total number of pots for gentamicin:

  1 crop x 4 levels x 2 growing media x 3 replicates x 1 antibiotic = 24 pots
- The required amount of antibiotics was diluted with 100 mL of distilled water
- Fertilizer rate added: 0.5g / 5 kg soil (20-20-20 + TE)
- Both soil mixtures were placed each in plastic bags and then the bags were placed in the pots to prevent leaching (no air spaces were done and water was added right enough)
- The pots were labeled clearly (antibiotics used, concentration level, pot number and date)

c. Antibiotic treatments

Cucumbers were separately administered with gentamicin and enrofloxacin whereas lettuces were administered with enrofloxacin only. Four different levels were applied: 0, 5, 10 and 20 mg/kg. The crops were planted in soil with manure and soil without manure (two different soil growing media) and three replications were done.

Therefore, 48 pots were prepared for enrofloxacin and 24 pots for gentamicin for every soil mix treatment.

4. Tissue analysis (extraction of antibiotics)

After harvesting the crops, each treatment with its respective level was placed into a labeled paper bag (marked with the crop, treatment, level and antibiotic). Tissue extraction took place directly after harvest. The extractants were stored in the fridge at 4 °C for analysis. The procedure adopted for all treatments is mentioned below:

- Weigh each whole crop alone (roots, leaves and fruits)
- Separate leaves, roots and fruits from each other and weigh each alone
- Wash the plant tissue and make sure that no soil remains on any part
- Blot the crop using clean paper towels
- Using a knife, finely chop the roots, leaves and fruits (plant materials)
- 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) – (2 g roots, 6 g fruits, 6 g leaves)
- Add their respective extractants (80% methanol, PBS and Mcllvain for enrofloxacin, tylosin and oxytetracycline respectively) at the right volume, following the ratio 1:3 (1 g plant tissue to 3 mL solvent)
- 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 trivial 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

As recommended by the manufacturer of the ELISA kits (ABRAXIS), distilled water and 80% methanol were used to respectively extract the available gentamicin and enrofloxacin in roots, leaves and fruits. A ratio of 1:3 (1 g plant tissue to 3 mL solvent) was taken.

B. Hydroponic experiment

The hydroponic experiment was performed using white plastic containers (length: 35 cm, width: 26 cm, height: 13 cm) brought and filled with 6 liters, Hoagland nutrient solution (Table 12) and spiked with antibiotics at 0, 5 and 10 mg/kg rates. The preparation of the plastic containers was done and set in the greenhouse area of AUB, on the 13th of October 2017. Aeration materials such as air perforated tubings, pumps, and tubes were brought from shopper's supermarket, Raouché branch, Lebanon. The container preparation follows an official experimental scheme, with defined replications, treatments and controls. The section below elaborates on the hydroponic system (system arrangement and structure, container preparation), crop description, antibiotics resorted to and concentrations added, planting (experimental scheme, planting scheme and antibiotic treatment), and at last tissue analysis.

1. Hydroponic system

a. System arrangement and structure

The system was placed in a greenhouse where the temperature was maintained at 24 °C. White containers (length: 35 cm, width: 26 cm, height: 13 cm) were used as the base for planting and perforated aeration tubings. The system and structure adopted to build the experimental setting are pictured in Figure 6 and it is as follows:

- Clean the white plastic containers (the containers are of white color to reduce heat absorption)
- Place each 3 containers one in front of the other (9 columns of containers, 3 containers each) (Figure 5)
- Add perforated aeration tubing into each container (one perforated aeration tubing per container – it helps to aerate the solution, hence the root system)
- Plug the perforated aeration tubing into tubes and the tubes into pump openings (one container needs one pump opening)
- Make sure that each two columns have an electricity plug near them
- Plug the pumps into the electricity
- Fill the containers with 6 liters of distilled water and add the right volume of the Hoagland nutrient solution (Table 12) to each container (Figure 6)
- Adjust a 25-cell seedling tray (length: 53 cm, width: 28 cm) to the container size and place it onto each container (it should cover the whole surface of the container) (Figure 7)
- Turn the electricity on and make sure that all perforated aeration tubings are working
- Turn the electricity off

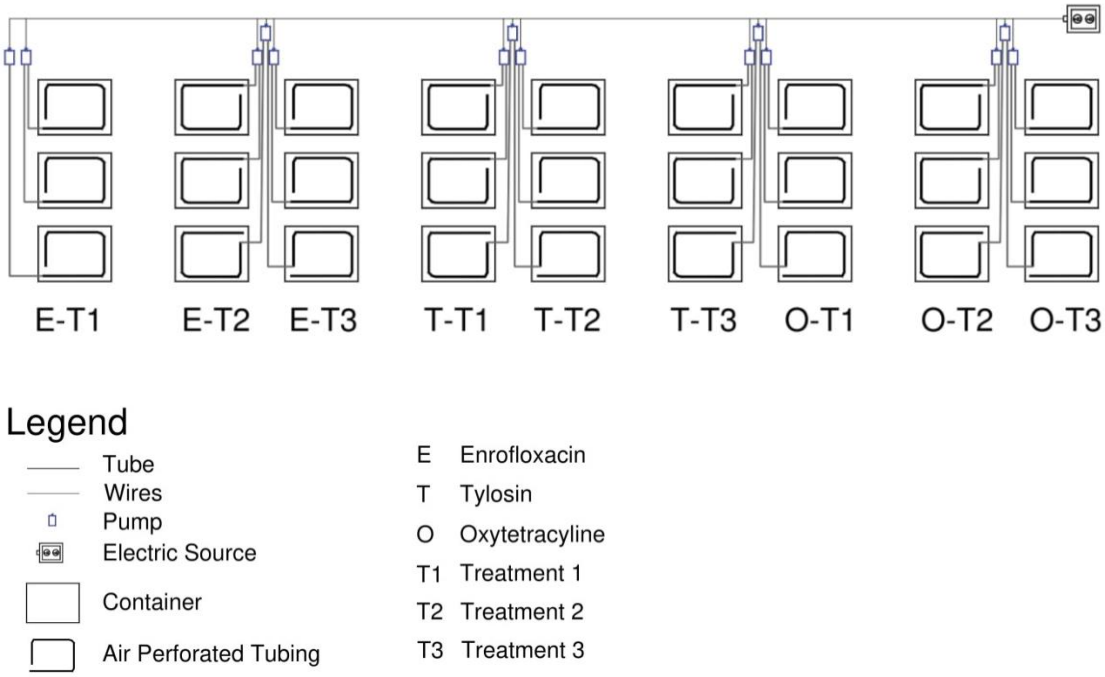


Figure 5. Hydroponic system scheme

Figure 6. Container setting of the hydroponic system (after adding water, antibiotic and Hoagland solution)

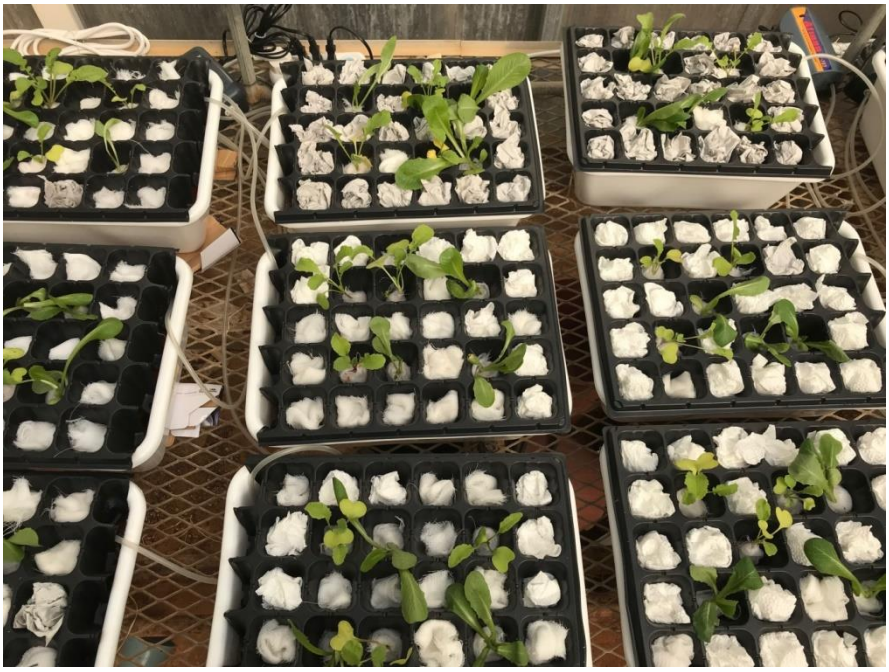


Figure 7. All tylosin treatments one day after transplanting

b. Container preparation

- Growing media:
  - Hoagland nutrient solution (Table 12) + antibiotic
- Total number of containers per antibiotic:

  1 crops x 3 levels x 1 growing medium x 3 replicates x 1 antibiotic = 9 containers
- The containers were labeled clearly (antibiotics used, concentration level, treatment and container number)

Table 12. Modified Hoagland nutrient solution added to the hydroponic experiment treatments

Macronutrients					
Compound	**Concentration of stock solution (M)**	**Concentration of stock solution (g/L)**	**Element**	**Volume of stock solution (mL/L)**	**Volume of stock solution (mL/6L)**
KNO_3	1.00	101.10	N	6.0	36.0
$Ca(NO_3)_2.4H_2O$	1.00	236.16	K	4.0	24.0
$NH_4H_2PO_4$	1.00	115.08	Ca	2.0	12.0
$MgSO_4.7H_2O$	0.50	123.245	P, S, Mg	2.0	12.0
Micronutrients					
Compound	**Concentration of stock solution (mM)**	**Concentration of stock solution (g/L)**	**Element**	**Volume of stock solution (mL/L)**	**Volume of stock solution (mL/6L)**
KCl	50	3.728	Cl	1.0	6.0
H_3BO_3	25	1.546	B		
$MnSO_4.H_2O$	2.0	0.338	Mn		
$ZnSO_4.7H_2O$	2.0	0.575	Zn		
$CuSO_4.5H_2O$	0.5	0.125	Cu		
$(NH_4)_6Mo_7O_{24}.4H_2O$ (57.71% Mo)	0.5	0.081	Mo		
Fe-EDDHSA	40	13.844	Fe	2.0	12.0

Source: (Epstein, 1972)

2. Crop description

To study the effect of residual antibiotics on plant growth as well as their uptake and accumulation site in plants grown in water, two crops were grown hydroponically: lettuce and radish. As mentioned earlier, the lettuce was chosen as a leafy green, whereas the radish was chosen as a root. The lettuces were brought as young seedlings from the nursery in Sidon (coastal Mediterranean climate like Beirut, thus no stress on the plants), whereas the radishes seedlings were grown at the greenhouse area at AUB. On October 13, 2017, radishes and lettuces were transplanted

into the filled containers, Hoagland nutrient solution (Table 12) and spiked with antibiotics at 0, 5 and 10 mg/kg. Each container received 3 seedlings of each crop (total of 6 crops in each container: 3 lettuces and 3 radishes), and the water level in each container was kept at 6 liters. The air conditioning was maintained at a temperature of 24°C. After harvesting on November 11, 12 and 13 2017, the extraction of antibiotics was done directly after harvest from the roots and leaves of both crops, and in the radish itself when permissible (grown enough).

3. Antibiotics included in the study and their concentrations

In the hydroponic experiment, enrofloxacin, tylosin and oxytetracycline, were used. The chemical properties of each are illustrated in Table 11. Each antibiotic was tested at three different levels (0, 5 and 10 mg/kg) and every treatment was replicated three times.

4. Planting

a. Experimental scheme

The design used in this experiment was a complete randomized design with 3 treatments and 3 replicates per treatment

Crops used: 2 crops (lettuce and radish)

Antibiotics used: 3 antibiotics (enrofloxacin, oxytetracycline and tylosin)

Growing media: hydroponic system

Antibiotic concentrations used: 3 levels (0, 5, and 10 mg/kg)

Replicates: 3 replicates per treatment

The total number of containers per antibiotic in this experiment is: 1 crop x 1 growing medium x 3 antibiotics x 3 levels x 3 replicates = 27 containers

NB: the number of crops is counted as 1 in the equation above as both crops are placed in the same container for each level

b. Planting scheme

After the lettuce and radish crops have become young seedlings ready to be transplanted, their roots were thoroughly washed with water to remove any peat moss or soil particles and then they were moved into their respective containers. The procedure followed for each treatment is explained below:

- Check if the electricity is off
- Cut 7 openings into the 25-cell seedling tray (one for each crop: 3 lettuces and 3 radishes per tray and an additional one for observation of the solution inside the container)
- Wash the crops roots with water thoroughly to remove any peat moss or soil particles (it is essential for roots to be very clean in order not to contaminate the solution)
- Wrap the space between the roots and the stem with a piece of cheese cloth (it will help to protect the roots from breakage or cuts)
- Place each physiologically similar crops into the same container (3 lettuces and 3 radishes per container)
- Wrap a piece of cotton around the seedlings to keep it standing straight

- Close the periphery of the containers with masking tape and the cell openings with cotton or tissue paper to prevent sunlight from entering and promoting algal growth as well as it helps to reduce evapotranspiration (Figure 7)
- Turn the electricity on and keep aeration going at a slow rate until the end of the experiment
- Maintain the containers water level to 6 liters by adding distilled water when needed

c. Antibiotic treatments

Both radishes and lettuces were administered with the three antibiotics: enrofloxacin, tylosin and oxytetracycline. The crops were transplanted as young seedlings into the solution filled containers. Three different levels were tested: 0, 5 and 10 mg/kg and were replicated three times. Therefore, 9 containers were prepared for each antibiotic (three containers per treatment).

5. Tissue analysis

After harvesting the crops, each treatment with its respective level was placed into a labeled paper bag (marked with the crop, treatment, level, antibiotic). Tissue extraction took place directly after harvest. The extractant was stored in the fridge at 4 °C for analysis. The followed procedure in all treatments is mentioned below:

- Weigh each whole crop alone (roots, leaves and bulbs)
- Separate leaves, roots and bulbs from each other and weigh each alone
- Wash the crop completely
- Blot the crop using clean paper towels

- Using a knife, finely chop the roots, leaves and bulbs (plant materials)
- Weigh some of the chopped material (representative sample) and place them in an extraction bag “universal” (number the bag indicating the plant material and antibiotic) – (2 g roots, 2 g bulbs, 6 g leaves)
- Add their respective extractant in the bag at the right volume, following the ratio 1:5 (1 g plant tissue to 5 mL solvent so the sample is diluted 6 times)
- Grind the fresh mixture directly using an ELISA grinder until all the plant material is fully cut and become a suspension (uniform grinding and mixing are trivial to get representative samples and accurate analytical results)
- Place the suspension in clean 50 mL falcon tube and close it (number the bag indicating the plant material, antibiotic, treatment and concentration)
- 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 (number the tube indicating the plant material, antibiotic, treatment and concentration).

As recommended by the manufacturer of the ELISA kits (ABRAXIS), 80% methanol, PBS buffer and Mcllvain buffer were used respectively for enrofloxacin, tylosin and oxytetracycline in roots, leaves and bulbs. A ratio of 1:5 (1 g plant tissue to 5 mL solvent) was taken.

C. Antibiotics persistence in soil

The pot preparation was set in the greenhouse area of AUB on the 20th of November 2017 and went on for two months until the 16th of January 2018. The pot preparation follows an official experimental scheme, with defined replications, treatments and controls. The same soil used in the previous study was used here and the antibiotics were brought from a veterinary store. The section below elaborates on the antibiotics included, their added concentrations, and soil mix (experimental scheme, pot preparation and antibiotics treatments).

1. Antibiotics included in the study and their concentrations

As enrofloxacin, tylosin and oxytetracycline are three extensively used antibiotics in animal fattening of livestock and poultry in Lebanon (Choueiri, 2008), thus it is important to know how long they persist in soil and the following experiment was conducted.

2. Soil mix

a. Experimental scheme

Antibiotics used: 3 antibiotics (enrofloxacin, tylosin and oxytetracycline)

Growing media: 1 growing medium (soil alone)

Antibiotic concentrations used: 2 levels (0 and 5 mg/kg)

Replicates: 3 replicates per treatment

b. Pot preparation

- One growing media:
    - Soil without manure: after sieving the soil in a 10-mm sieve, 5 kg were added into 5 kg capacity pots
    - The soil was kept moist, hence watered regularly by maintaining the weight to 5.4 kg
- Total number of pots per antibiotic:

  1 level x 1 growing medium x 3 replicates x 1 antibiotic + 1 control level = 4 pots

NB: the level is counted as 1 rather than 2 in the equation above as the control of every antibiotic was not replicated (Figure 8).

- The required amount of antibiotics was diluted with 100 mL of water
- The soil mixtures were placed in plastic bags and then the bags were placed in the pots to prevent leaching
- The pots were labeled clearly (antibiotics used, concentration level, pot number and date)

c. Antibiotics treatments

Enrofloxacin, tylosin and oxytetracycline were added each to their respective pots at a concentration of 5 mg/kg. Each pot contained 5 kg of soil so 25 mg of antibiotic per pot. Two levels were tested: the control (0 mg/kg) and 5 mg/kg. In addition to the control (not replicated per treatment), three replications were done for each antibiotic; therefore 4 pots were prepared for each antibiotic (total of 12 pots) (Figure 8).

Figure 8. Soil Persistence experimental design setting

d. Soil Sampling

Soil samples were collected consecutively from day one, week one until week 8. The collected soil samples were extracted with water at a ratio of 1:5 (1g soil : 5 mL water). The extractants were kept in clean and closed falcon tubes at a temperature of 4 °C for analysis.

3. Antibiotic extraction from soil

To know an estimate of the persistence of enrofloxacin, tylosin and oxytetracycline, the latter were added each to their respective pots at a concentration of 5 mg/kg (25 mg/pot) and soil samples were taken one day after setting the experiment and then every additional week for two months. The pot preparation took place on November 20, 2017; the first soil sampling was taken one day later on November 21, 2017 and then every additional week until January 16, 2018 a soil sample was taken. Extraction is done directly after collecting the soil samples. The weekly procedure followed for the extraction of the antibiotics from the soil is thoroughly described below:

- Take a soil sample of ~50 g from each pot and place it in a 50 mL falcon tube (number the tubes indicating the antibiotic and pot number)
- Take 5 g of the initial soil sample and place it in a new falcon tube (number the tube with the antibiotic and pot number)
- Add water at a ratio of 1:5 (1 g of soil to 5 mL of water so the sample is diluted 6 times)
- Shake the tubes for 45 minutes at 500 RPM on a shaker (Uniform mixing is trivial to get a representative sample and accurate analytical results)
- Filter each mixture into its respective new 50 mL falcon tube using (F40 Whatman) filter papers (number the tube with the date of extraction, antibiotic and pot number)
- Wait for all the mixture to filtrate fully
- Close the tubes and place them in the refrigerator at a temperature of 4°C

D. Methods of analysis

1. Soil

This study is a continuation for a previous MSc. thesis "Uptake of gentamicin, tylosin and oxytetracycline by lettuce and radish plants" by Youssef (2016) and the reported soil analysis results are directly taken from the same thesis. The methods followed for the physical and chemical properties of the soil are displayed in Table 13.

Table 13. Methods followed to determine the physical and chemical properties of the soil

Soil Property	Method Or Instrument used
Soil moisture content	Gravimetric method
Soil texture	Bouyoucous hydrometer method
Soil pH	pH meter (soil:water; 2:1 ratio)
Soil salinity (Electrical Conductivity)	Electrical conductivity meter (saturated paste)
Available phosphorus	Olsen modified methods
Available sodium and potassium (1N NH_4OAC solution)	K^+ and Na^+ by Flame photometer (BWB Technologies, XP 2011)
DTPA – Extractable micronutrient (Fe, Zn, Cu+Mn)	DTPA (diethylenetriaminepentaacetic acid) extraction method
Total free calcium carbonate	Calcimeter method

Source: Information gathered from (Youssef, 2016)

2. Tissue analysis (extraction of antibiotics)

After harvesting the crops, each treatment with its respective level was placed into a labeled paper bag (marked with the crop, treatment, level and antibiotic). Tissue extraction took place directly after harvest. The extractants were stored in the fridge at 4 °C for analysis. The procedure adopted for all treatments is mentioned below:

- Weigh each whole crop alone (roots, leaves and fruits)
- Separate leaves, roots and fruits from each other and weigh each alone
- Wash the crop completely
- Blot the crop using clean paper towels
- Using a knife, finely chop the roots, leaves and fruits (plant materials)
- 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) – (2 g roots, 2g bulbs, 6 g cucumbers, 6 g leaves)

- Add their respective extractants (distilled water, 80% methanol, PBS and Mcllvain for gentamicin, enrofloxacin, tylosin and oxytetracycline respectively) at the right volume, following the ratios:
    - In soil: 1 g plant tissue to 3 mL solvent (sample diluted 4 times)
    - In nutrient solution: 1 g plant tissue to 5 mL solvent (sample diluted 6 times)
- 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 trivial 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 (number the tube indicating the plant material, antibiotic, treatment and concentration)

As recommended by the manufacturer of the ELISA kits (ABRAXIS), distilled water, 80% methanol, PBS buffer and Mcllvain buffer were used to respectively extract the available gentamicin, enrofloxacin, tylosin and oxytetracycline from roots, leaves and fruits. Different ratios were taken in crops grown in soil and in nutrient solution:

- In soil for gentamicin and enrofloxacin: 1 g plant tissue to 3 mL solveny
- In nutrient solution for enrofloxacin, tylosin and oxytetracycline: 1 g plant tissue to 5 mL solvent

3. Statistical analysis

Data in all trials were pooled and analyzed using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS) version 9, year 2008 . Means were compared with Student-Newman-Keuls Test (SNK) where applicable.

In the pot experiment, a 2 x 4 factorial arrangement of treatments with interaction in a complete randomized design was used to test the effect of soil manure (0 and 5%) and antibiotic concentration (0, 5, 10 and 20 mg/kg) and their interaction on the concentration of these antibiotics in roots, leaves and fruits of lettuces and cucumbers during a 5 weeks period.

The design of the hydroponic trial was a complete randomized design with 3 treatments consisting of antibiotic concentration in water (0, 5 and 10 mg/kg). Each treatment was replicated three times (three containers) where three plants of lettuces and radishes were transplanted into the same container. To study the effect of antibiotic concentration in leaves, roots of lettuces in addition to the bulb in radishes, three antibiotics were used, namely: enrofloxacin, oxytetracycline and tylosin. Also their effect on plant weights (total, roots, leaves and bulbs) were recorded.

E. Antibiotic analysis (ELISA procedure)

The ELISA assay procedure was applied to identify and detect the presence of antibiotics in samples. It is renowned for its sensitivity, convenience to reliably quantify concentration availability of antibiotics, safety, economical and not time consuming (Dolliver et al., 2007; Nagel, Manners, & Birch, 1992). The test principle for gentamicin, tylosin, enrofloxacin and oxytetracycline will be stated, followed by the

reagents and standards used, and last the ELISA assay preparation and procedure for the measurement of antibiotic concentration.

1. Test principle for gentamicin, tylosin, enrofloxacin and oxytetracycline

Based on the antibiotics ELISA kits: enrofloxacin (product no. 522511), tetracycline (product no. 52254BA), gentamicin (product no. 511GEN1A) and tylosin (product no. 52256B) brought from ABRAXIS, the test principle they follow is cited below.

"This test is a direct competitive ELISA based on the recognition of antibiotic by specific antibodies. Antibiotic once present in a sample and an antibiotic-enzyme conjugate compete for the binding sites of anti-antibiotic antibodies which are immobilized on the wells of the microtiter plate. After washing and addition of the substrate solution, a color signal is produced. The intensity of the color is inversely proportional to the concentration of the used antibiotic present in the sample. The color is later stopped and evaluated using ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run." The list of standards used for each antibiotic is mentioned in Table 15.

2. Reagents and standards used

a. Reagents prepared

As mentioned by the manufacturers, the Mcllvain buffer was prepared for the oxytetracycline ELISA kit, PBS was prepared for the tylosin ELISA kit and 80% methanol was prepared for the enrofloxacin ELISA kit. Distilled water was used as the extractant for the gentamicin ELISA kit. The reagents were prepared at the lab and they

were stored in the fridge at 4°C. Prior to usage they were removed one to two hours and brought up to room temperature (20 to 25°C).

b. Reagents provided

The reagents provided by the ELISA kits manufacturers and the ones used are mentioned in Table 14. When received, they were kept in the refrigerator at a temperature of 4°C.

Table 14. Reagents provided by the manufacturer (ABRAXIS) of the ELISA kits for enrofloxacin, gentamicin, oxytetracycline and tylosin

Enrofloxacin	**Gentamicin**	**Oxytetracycline**	**Tylosin**
Standards (7)	Standards (6)	Standards (7)	Standards (6)
-	Assay buffer, 6 mL	Assay buffer, 6 mL	-
Sample diluent, 25 mL, used to dilute samples	Sample diluent (10X) concentrate, 25 mL (must be diluted before use)	Sample diluent (10X) concentrate, 2 bottles (must be diluted before use)	Sample diluent (10X) concentrate, used to dilute the sample
Enrofloxacin-HRP conjugate, 6 mL	Gentamicin-HRP conjugate solution, 12 mL	Tetracycline-HRP conjugate, 2 vials (lyophilized)	Tylosin HRP Enzyme conjugate, 7 mL
Rabbit Anti-Enrofloxacin antibody solution, 6 mL	-	-	Anti-tylosin antibody, 7 mL
-	-	Conjugate diluent, 2 bottles, 12 mL each	-
Wash solution (5X) concentrate, 100 mL	Wash solution (5X) concentrate, 100 mL	Wash solution (5X) concentration, 100 mL	Wash solution (5X) concentration, 100 mL, need to be further diluted
Color (substrate) solution (TMB), 12 mL	Color (substrate) solution (TMB), 12 mL	Color (substrate) solution (TMB), 16 mL	Substrate solution, 14 mL
Stop solution, 12 mL	Stop solution, 12 mL	Stop solution, 12 mL	Stop solution, 14 mL

c. Standards provided

As soon as the ELISA kits were received, they were kept in the refrigerator at 4°C. Prior to using them, they were removed one to two hours before and brought up to room temperature (20 to 25°C). The standards provided by each manufacturer are stated in Table 15.

Table 15. Antibiotics standard concentrations provided by the manufacturer (ABRAXIS) of the enrofloxacin, gentamicin, tylosin and oxytetracycline ELISA kits

Concentrations of antibiotics (ng/mL)			
Enrofloxacin	**Gentamicin**	**Oxytetracycline**	**Tylosin**
0	0	0	0
0.025	0.25	0.10	0.05
0.05	0.50	0.20	0.1
0.125	1.0	0.30	0.50
0.25	2.5	0.40	1.0
0.5	5.0	0.60	5.0
1.0	-	0.80	-

3. ELISA assay preparation and procedure for the measurement of antibiotic concentrations

Every ELISA kit contained the ELISA assay preparation and procedure specific to it. The chronological steps to complete the ELISA assay is almost the same for all four antibiotics; however, a couple of steps differ. The latter is mainly in the incubation period, volume of reagents used and dispensed into the plate wells and number of standards. The main steps of the preparation and the procedure are summarized in the sequence below:

Test preparation:

- Dilute the wash buffer concentrate at a ratio of 1:4 (1 mL wash buffer to 4 mL deionized or distilled water).

- Dilute the initial tissue sample extracts at a ratio of:
    - 1 mL sample to 1 mL sample diluent for gentamicin (diluted two times)
    - 1 mL sample to 2 mL sample diluent for enrofloxacin in pot experiment (diluted three times)
    - 1 mL sample to 4 mL sample diluent for enrofloxacin in hydroponic experiment (diluted 5 times)
    - 1 mL sample to 9 mL sample diluent for tylosin (diluted 10 times)
    - 1 mL sample to 9 mL sample diluent for oxytetracycline (diluted 10 times)

Test procedure:

- Add (25 μL of gentamicin and 50 μL oxytetracycline) of assay buffer solution to the individual wells successively. This step is excluded in the enrofloxacin and tylosin assay procedures.
- Add (25 μL of gentamicin, 50 μL of enrofloxacin, 100 μL of oxytetracycline and 50 μL of tylosin) of the standard solutions and plant tissue sample extract (1 g sample + 5 mL solvent = 6x) into the wells. If possible, duplicate the samples.
- Add (100 μL of gentamicin, 50 μL of enrofloxacin, 50 μL of oxytetracycline and 50 μL of tylosin) enzyme conjugate solution to the individual wells successively using a multichannel pipette
- Add 50 μL of antibody solution into each test well successively using a multichannel pipette (only for enrofloxacin and tylosin)

- Cover the wells with parafilm and mix the content by moving the strip holder in a circular motion on the bench for 30 seconds
- Incubate the strips for (30 minutes for gentamicin and 60 minutes for oxytetracycline, enrofloxacin and tylosin) at room temperature
- After incubation, remove the covering and briskly dispose of the contents of these wells into sink
- Wash the strips three times using 1X washing buffer solution. In each washing step, use 250 μL of washing buffer in each well
- Add (100 μL gentamicin, 100 μL enrofloxacin, 100 μL tylosin and 150 μL oxytetracycline) substrate color solution to the wells. Cover the plate, shake it and incubate for 20 to 30 minutes
- Add 100 μL of stop solution to the gentamicin, enrofloxacin, oxytetracycline and tylosin plates separately
- Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stop solution

CHAPTER 4
RESULTS AND DISCUSSION

This chapter elaborates on the overall results and analysis obtained from this study and it is divided into five major parts. As this study is a continuation of a previous MSc thesis, the first part will illustrate the soil analysis results previously obtained (Youssef, 2016). The second part will show the results of enrofloxacin and gentamicin uptake and accumulation by lettuce and cucumber administered with four different antibiotic concentrations (0, 5, 10 and 20 mg/kg) grown in two growth media (soil without manure and soil with 5% manure). The third part will show the results of the enrofloxacin, tylosin and oxytetracycline uptake and accumulation in lettuce and radish crops administered with three different antibiotic concentrations (0, 5 and 10 mg/kg) grown hydroponically and the fourth part will illustrate the effect of these antibiotics on plant growth of lettuce and radish crops. The fifth part will demonstrate the persistence of enrofloxacin, tylosin and oxytetracycline in soil administered with 5 mg/kg (25 mg/5 kg pot) of each antibiotic separately.

A. Soil type

Referring to the conventional international procedures, the physical and chemical properties of the soil free from antibiotics was analyzed. The same soil was used in a previous thesis and was analyzed earlier (Table 16**).** The soil analysis results indicate that the soil is sandy loam composed of 75% sand, 20% Clay and 5% silt. It is non saline, slightly alkaline and highly calcareous. It holds a dark red color on dry basis (dry 7.5R 3/6) and dusky red on wet basis (wet 10R 3/4). This demonstrates that the soil

is principally under aerobic condition and belongs to the soil order of Aridosol. The available nutrient levels in the soil were low in phosphorous, iron, zinc and manganese and medium in potassium and copper. Consequently, in the pot experiment, to provide the lettuces and cucumbers with a sufficient amount of nutrients to grow, 0.5g of (20-20-20 + TE) fertilizer per pot (5 kg of soil) were added to all treatments.

Table 16. Soil sample physical and chemical characteristics

Characteristic	Value
Soil Texture	Sandy Loam
Sand %	75
Silt %	5
Clay %	20
pH (1:2)	7.47
EC (1:2)	0.34 dS/m
Free $CaCO_3$	50 %
$NaHCO_3$-P	6.83 mg/kg
NH_4OAC-Na	165 mg/kg
NH_4OAC-K	250 mg/kg
DTPA-Fe	0.764 mg/kg
DTPA-Zn	0.248 mg/kg
DTPA-Cu	0.38 mg/kg
DTPA-Mn	0.028 mg/kg

Source: (Youssef, 2016)

B. Antibiotic uptake by cucumber and lettuce grown in soil

The section below describes the antibiotic uptake and accumulation by lettuce and cucumber; it is divided into three parts for each crop. The two first parts elaborate on the uptake and accumulation of enrofloxacin and gentamicin in the respective parts of lettuce (roots and leaves) and cucumber (roots, leaves and fruits) whereas the third part provides a comparison between the two antibiotics in lettuce and cucumber parts. In the statistical analysis, to observe the effect of manure on the uptake of antibiotics and their accumulation sites, the average of all four antibiotic levels (0, 5, 10 and 20

mg/kg) were taken for each manure level (0 and 5%) in roots and leaves for lettuce and in roots, leaves and fruits for cucumber. Also, to discern the uptake and accumulation site of the antibiotic in the crop parts, the average of the soil with 5% manure and soil without manure were averaged and assigned to each antibiotic level treatment (0, 5, 10 and 20 mg/kg).

1. Antibiotic uptake by lettuce

In part of the soil pot experiment, the uptake and accumulation of enrofloxacin in lettuces at four different concentrations (0, 5, 10 and 20 mg/kg) and in two different growth media (soil without manure and soil mixed with 5% manure) was tested. An earlier study had proved that gentamicin was absorbed and accumulated by lettuces grown in manured and non manured soil, thus the experiment was not performed again (Youssef, 2016). The results of the statistical analysis were plotted on bar graphs where means with different superscripts are significantly different and means with same superscripts are not significantly different.

a. Enrofloxacin accumulation in lettuce leaves and roots

The concentrations of enrofloxacin in lettuce roots and leaves are reported in Figure 9, whereas the concentration of enrofloxacin in lettuce roots and leaves with respect to the presence or absence of cow manure is represented in Figure 10. Both are tabulated in Table 18 of the appendix.

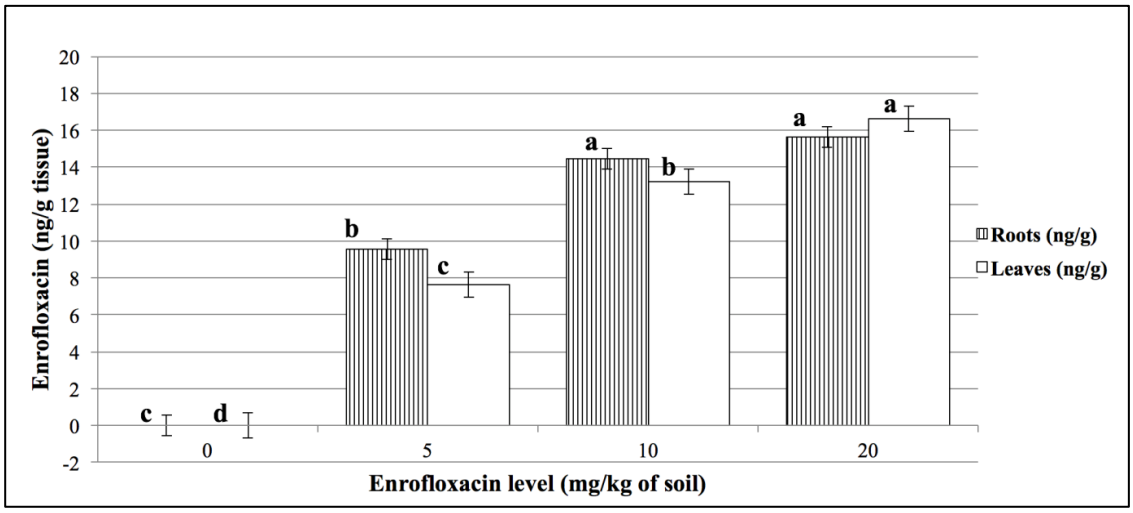


Figure 9. Concentration of enrofloxacin in lettuce roots and leaves

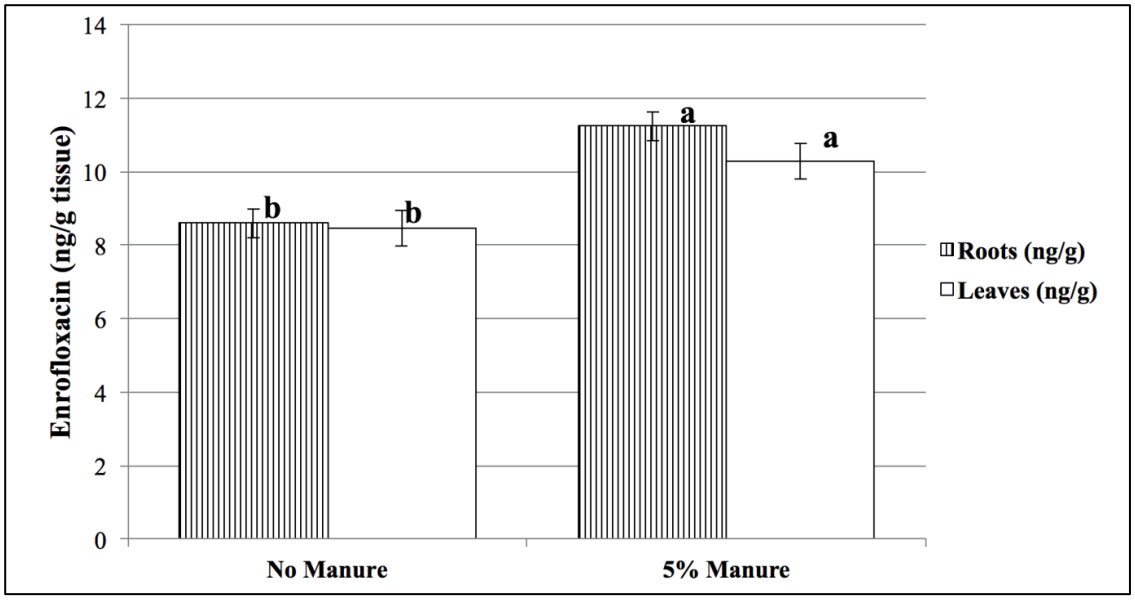


Figure 10. Concentration of enrofloxacin in lettuce roots and leaves planted in two growth media

Based on the obtained results in Figure 9 (Table 18 in appendix), there is a significant difference between the control and the three different concentration levels (5, 10 and 20 mg/kg) as well as between the different antibiotic levels. Consequently, irrespective of the enrofloxacin level, the lettuce leaves and roots absorb and accumulate enrofloxacin in its presence. This result agrees with the findings of

Lillenberg et al. (2010) who stated that barley, lettuce and cucumber administered with 50, 200 and 500 µg/g of enrofloxacin all accumulated the antibiotic in them. Also, it is observed that the higher the level of enrofloxacin in soil, the higher its concentration in tissue. This is in accordance with Chander, Kumar, Goyal, and Gupta (2005) who pointed out that the higher the antibiotic level in the media, the higher its concentration in plant tissue.

At 5mg/kg and 10 mg/kg (Figure 9), the roots and leaves accumulated more enrofloxacin as the concentration at 10 mg/kg was significantly higher than that at 5 mg/kg. Between the 10 and 20 mg/kg levels, there was no significant difference between the accumulation of enrofloxacin in roots. There was a significant increase between 5, 10 and 20 mg/kg; hence, a higher concentration of enrofloxacin leads to a greater accumulation in lettuce leaves, but not in lettuce roots. This also demonstrates that roots accumulated enrofloxacin to a certain limit. Lillenberg et al. (2010) studied the mobility of ciprofloxacin and enrofloxacin from soil to plant and concluded that fluoroquinolones reach the plant and accumulate there while maintaining its antimicrobial activity; he also stated that when the vegetation period is longer, antibiotics accumulate in the plant more. In his experiment, ciprofloxacin was added to the soil at a concentration of 10 µg/g and the ciprofloxacin content of the lettuce was 44 µg/g (Lillenberg et al., 2010).

In figure 10 (Table 18 in appendix), it is observed that there is a significant difference between the concentration of antibiotic in the roots and leaves grown in two media: soil with no manure and the one amended with 5% manure. The roots and leaves grown in presence of manure accumulated a greater amount of enrofloxacin than the ones grown in the absence of manure. This indicates that the presence or the addition of

manure in soil increases the absorption and accumulation of enrofloxacin by the plant. Accordingly, Youssef and Bashour (2017) verified that the addition of manure increased the absorption of gentamicin, tylosin and oxytetracycline by lettuce and radish; the concentration of gentamicin in lettuce roots increased from 4.41 ng/g in absence of manure to 16.4 ng/g with 5% manure.

Additionally, to concretize the above mentioned results, studies on limits of MRL of antibiotics in crops have not been specified yet (Ahmed et al., 2015). Nevertheless, X. Yu et al. (2018) state that MRL in muscle is of 100 µg/kg and an estimate of the MRL in leafy vegetables falls between 1/6.5 to ½ of that in muscles. This represents an interval of 15.4 to 50 µg/kg MRL limit in leafy greens. The European Community defines MRLs as the highest concentration of residue of a veterinary product available in products of animal origin, which may be consumed daily without causing any toxicological threat to human health (Pérez-Rodríguez, Pellerano, Pezza, & Pezza, 2018). Based on our results, the highest accumulated level of enrofloxacin in lettuce was of 16.62 µg/kg in the leaves. This concentration falls within the MRL interval, hence it could be interpreted that upon consumption, it does not cause health hazards to humans.

b. Gentamicin accumulation in lettuce leaves and roots

The graphs below are taken from a previous MS thesis experiment (Youssef, 2016) and show the measured concentration levels of gentamicin absorbed and accumulated in roots and leaves of lettuce grown in soil without manure and soil with 5% manure (Figure 11 and Figure 12).

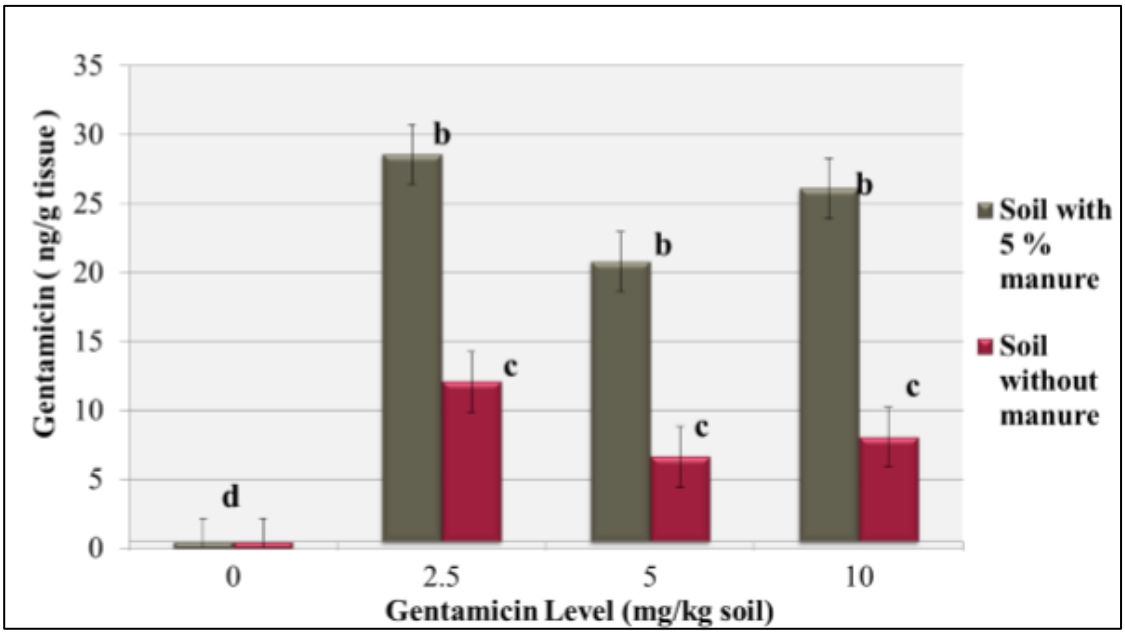


Figure 11. Concentration of gentamicin in lettuce roots grown in soil (without and with 5% manure) (Youssef, 2016)

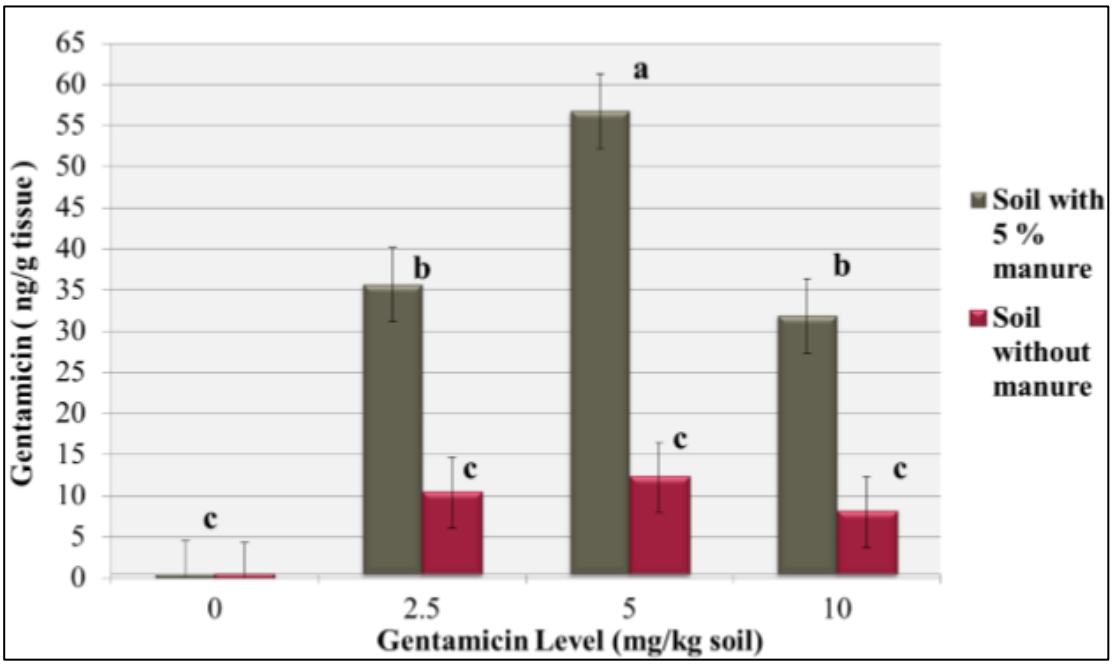


Figure 12. Concentration of gentamicin in lettuce leaves grown in soil (without and with 5% manure) (Youssef, 2016)

In a previous experiment done by Youssef (2016) on the uptake of gentamicin by lettuce grown in two different growth media (soil without manure and soil with 5%

manure) at 4 different gentamicin levels (0, 2.5, 5 and 10 mg/kg), it was concluded that irrespective of the antibiotic level, gentamicin was absorbed by lettuce leaves (Figure 12) and roots (Figure 11).

As the enrofloxacin lettuce results indicated (Figure 10), in this case as well, the presence of manure in the soil increased the uptake of gentamicin by lettuce. Nevertheless, the previous data also demonstrated that the increase of gentamicin level in both growing media (soil with and without manure) did not cause an increase in the sorption of gentamicin by lettuce leaves (Figure 12). This is not in accordance with our results where an increase in enrofloxacin level led to a significant increase of its accumulation in lettuce leaves (Figure 10). Our findings are not in accordance with Azanu et al. (2016) results where carrots and lettuces absorbed antibiotics from irrigation water at all tested concentrations: 0.1, 1.0, 10.0 and 15.0 mg/L. Significant differences were perceived in the absorption of amoxicillin at the four treatments for both lettuce and carrot. Amoxicillin was identified in lettuces from 13.7 to 33.6 ng/g and in carrots from 14.3 to 45.2 ng/g. Tetracycline was identified in lettuces at 4.4 to 28.3 ng/g and in carrots at 12.0 to 36.8 ng/g.

Additionally, the highest accumulated concentration of gentamicin in lettuce leaves was around 55 µg/kg in presence of manure and around 12 µg/kg in absence of manure (Figure 12). Based on the previous MRL estimations, the presence of manure causes an accumulation of gentamicin in lettuce leaves (edible part) greater than the upper limit of the MRL mentioned earlier (15.4 - 50 µg/kg) (X. Yu et al., 2018) whereas in absence of manure the concentration is lower than the MRL interval.

2. Antibiotic uptake by cucumbers

In this part, the uptake and accumulation of enrofloxacin and gentamicin in cucumber root, leaf and fruit was tested and analyzed at four different concentrations (0, 5, 10 and 20 mg/kg) and in two different growth media (soil without manure and soil with 5% manure). At last, the accumulation of enrofloxacin and gentamicin in the cucumber was compared. The results of the statistical analysis were plotted on bar graphs (Figure 13) where means with different superscripts are significantly different and means with same superscripts are not significantly different.

a. <u>Enrofloxacin accumulation in cucumber roots, leaves and fruits grown in soil and soil + 5% manure</u>

The concentrations of enrofloxacin in cucumber roots, leaves and fruits are indicated in Figure 13 whereas the effect of manured soil on cucumber growth is organized in Figure 14. Both are also tabulated in Table 19 in the appendix.

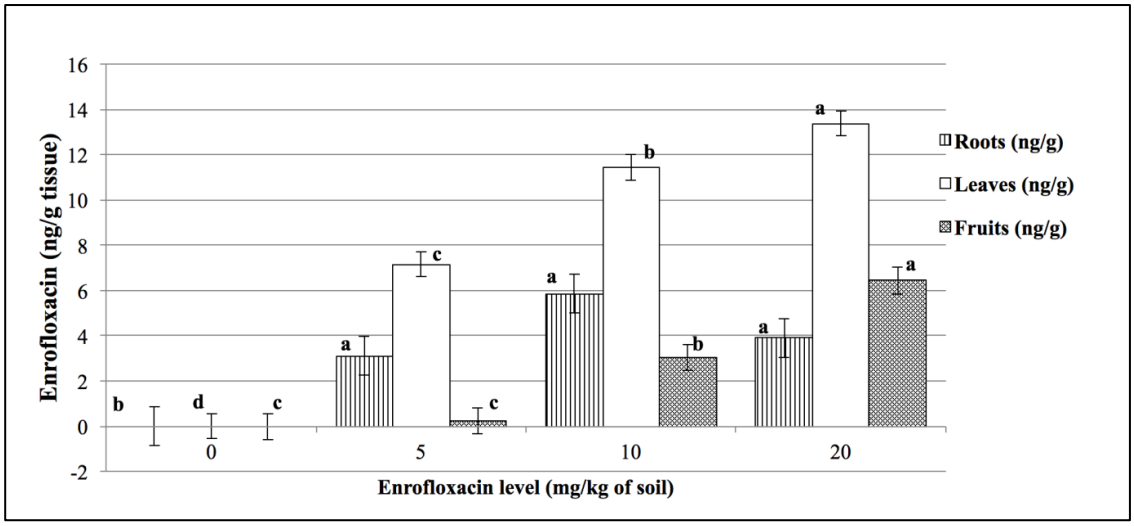


Figure 13. Concentration of enrofloxacin in cucumbers roots, leaves and fruits grown in soil with and without manure

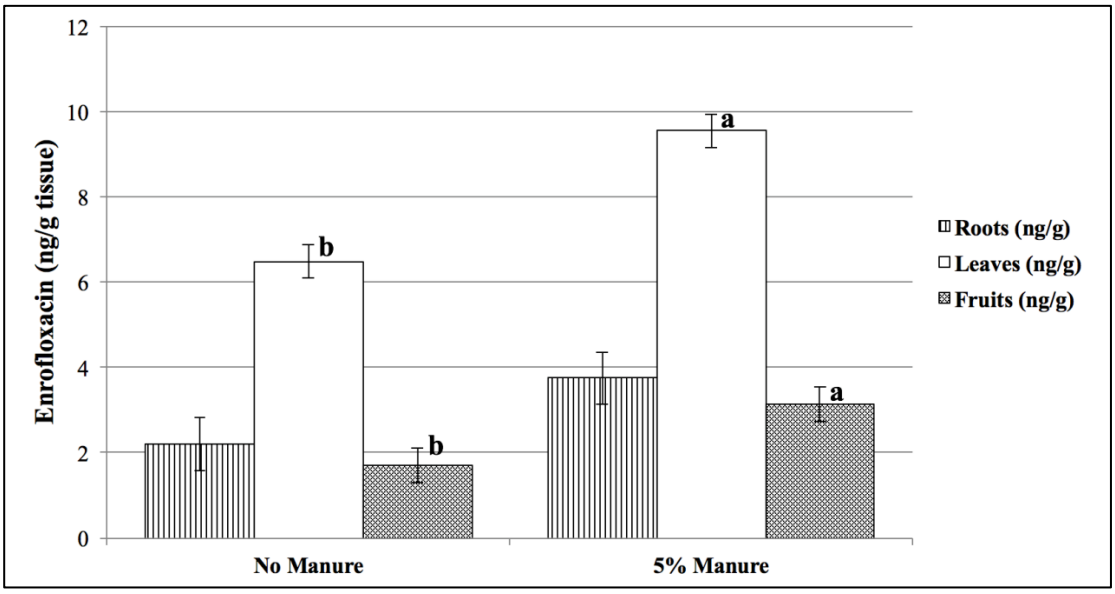


Figure 14. Concentration of enrofloxacin in cucumber roots, leaves and fruits planted in two growth media (soil and soil + 5% manure)

As observed on Figure 13 and Table 19 of the appendix, there is a significant difference between the control and the three different enrofloxacin levels (5, 10 and 20 mg/kg) and irrespective of the enrofloxacin level, the cucumbers roots, leaves and fruits growing medium accumulated enrofloxacin. As the concentration of enrofloxacin increased in the growing media, its accumulation in leaves and fruits increased significantly. This indicates that roots translocate the enrofloxacin up to the vegetative part of the plant. This results agrees with the report of Pan and Chu (2017c) who asserted that the translocation factor of norfloxacin is generally greater than 1, thus indicating that it is transferred from roots to shoots.

Also, results show that as the antibiotic concentration increases, its accumulation in leaves and fruits increases. Pan and Chu (2017c) confirms that a soil contaminated with greater levels of antibiotics, enhances the accumulation of the antibiotic in crops (lettuce, carrot and tomato); the highest concentration of antibiotics (norfloxacin, chloramphenicol and tetracycline) was found in the crops grown in a soil

administered with 2,000 µg/g antibiotic than with 200 µg/g antibiotic and in crops administered with wastewater containing 20 µg/g antibiotic than with 2 µg/g antibiotic through.

In Figure 14 tabulated in Table 19 of the appendix, it is shown that there is a significant difference between the amounts of antibiotic accumulated by leaves and fruits of cucumbers grown in soil with no manure and the one amended with 5% manure, whereas no significant difference was observed on roots grown in these two-distinctive media. The roots, leaves and fruits accumulated a greater amount of enrofloxacin than the ones grown in absence of manure. Accordingly, the administration of manure onto soil increases the absorption and accumulation of enrofloxacin by the plant. The results are in accordance with (Sukul, Lamshöft, Zühlke, & Spiteller, 2008) stating that the presence of manure significantly increases the accumulation of antibiotics. Kang et al. (2013) affirms that organic compounds degrade over time; hence conventional fresh manure should be administered onto soil as composted manure. This will promote and help the degradation of antibiotics, thus lowering their concentration for plant uptake.

Additionally, the highest accumulated concentration of enrofloxacin in cucumber fruits was 13.37 µg/kg (Figure 13). In presence of manure 3.17 µg/kg accumulated whereas in absence of manure 1.7 µg/kg accumulated in the cucumber fruit (Figure 14). Consequently all the above mentioned concentrations fall below the lower limit of the MRL (15.4 – 50 µg/kg) (X. Yu et al., 2018).

b. Gentamicin accumulation in cucumber roots, leaves and fruits grown in soil and soil + 5% manure

In the graphs below, Figure 15 specifies the measured concentration levels of gentamicin absorbed and accumulated in different parts of cucumbers (roots, leaves and fruits) and Figure 16 demonstrates the average accumulated gentamicin in roots, leaves and fruits of cucumbers grown in two different media (soil without and soil with 5% manure). Both figures are tabulated in Table 20 of the appendix.

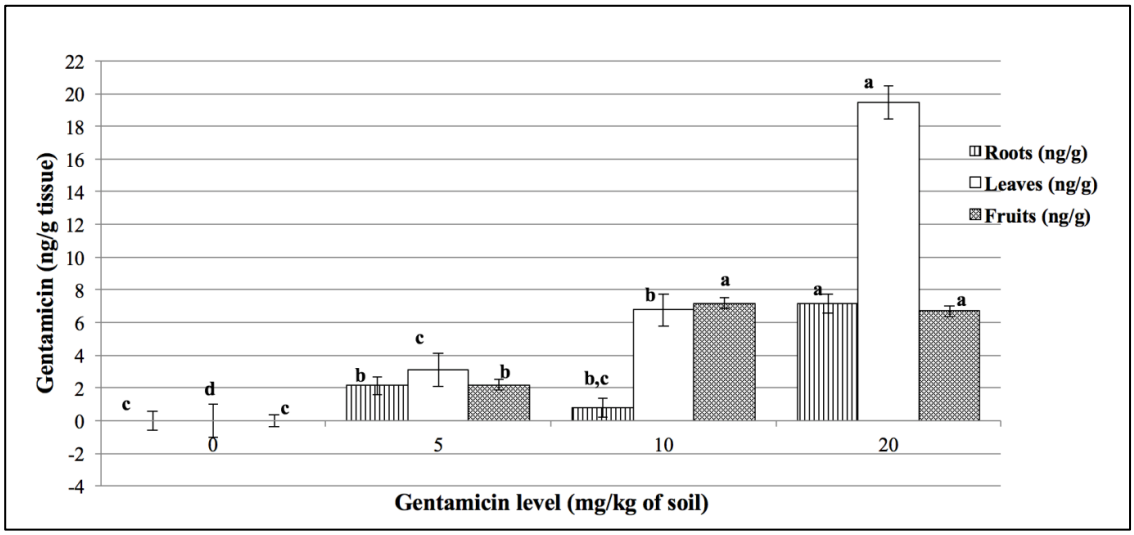


Figure 15. Concentration of gentamicin in cucumbers roots, leaves and fruits grown in soil and soil + 5% manure

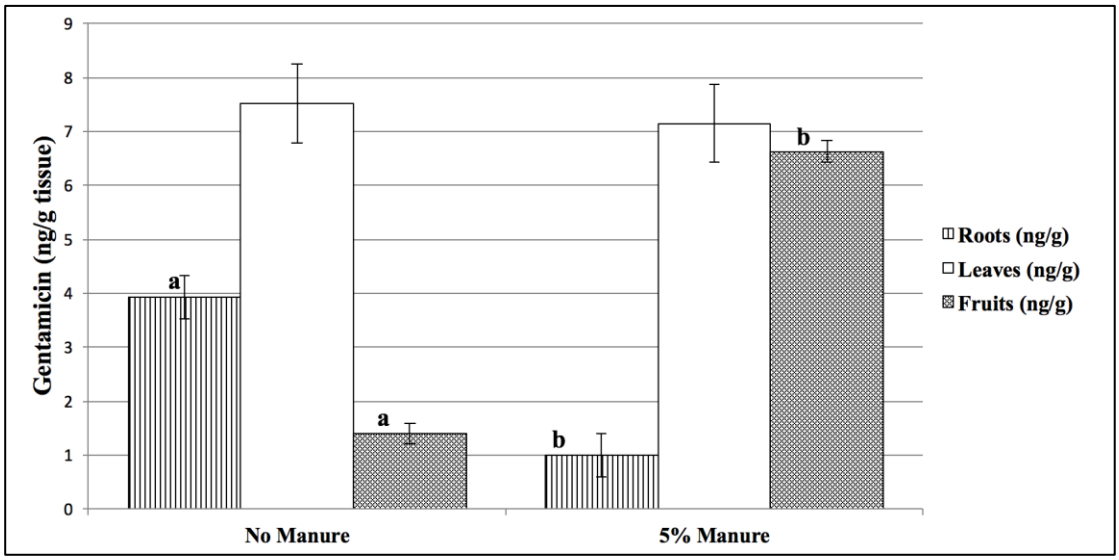


Figure 16. Concentration of gentamicin in cucumbers roots, leaves and fruits planted in two growth media (soil and soil + 5% manure)

As the results indicate in Figure 15 and Table 20 of the appendix, there is a significant difference between the control and the three other gentamicin concentrations (5, 10 and 20 mg/kg). The results indicate that roots accumulate and translocate the antibiotic to shoots irrespective of the antibiotic level. There is a significant difference between the three different concentration levels, but not in the fruits of the treatments 10 and 20 mg/kg. Consequently, gentamicin was mainly translocated and accumulated in leaves and a higher concentration of it led to a greater accumulation in the fruit. This is affirmed by Mitchell (1954) whose study of streptomycin in beans indicated that 67% of the drug was translocated within the first 5 days of applications to the primary leaves; no evidence of reverse direction translocation was noted.

The effect of manure on gentamicin uptake is represented in Figure 16 tabulated in Table 20 of the appendix. The results show a significant difference between the amount of antibiotic accumulated in the roots and fruits of cucumbers grown in soil with and without manure, whereas no significance is noted in leaves. The most distinguished change is in the fruit where there was a 374% increase in the

accumulation of the drug; six times more than in the absence of manure. Consequently, a manured soil increased the accumulation of gentamicin in cucumbers; especially in the edible part. Dolliver et al. (2007) affirms that potato tubers cultivated in manure amended soil, absorbed more sulfamethazine as the manure concentration increased.

Additionally, the highest accumulated concentration of gentamicin in cucumber fruits was 19.47 µg/kg (Figure 15). In presence of manure 6.63 µg/kg accumulated whereas in absence of manure 1.4 µg/kg accumulated in the cucumber fruit (Figure 16). Consequently all the above mentioned concentrations fall between and below the lower limit of the MRL (15.4 – 50 µg/kg) (X. Yu et al., 2018).

c. Comparison of enrofloxacin and gentamicin in cucumbers grown in soil and soil + 5% manure

The graph below (Figure 17) compares the average concentration levels of enrofloxacin and gentamicin absorbed and accumulated in different parts of cucumbers (roots, leaves and fruits) grown in soil at 4 different levels (0, 5, 10 and 20 mg/kg of soil) in the presence and absence of manure. As mentioned previously, the statistical analysis for the uptake of antibiotics by the crops was done by considering both growing media (soil with and without manure) together.

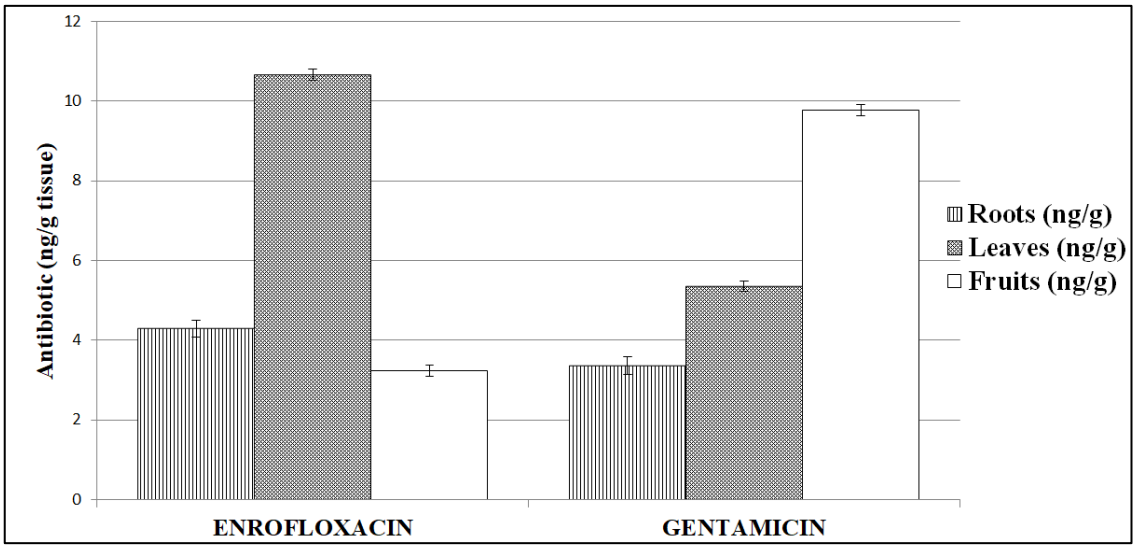


Figure 17. Comparison of enrofloxacin and gentamicin levels in cucumbers roots, leaves and fruits grown in soil without manure and soil + 5% manure

The results show that enrofloxacin was absorbed and accumulated in roots at an average of 4.29 ng/g and in fruits and leaves at an average of 3.23 and 10.66 ng/g respectively. Similarly, gentamicin was absorbed and accumulated in roots at an average of 3.36 ng/g and translocated to leaves and fruits at an average of 5.36 ng/g and 9.78 ng/g respectively. Therefore, in enrofloxacin and gentamicin, the highest accumulation was in leaves and fruits respectively. In a decreasing order, the accumulation of enrofloxacin in cucumbers is: leaf>root>fruit whereas for gentamicin it is fruit>leaf>root.

As for the MRL, it is noticed that the highest concentration of antibiotic accumulated in the cucumber fruit does not exceed 10 µg/kg, which is below the MRL (15.4 – 50 µg/kg) (X. Yu et al., 2018).

C. Antibiotic uptake by lettuce and radish grown hydroponically

The section below describes the antibiotic uptake and accumulation by lettuce and radish grown hydroponically at three different concentrations (0, 5 and 10 mg/kg);

it is divided into four parts for each crop. The first three parts elaborate on the uptake and accumulation of enrofloxacin and gentamicin in the respective parts of lettuce (roots and leaves) and radish (roots, leaves and bulbs) whereas the third part provides a comparison between the two antibiotics in lettuce and radish parts. In the statistical analysis, to observe the uptake of antibiotics, their accumulation sites and effect of their charge (if any), the levels of each stored antibiotic in the different parts of the crops were quantified and plotted into graphs.

1. Antibiotic uptake by lettuce

In this part, the uptake and accumulation of enrofloxacin, tylosin and oxytetracycline in lettuce roots and leaves was tested at three different concentrations (0, 5, and 10 mg/kg). Also, the accumulation of these antibiotics in lettuce was compared, and the effect of antibiotic charge was pointed out. The results of the statistical analysis were plotted on bar graphs where means with different superscripts are significantly different and means with same superscripts are not significantly different.

a. Enrofloxacin accumulation in lettuce leaves and roots grown in nutrient solution

The graph below (Figure 18 and Table 21 in the appendix) show the concentrations of enrofloxacin in lettuce roots and leaves while being subjected to different enrofloxacin levels in water (0, 5 and 10 mg/kg).

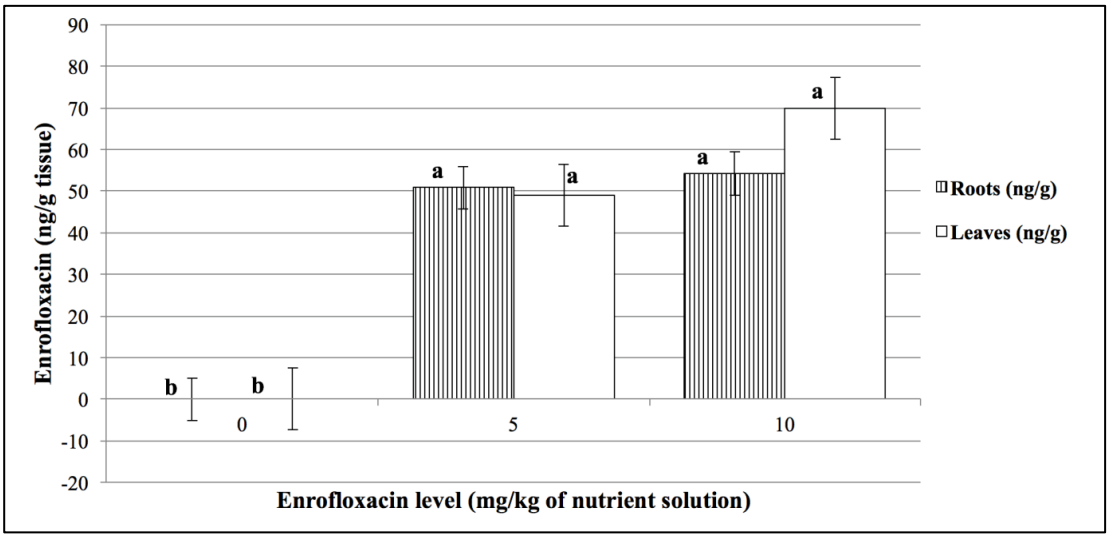


Figure 18. Concentration of enrofloxacin in lettuce grown hydroponically

As in the soil experiment, lettuce grown in nutrient solution spiked with enrofloxacin absorbed it irrespective of its concentration. As observed in Figure 18, there is a significant difference between the control and the two enrofloxacin concentrations (5 and 10 mg/kg), but despite the higher accumulation of enrofloxacin in leaves at 10 mg/kg, the difference between 5 and 10 mg/kg treatments is not significant. As mentioned earlier, this indicates that the roots and leaves of lettuce accumulate enrofloxacin at similar concentrations regardless of the concentration of enrofloxacin if it is 5 or 10 mg/kg in the nutrient solution. Also, these results indicate that a higher enrofloxacin concentration does not lead to a higher accumulation in lettuce tissue. This contradicts Youssef (2016) and L. Liu et al. (2013) results, where *Phragmites australis* cultivated in water, accumulated a greater amount of ciprofloxacin under an increasing level of the drug. The concentration of enrofloxacin in lettuce roots and leaves grown in nutrient solution (50 – 60 ng/g in Figure 18) was much higher than in lettuce grown in soil (6 - 16 ng/g in Figure 9).

Additionally, the highest accumulated concentration of enrofloxacin in lettuce leaves was 48.99 and 69.78 µg/kg at 5 and 10 mg/kg nutrient solution (Figure 18).

Consequently, as the level of enrofloxacin increases in the nutrient solution media, its accumulation in term of MRL increases as well, reaching a level greater than the MRL upper level (15.4 – 50 µg/kg) (X. Yu et al., 2018); however, at 5 mg/kg of nutrient solution, its accumulation is acceptable.

b. Tylosin accumulation in lettuce leaves and roots grown in nutrient solution

Figure 19 and Table 22 in the appendix designate the concentration of tylosin accumulated by lettuce roots and leaves while being subjected to different tylosin levels in water (0, 5 and 10 mg/kg)

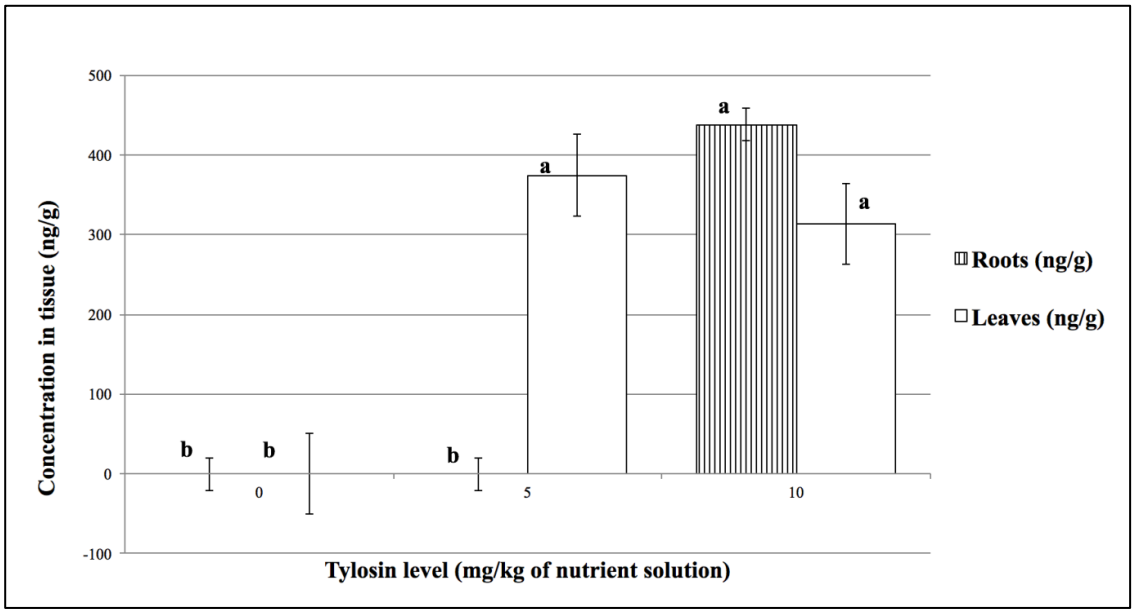


Figure 19. Concentration of tylosin in lettuce grown hydroponically

As observed in Figure 19, there is a significant difference in the concentration of tylosin in the roots between the control of tylosin and the 10 mg/kg treatment and for the leaves, between the control and the 5 mg/kg treatment. For leaves, there is only a significant difference between the control and the other two concentrations (5 and 10

mg/kg) but none between the two latter levels. Consequently, this infers that the amount of tylosin absorbed by lettuce roots was completely translocated to leaves at 5 mg/kg and that the excess at 10 mg/kg accumulated in leaves and roots. A previous study revealed that the accumulation of tylosin in lettuce at 0, 2.5, 5 and 10 mg/kg was not significant and in radish, the translocation of tylosin from roots to leaves readily occurred at all levels (Youssef, 2016). Nevertheless, there is lack of research on tylosin uptake and accumulation in crops grown hydroponically. Kang et al. (2013) justifies the absence of tylosin absorption by onions, cabbage and corn grown in soil to be due to the large molecular size of tylosin. As mentioned previously in Table 11, tylosin holds a positive charge. Also, our results demonstrate that tylosin accumulated in both lettuce and radish (Figure 19 & Figure 20). Consequently, it can be deduced that the lack of accumulation of tylosin in crops grown in soil media could be due to its charge rather than large molecular size. It is recommended to repeat this experiment to confirm the above results, mainly the concentration of tylosin in roots at 5 mg/kg level.

Additionally, the highest accumulated concentration of tylosin in lettuce leaves was 374.47 and 313.19 µg/kg at 5 and 10 mg/kg nutrient solution (Figure 19). As mentioned previously, tylosin is greatly accumulated in lettuce leaves, and at both levels, the accumulated concentration is drastically greater than the MRL upper limit (15.4 – 50 µg/kg) (X. Yu et al., 2018).

c. Oxytetracycline accumulation in lettuce leaves and roots grown in nutrient solution

Figure 20 and Table 23 in the appendix display the concentration of oxytetracycline accumulated by lettuce roots and leaves while being subjected to different oxytetracycline levels in nutrient solution (0, 5 and 10 mg/kg).

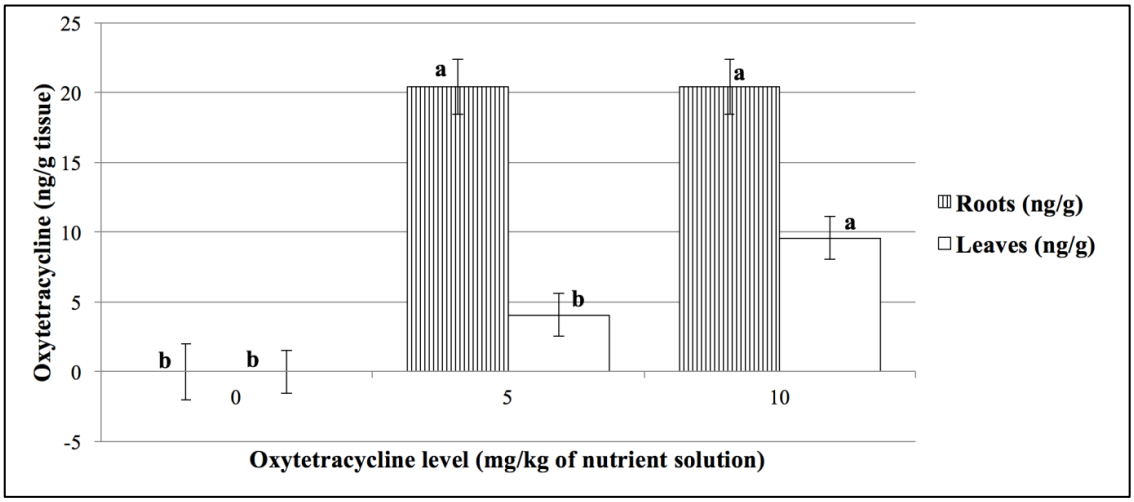


Figure 20. Concentration of oxytetracycline in lettuce grown hydroponically

As observed in Figure 20, there a significant difference between the control and the roots grown at both oxytetracycline levels (5 and 10 mg/kg); however, no significance was found between the roots of plants grown in the 5 and 10 mg/kg treatments. Leaves grown in the 5 mg/kg treatment, accumulated little quantity of oxytetracycline, but the increase was not significant. This may imply that roots accumulate oxytetracycline to a certain limit and then the excess would be translocated to the leaves, hence the significant greater storage of the drug in the leaves at 10 mg/kg. In a hydroponic experiment conducted by L. Liu et al. (2013), results demonstrated that *Phragmites australis* grown in water spiked with ciprofloxacin, sulfamethazine and oxytetracycline absorbed the drugs. Another finding from L. Liu et al. (2013) is that

there is a positive correlation between the antibiotic level and its accumulated concentration in *Phragmites australis*: total stored content of oxytetracycline in the crop at 1,000 µg/L was 6,901 ng/g dry weight whereas at 0.1 µg/L it was 165 ng/g dry weight.

Comparing these results with previous crops grown in soil spiked with antibiotics and no availability of oxytetracycline in the crop, indicates that the charge of the oxytetracycline could be the reason why it was not accumulated by crops grown in soil. Nevertheless, when grown in nutrient solution, it mainly got stored in roots. This could be explained by the fact that it got adsorbed by the charge within the roots. L. Liu et al. (2013) findings also showed that the distribution of oxytetracycline in a decreasing order in the crop was root>leaf>stem. Contrarily to our results, Pan and Chu (2017c) claim that when absorbed, oxytetracycline is available in its neutral form in the crop, hence it has a greater translocation potential and a greater storage level in leaves and fruits.

Additionally, the highest accumulated concentration of oxytetracycline in lettuce leaves was 20.4 µg/kg at both levels 5 and 10 mg/kg nutrient solution (Figure 19). These accumulated concentrations fall in the interval of the estimated acceptable MRL limits (15.4 – 50 µg/kg) (X. Yu et al., 2018).

d. Comparison of enrofloxacin, tylosin and oxytetracycline in lettuce

Figure 21 compares the accumulation levels of enrofloxacin, tylosin and oxytetracycline absorbed and stored in different parts of lettuce (roots and leaves).

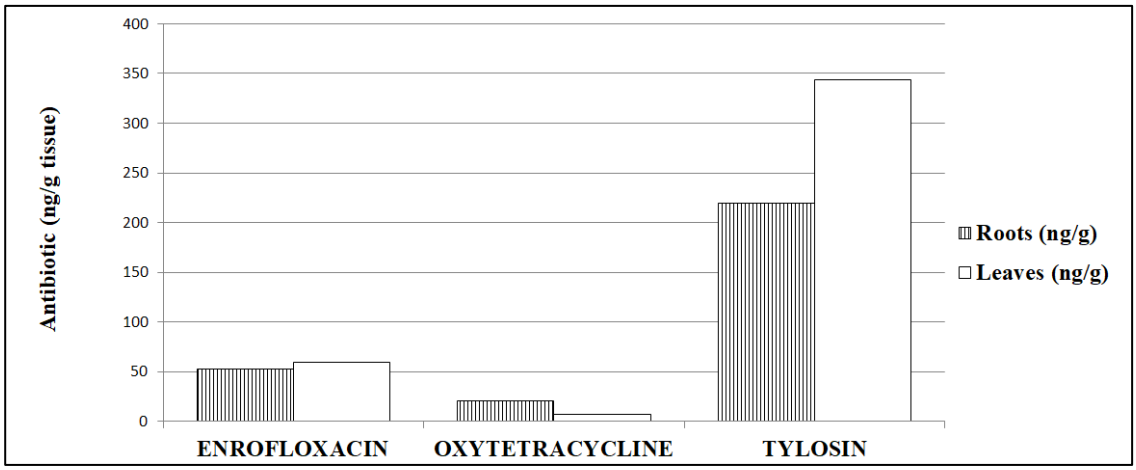


Figure 21. Comparison of enrofloxacin, oxytetracycline and tylosin in lettuce grown hydroponically

Based on Figure 21 and irrespective of the spiked level, oxytetracycline and enrofloxacin are the two antibiotics that accumulated the least in lettuce, while tylosin concentration was much higher. Tylosin accumulated at a level of 145.96 ng/g in roots and 229.22 ng/g in leaves. The sequence of distribution in a decreasing order in the radish goes as follows: tylosin>enrofloxacin>oxytetracycline. Our findings are in concordance with L. Liu et al. (2013) results whereby they stated that the total antibiotics in plants followed this sequence in an ascending order: ciprofloxacin>oxytetracycline>sulfamethazine.

Also, only oxytetracycline and enrofloxacin (level 5 mg/kg of nutrient solution) fall within the MRL limits (15.4 – 50 µg/kg) whereas tylosin and enrofloxacin (level 10 mg/kg nutrient solution) fall above the MRL upper limit (X. Yu et al., 2018).

2. Antibiotic uptake by radish

In this part, the uptake and accumulation of enrofloxacin, tylosin and oxytetracycline in radish roots, bulbs and leaves was tested and analyzed at three different concentrations (0, 5, and 10 mg/kg). Also, the accumulation of these

antibiotics in radish was compared, and the effect of antibiotics charge was discussed. The results of the statistical analysis were plotted on bar graphs where means with different superscripts are significantly different and means with same superscripts are not significantly different.

a. Enrofloxacin accumulation in radish leaves, roots and bulbs grown in nutrient solution

Figure 22 tabulated in Table 24 in the appendix arrange the concentration of enrofloxacin accumulated by radish roots, bulbs and leaves while being subjected to different enrofloxacin levels in water (0, 5 and 10 mg/kg).

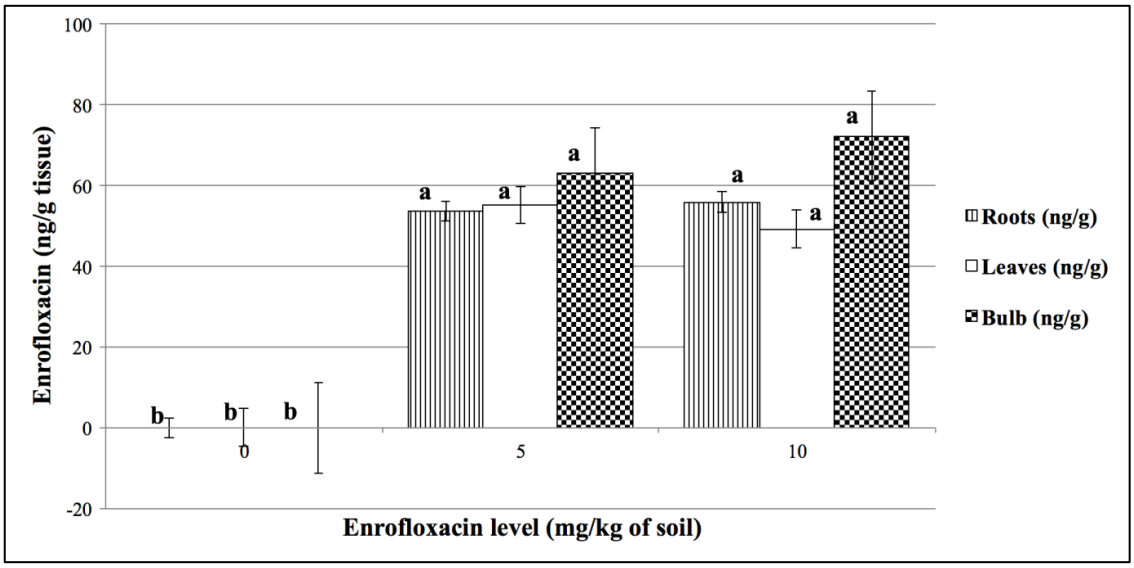


Figure 22. Concentration of enrofloxacin in radish grown hydroponically

Based on Figure 22, radishes absorbed enrofloxacin in all their parts at similar concentrations in hydroponics. The highest stored concentration is in the edible part of the radish. There is a significant difference between the control and the two other enrofloxacin levels (5 and 10 mg/kg) but no significant difference between the 5 and 10

mg/kg level or between the roots, bulbs or leaves. This indicates again that as the concentration of enrofloxacin increases in the media, its uptake and accumulation does not significantly increase and is distributed all through the plant parts root:bulbs:leaves at 1:1:1 ratios (uniform concentrations).

Additionally, the highest accumulated concentration of enrofloxacin in radish bulbs and leaves was above 50 µg/kg at 5 and 10 mg/kg nutrient solution (Figure 22). Consequently, both enrofloxacin levels led to an accumulated concentration in the edible parts of the radish (bulb and leaves) to be greater than the MRL upper limit (15.4 – 50 µg/kg) (X. Yu et al., 2018). This is in accordance with our previous results of lettuce grown in a nutrient solution in presence of enrofloxacin (Figure 18). Nevertheless, for cucumber fruits grown in soil administered with enrofloxacin, the antibiotics accumulation did not exceed the MRL limit (Figure 13 & Figure 14).

b. Tylosin accumulation in radish leaves, roots and bulbs grown in nutrient solution

Figure 23 tabulated in Table 25 in the appendix designate the concentration of tylosin accumulated by radish roots, bulbs and leaves while being subjected to different tylosin levels in water (0, 5 and 10 mg/kg).

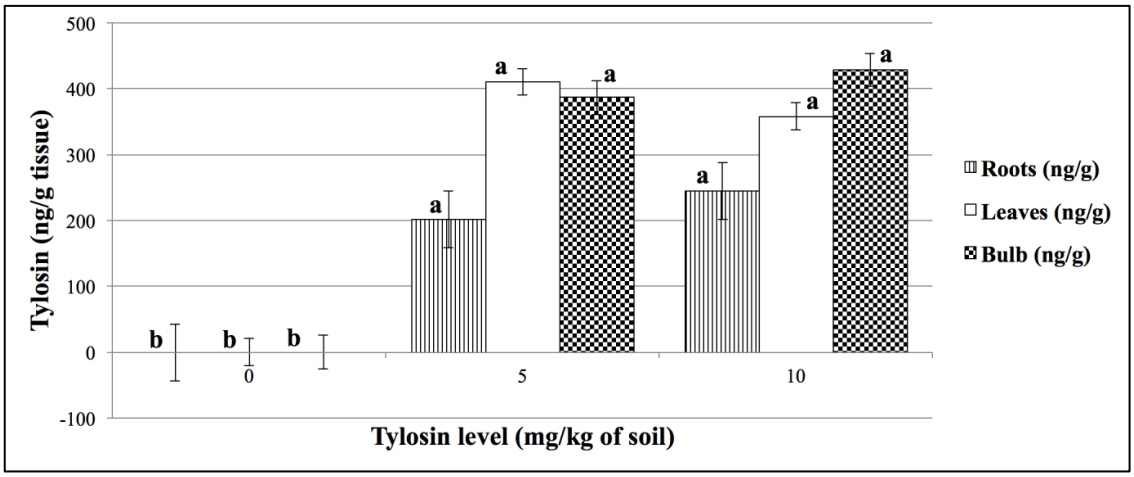


Figure 23. Concentration of tylosin in radish grown hydroponically

Referring to Figure 23, there is a significant difference between the control and the two other levels of tylosin (5 and 10 mg/kg). The uptake and accumulation of tylosin was not significant between levels: 5 and 10 mg/kg. Despite the mild increase in the drug accumulation in roots, leaves and bulbs at 10 mg/kg, no significance appeared between the two tylosin concentrations. Consequently, as observed previously: as the concentration of the antibiotic increases, its accumulation does not significantly increase.

As tylosin was present in all the parts of the radish crop, this shows that the antibiotic is translocated all over the crop, but mainly accumulates in the edible parts of the crop: bulb and leaves.

Additionally, the highest accumulated concentration of tylosin in radish bulbs and leaves varied from 200 to 410 µg/kg at 5 and 10 mg/kg nutrient solution (Figure 23). Consequently, regardless of tylosin level in the nutrient solution, its accumulation in radish edible parts (bulb and leaves) greatly exceeds MRL range (15.4 – 50 µg/kg) (X. Yu et al., 2018). This is in accordance with our previous results of lettuce grown in a nutrient solution in presence of tylosin (Figure 19).

c. Oxytetracycline accumulation in radish leaves, roots and bulbs grown in nutrient solution

Figure 24 and Table 26 in the appendix describe the concentration of oxytetracycline accumulated by radish roots, bulbs and leaves while being subjected to different oxytetracycline levels in water (0, 5 and 10 mg/kg).

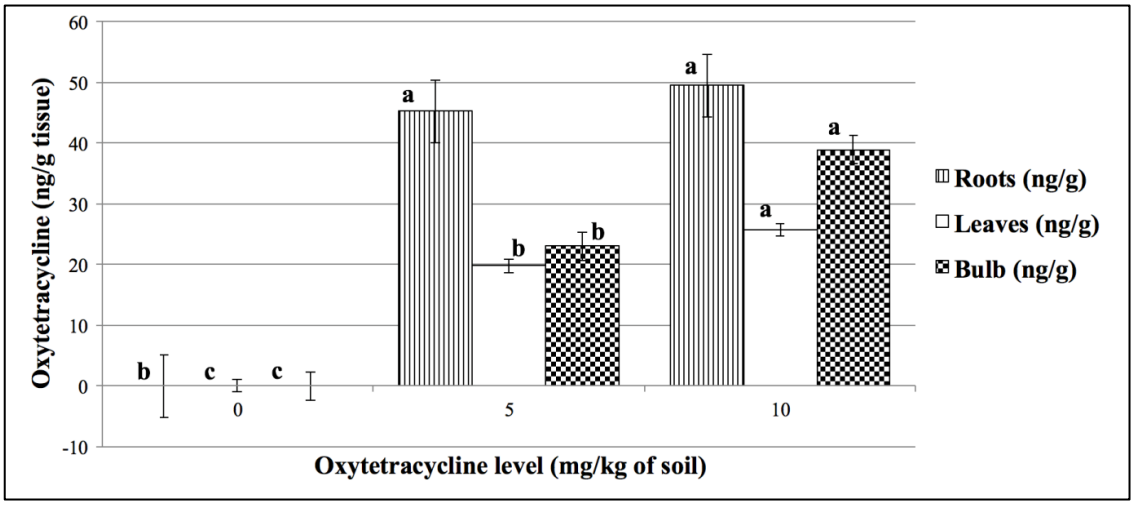


Figure 24. Concentration of oxytetracycline in radish grown hydroponically

The results tabulated in Figure 24 show a significant difference between the control and the two other levels of oxytetracycline (5 and 10 mg/kg). Here as well, results also indicate that irrespective of the concentration, oxytetracycline was accumulated by radish crops, thus speculating that the absence of its accumulation in crops grown in soil spiked with oxytetracycline could be due to its adsorption to soil charged particles (soil oxides, organic matter and clays). Compared to leaves and bulbs, roots were the ones that accumulated the highest level of oxytetracycline. Consequently, like in radishes grown in soil, oxytetracycline mainly accumulated in the roots (Youssef, 2016). The sequence of accumulation of oxytetracycline in radish crops in a decreasing order goes as follows: roots>bulbs≥leaves (Figure 24). (L. Liu et al., 2013)

confirmed in his article that roots stored the highest amount of antibiotics, followed by leaves and stems.

At both 5 and 10 mg/kg levels, the accumulation of oxytetracycline in roots slightly increased with absence of significance, whereas leaves and bulbs significantly accumulated more of the drug at 10 mg/kg. This demonstrates that as the antibiotic concentration increases in the nutrient solution, its uptake and accumulation increases as well.

Additionally, the accumulated concentration of oxytetracycline in radish bulbs and leaves did not exceed the MRL upper limit 50 µg/kg (X. Yu et al., 2018) at 5 and 10 mg/kg nutrient solution (Figure 24). Consequently, oxytetracycline accumulation in radish edible parts (bulb and leaves) does not exceed the MRL range (15.4 – 50 µg/kg) and this is in accordance with our previous results (Figure 20).

d. Comparison of enrofloxacin, tylosin and oxytetracycline in radish grown in nutrient solution

Figure 25 compares the accumulation levels of enrofloxacin, tylosin and oxytetracycline absorbed and accumulated in different parts of radish (roots, bulbs and leaves).

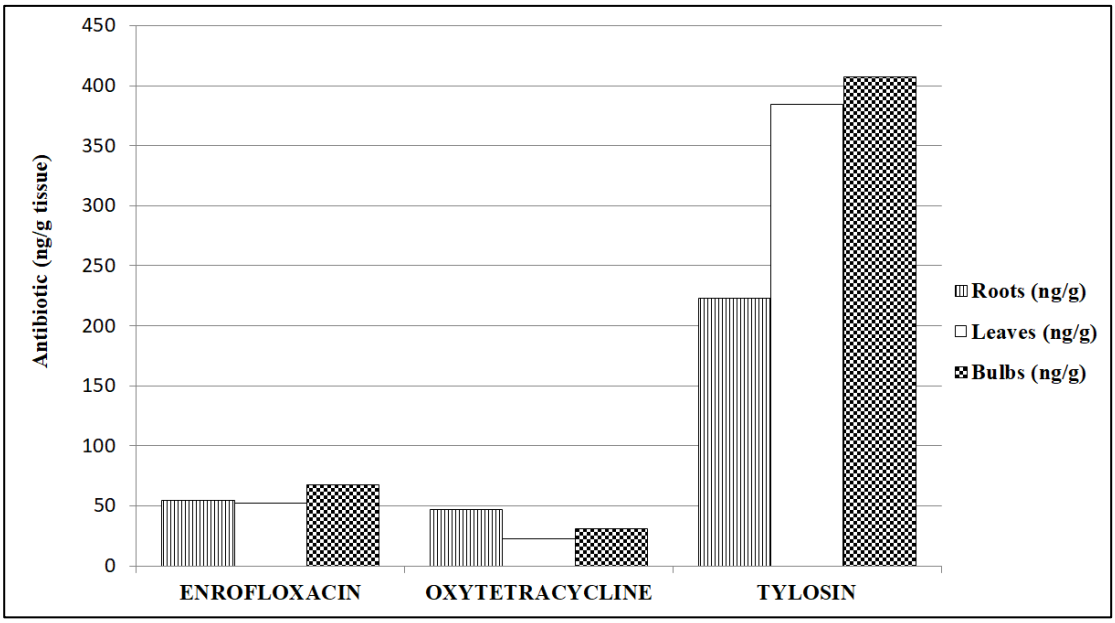


Figure 25. Comparison of enrofloxacin, oxytetracycline and tylosin in radish grown hydroponically

Based on Figure 25 and irrespective of the treatment, oxytetracycline and enrofloxacin are the two antibiotics that accumulated the least in the radish tissue (less than 67 ng/g) compared to tylosin (more than 220 ng/g). Tylosin accumulated at a level of 407.45 ng/g in bulbs whereas enrofloxacin and oxytetracycline accumulated at a level of 67.68 ng/g and 30.98 ng/g respectively. The sequence of distribution in a decreasing order in the radish goes as follows: tylosin>enrofloxacin>oxytetracycline.

As X. Yu et al. (2018) informed, in vegetables, the MRL range falls between 15.4 and 50 µg/kg. In Figure 25, it is observed that tylosin greatly exceeded the range upper limit (50 µg/kg), followed by enrofloxacin and at last oxytetracycline which fell within the interval.

D. Antibiotic effect on lettuce and radish growth

The section below describes the effect of enrofloxacin, tylosin and oxytetracycline on radish and lettuce grown hydroponically at three different

concentrations (0, 5 and 10 mg/kg). The graphs below show the average weight of the roots and leaves of lettuce and the bulbs for radish. In the statistical analysis, to observe the effect of the three antibiotics on plant growth, the average of the weight of all three lettuces and radishes grown at each level were taken separately for roots, leaves, bulbs and overall plant weight.

1. Antibiotic effect on lettuce growth

In the hydroponic experiment, the effect of enrofloxacin, tylosin and oxytetracycline on lettuce roots, leaves and overall growth was measured at three different concentrations (0, 5 and 10 mg/kg). Also, the average of the three levels for each antibiotic was computed and a comparison of their effect on lettuce is conducted and discussed. The results of the statistical analysis were plotted on bar graphs where means with different superscripts are significantly different and means with same superscripts are not significantly different.

a. Enrofloxacin effect on lettuce growth

The effect of enrofloxacin on growth of lettuce is presented in Figure 26 at three different concentrations (0, 5 and 10 mg/kg) and in Table 27 in the appendix.

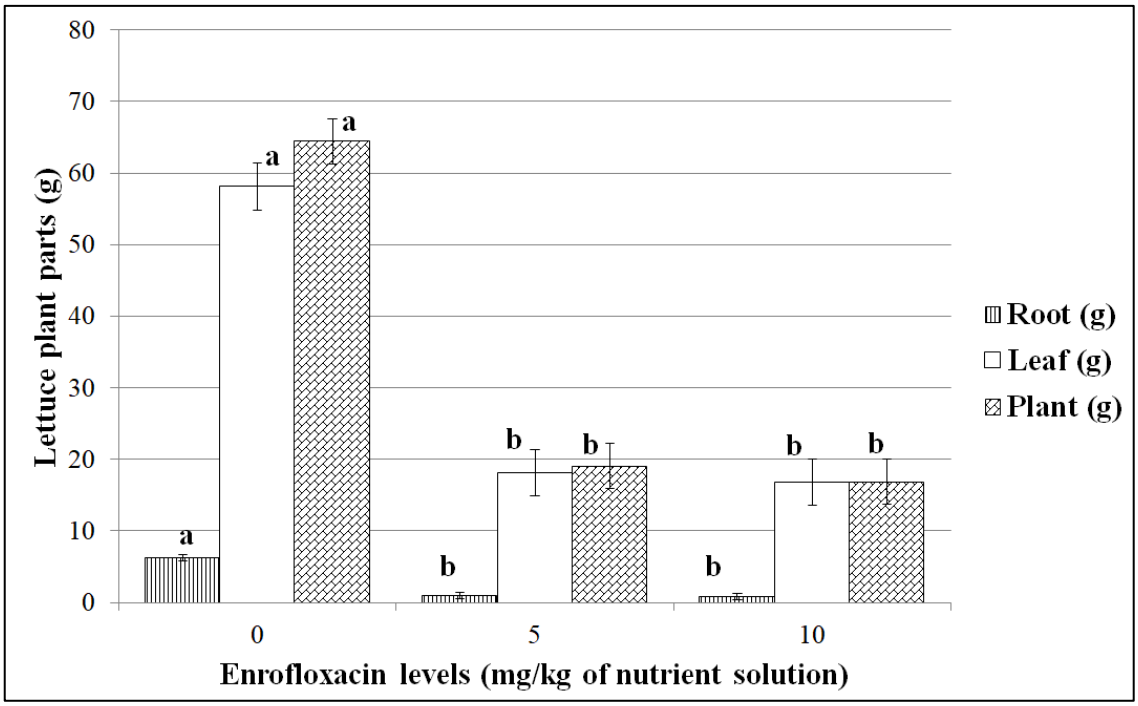


Figure 26. Weight of lettuces administered with enrofloxacin and grown hydroponically

The results (Figure 26) show that the application of enrofloxacin to the nutrient solution severely reduced the growth of the lettuce plants by about 70%. Nevertheless, the higher concentration of enrofloxacin did not further affect the lettuce weight; no significant difference appeared between the 5 and 10 mg/kg levels. Nickell and Finlay (1954) confirmed in their article that not all antibiotics such as bacitracin and penicillin G promote crop growth. Other antibiotics such as neomycin, netropsin and polymyxin inhibited growth depending on the concentration applied, the duration of the growth period and the experimental conditions. At four different levels (1, 5, 10 and 20 mg/kg), neomycin decreased plant weight by 10, 85, 90 and 95% respectively whereas citrinin increased it by 120, 100% then decreased it by 20 and 98% respectively.

Visually, enrofloxacin appeared to have a great phytotoxic effect on lettuces; growth was weak, roots short and leaves thin, white and small. As the concentration of enrofloxacin and length period increased, the phytotoxicity was more pronounced

(Figure 27), and crops were witnessed to weigh less (Figure 27). Migliore, Cozzolino, and Fiori (2003) confirmed our results, stating that at 100 µg/L of enrofloxacin a toxic and hermetic effect were apparent; the toxic effect increased with time and was observed on the leaves of bean and radish as well as in the primary roots of lettuce and cucumber. They also suggested that crops grown in a high enrofloxacin concentration (5,000 µg/L) partially metabolize the latter into ciprofloxacin.

Figure 27. Comparison between the three treatments of lettuce in enrofloxacin at harvest (a) control (b) 5mg/kg (c) 10 mg/kg

b. Tylosin effect on lettuce growth

The effect of tylosin on lettuce growth is graphed (Figure 28) indicating the average weights of the lettuces, roots and leaves in the three treatments (0, 5 and 10 mg/kg) and in Table 28 in the appendix.

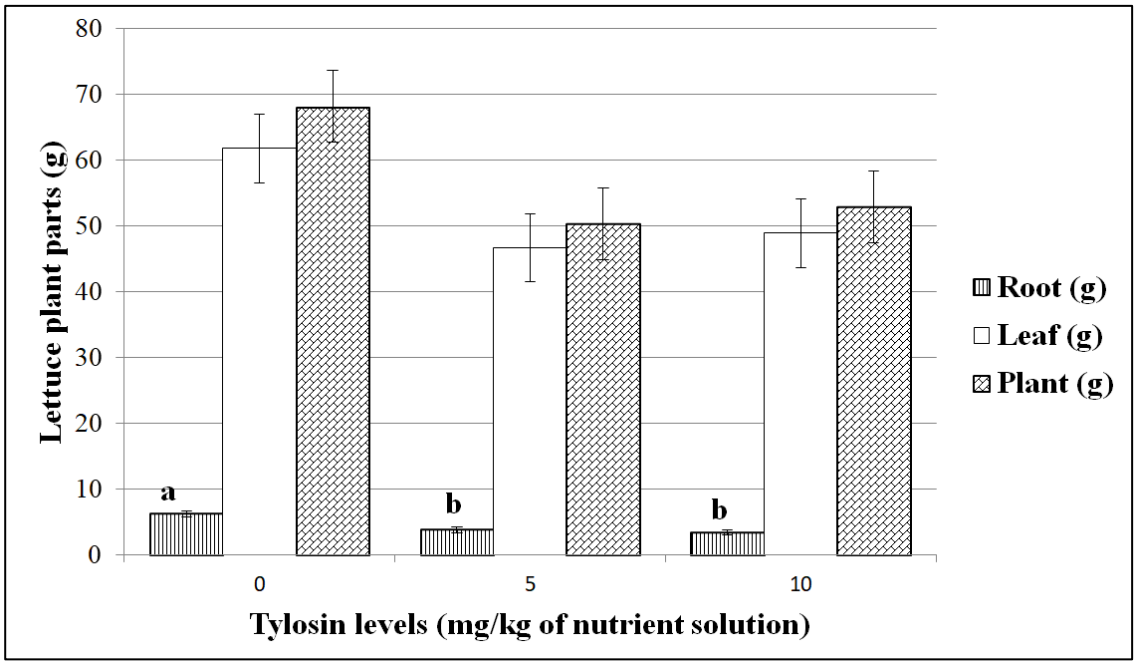


Figure 28. Weight of lettuces administered with tylosin and grown hydroponically

Our results charted in Figure 28 show that there is a significant difference on lettuce growth between treatment 0 (control) and treatments 5 and 10 mg/kg; however, no significant difference between treatment 5 and 10 mg/kg. About 20% reductions were observed. Azanu et al. (2016) asserts that antibiotics primarily accumulate in roots, thus affecting their growth. As for the edible part of the lettuce, no significant difference is noted between the whole lettuces and leaves weight; hence it is speculated that tylosin has no prominent effect on the weight of comestible parts of the crop. Correlating that to the visual appearance of grown lettuces in the hydroponic system, only a slight difference could be observed on root and leaf growth (Figure 29).

Associating these results with the accumulation site of tylosin in lettuce (Figure 19), the storage of the drug in leaves at 5 and 10 mg/kg had no effect on the crop weight. Our results are in accordance with Hillis et al. (2011) where there was an absence of significance for tylosin opposing effect on lettuce or alfalfa, however the

growth of carrot roots decreased significantly when administered with 10,000 µg/L of tylosin.

Figure 29. Comparison of lettuce crops at harvest in all three treatments of tylosin (a) control (b) 5 mg/kg (c) 10 mg/kg

c. Oxytetracycline effect on lettuce growth

Figure 30 show the variation of root, leaf and whole lettuce weight with respect to the level of oxytetracycline it is supplied with (0, 5 and 10 mg/kg) and in Table 29 in the appendix.

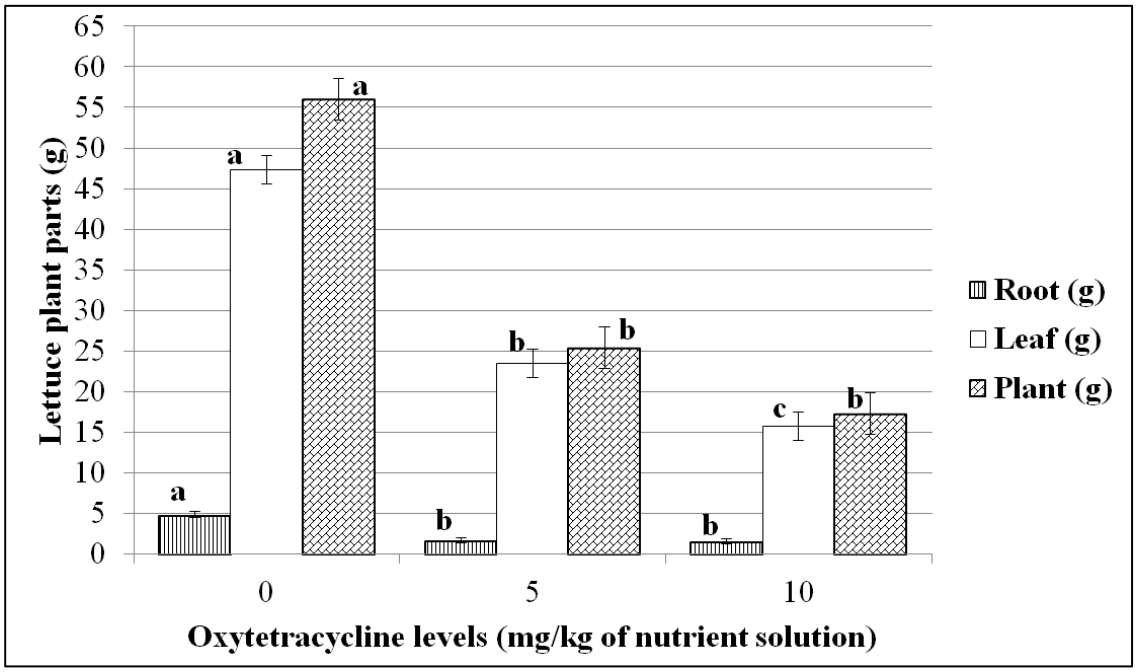


Figure 30. Weight of lettuces administered with oxytetracycline and grown hydroponically

Our results charted in Figure 30 show that there is a significant difference between the control and the two other oxytetracycline concentrations (5 and 10 mg/Kg). The edible part of the lettuce is the one that was the most affected as both oxytetracycline concentrations significantly decreased its weight. This change was also perceived visually throughout the experiment (Figure 31). Our results are in accordance with Ahmed et al. (2015) findings where tetracyclines added to soils at 5, 10 and 20 mg/kg inhibited the growth of cucumber, lettuce and tomato. Also, L. Liu et al. (2013) demonstrated that high antibiotic levels (greater than 10 µg/L) exerted a toxic effect on root activity as well as the exposure of the crop to higher sulfamethazine, ciprofloxacin and oxytetracycline concentrations led to a greater amount in the crops (grown hydroponically). Consequently, as mentioned previously, the highest accumulation of oxytetracycline in lettuces and radishes is in their roots (Figures & 24), hence the lowest weight tabulated here.

Figure 31. Comparison of lettuce crops at harvest in all three treatments of oxytetracycline (a) control (b) 5 mg/kg (c) 10 mg/kg

d. Comparison of enrofloxacin, tylosin and oxytetracycline on lettuce growth

Figure 32 compares between the effect of enrofloxacin, tylosin and oxytetracycline on lettuce weight in its different plant parts: roots and leaves. The average weight of the three levels in each antibiotic is tabulated on the graph below.

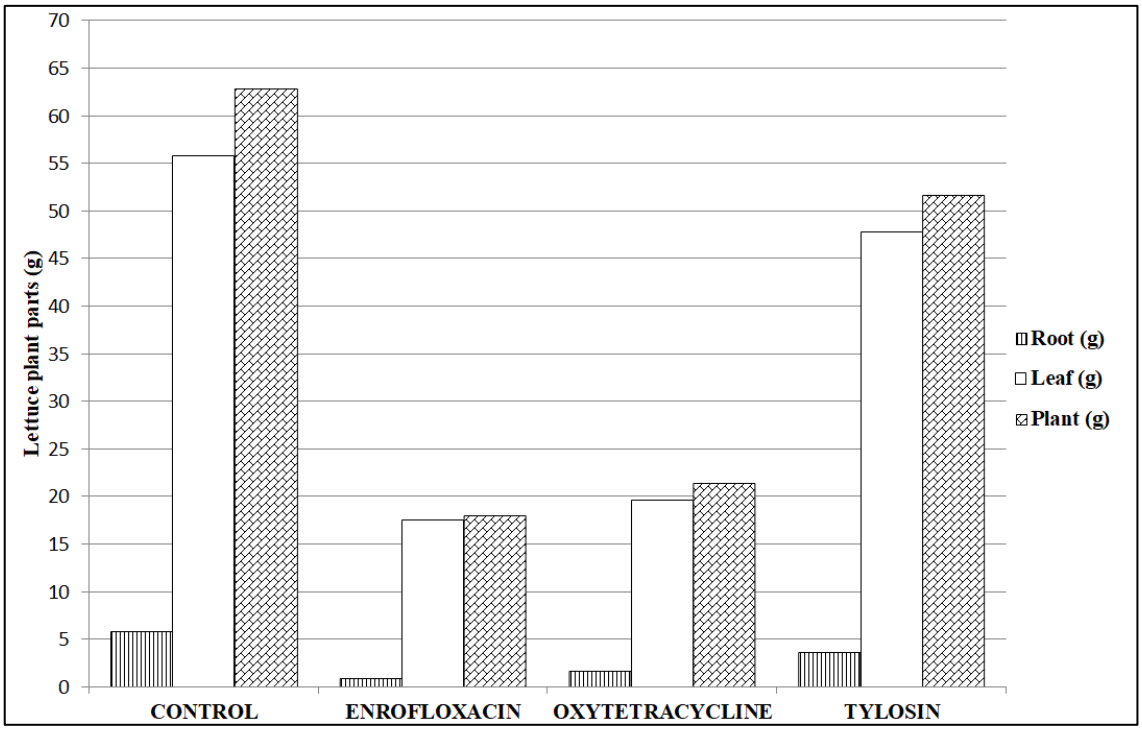


Figure 32. Comparison of enrofloxacin, oxytetracycline and tylosin effect on lettuce weight grown hydroponically

Figure 32 shows that the three antibiotics led to a decrease in the yield of lettuce. Enrofloxacin and oxytetracycline appear to have a similar negative impact on the leaves and roots, hence on the total weight of the plant: they are the two that mostly affected lettuce growth. Compared to the control, enrofloxacin caused a 71.36% decrease, oxytetracycline 65.97% and tylosin 17.89%. As mentioned earlier, tylosin only had a negative impact on the lettuce roots.

Referring to our previous results, the antibiotics accumulated the most in lettuce roots. Correspondingly, in Figure 32, the lowest percentage decrease is observed in leaves and the highest in roots; 68.66%, 64.81% and 14.28% decrease in enrofloxacin, oxytetracycline and tylosin leaves respectively and 84.54%, 72.03% and 37.29% decrease in their roots respectively.

2. Antibiotic effect on radish growth

In the hydroponic experiment, the effect of enrofloxacin, tylosin and oxytetracycline on radish roots, bulbs, leaves and overall growth was tested at three different concentrations (0, 5 and 10 mg/kg). The average of the three levels for each antibiotic is computed and a comparison of their effect on radishes is highlighted and discussed. The results of the statistical analysis were plotted on bar graphs where means with different superscripts are significantly different and means with same superscripts are not significantly different.

a. Enrofloxacin effect on radish growth

Figure 33 shows the effect of three enrofloxacin concentrations (0, 5 and 10 mg/kg) on the entire radish weight, root, bulb and leaf and in Table 30 in the appendix.

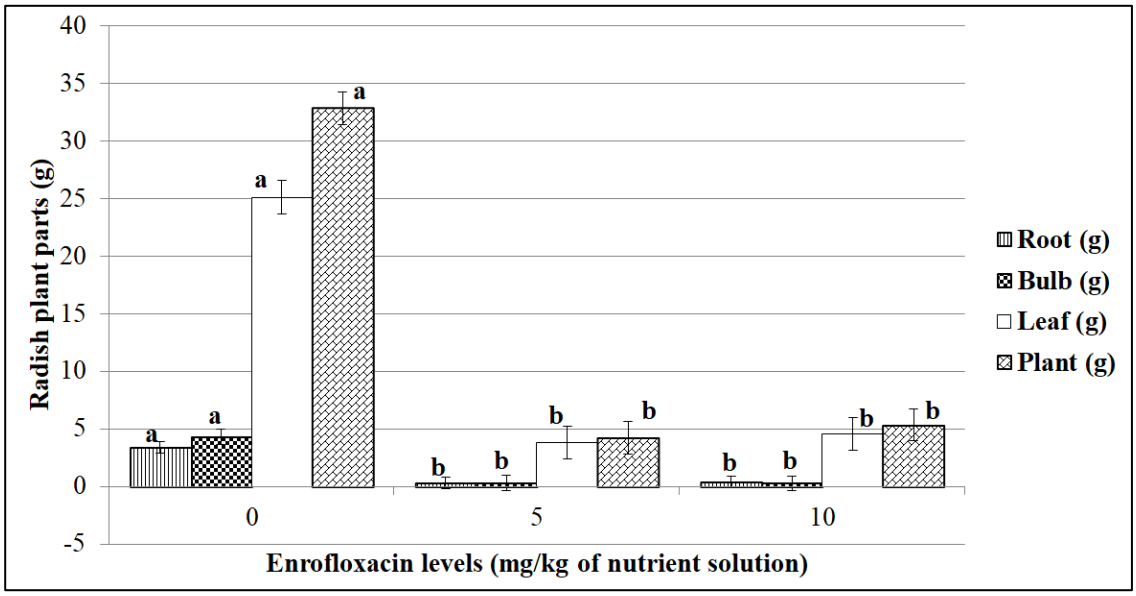


Figure 33. Weight of radish crops administered with enrofloxacin grown hydroponically

Figure 33 portrays that there is only a significant difference between the control and the two other enrofloxacin concentrations (5 and 10 mg/kg). This indicates that the presence of enrofloxacin at 5 and 10 mg/kg caused a 90% decrease in the weight of the roots and bulbs weight; a higher enrofloxacin level did not lead to a harsher effect on radish growth. The findings of Adomas, Antczak-Marecka, Nałęecz-Jawecki, and Piotrowicz-Cieślak (2013) contradict our results whereby an increasing level of enrofloxacin caused a harsher root inhibition and minor increase in the dry mass. At 0.5 mM of enrofloxacin, the plant morphological organs were already affected. At 10 mM of enrofloxacin, a notable decrease in stem and root elongation was observed and at 50 mM of enrofloxacin, root elongation was inhibited by an average of 98% and stem elongation was completely stopped (100%). Also, our results are in accordance with Migliore et al. (2003) where growth media of concentrations between 0.05 and 5 mg/L led to reduced plant growth (length of primary root, number and length of leaves).

Visually, the difference in weight of radishes is greatly observed while radishes were harvest (Figure 34). Again, phytotoxicity was greatly pronounced on radish leaves (Figure 34).

Figure 34. Comparison of radish crops at harvest in all three treatments of enrofloxacin (a) control (b) 5 mg/kg (c) 10 mg/kg

b. Tylosin effect on radish growth

Figure 35 displays the effect of three tylosin concentrations (0, 5 and 10 mg/kg) on the entire radish weight, root, bulb and leaf and in Table 31 in the appendix.

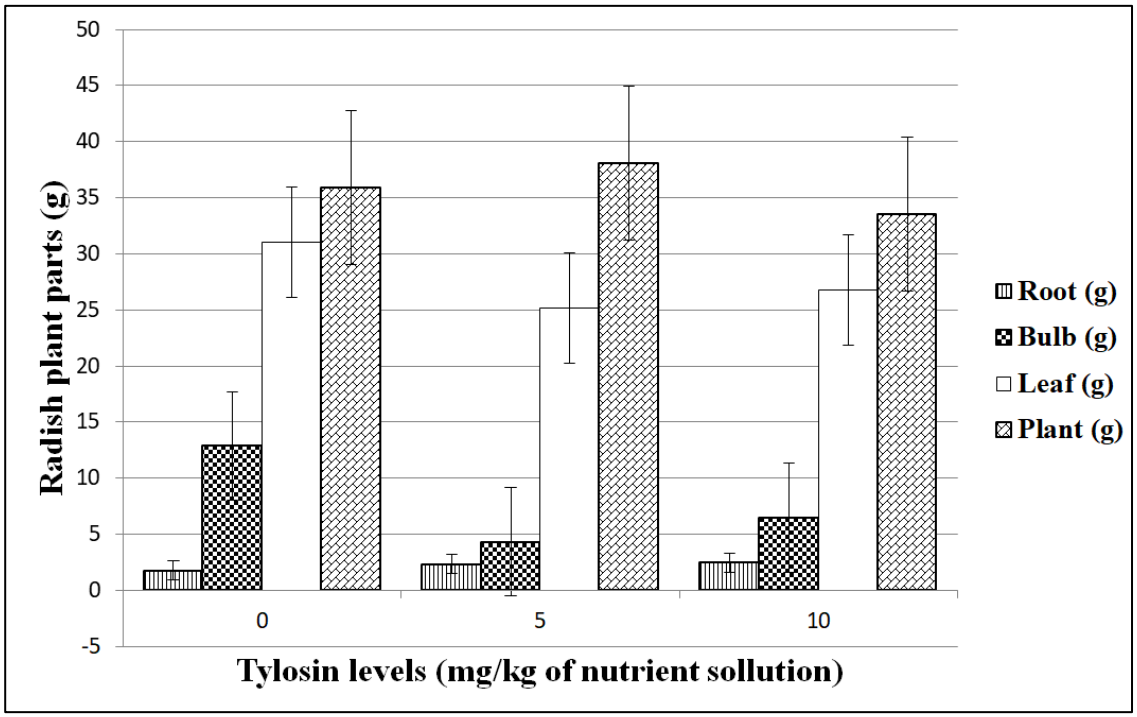


Figure 35. Weight of radish administered with tylosin and grown hydroponically

Despite the fact that radishes accumulated tylosin in all parts at 5 and 10 mg/kg (Figure 23), it did not present any significant effect on its weight. Based on Figure 35, irrespective of the concentration of tylosin, no significant weight change in radishes took place. Nevertheless, compared to the control some decrease in bulb and leaf weight took place similarly at 5 and 10 mg/kg levels. This lack of significant difference in weight was greatly observed on radishes at harvest (Figure 36). F. Liu et al. (2009) states that among two tetracyclines and tylosin, the latter was the one who detained the lowest toxicity, specifically on cucumber and rice seeds.

Figure 36. Comparison of radish crops at harvest in all three treatments of tylosin (a) control (b) 5 mg/kg (c) 10 mg/kg

c. Oxytetracycline effect on radish growth

Figure 37 displays the effect of three oxytetracycline concentrations (0, 5 and 10 mg/kg) on the entire radish weight, root, bulb and leaf and in Table 32 in the appendix.

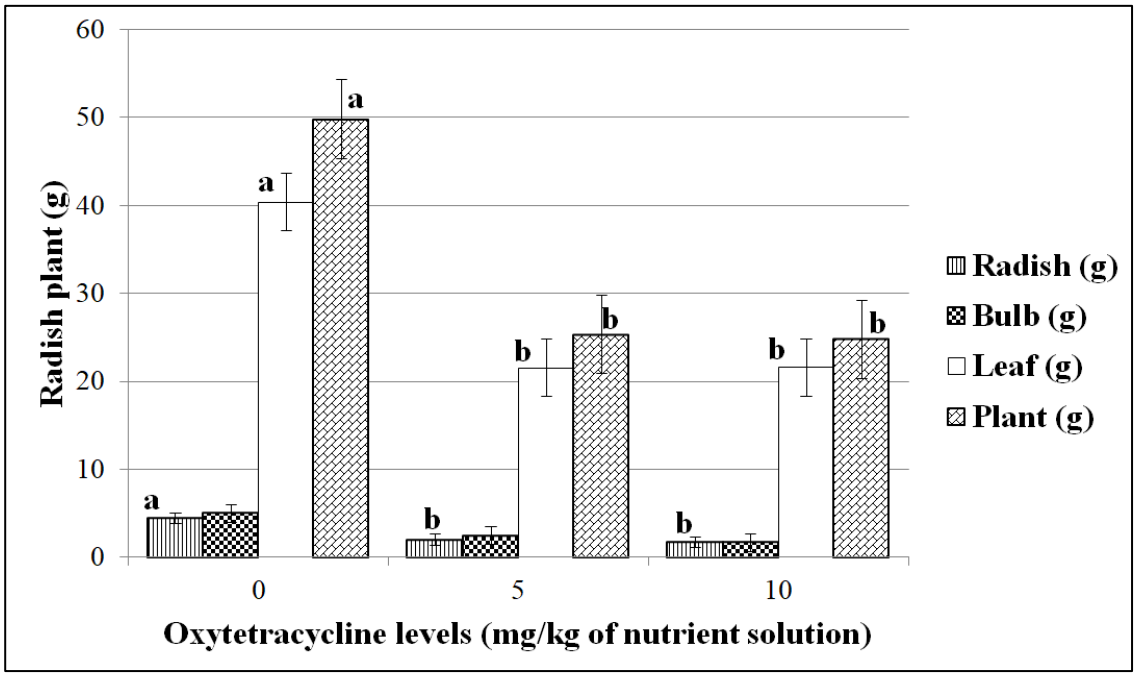


Figure 37. Weight of radishes administered with oxytetracycline and grown hydroponically

The results in Figure 37 show that there is a significant decrease between the control and the other two levels (5 and 10 mg/kg), except for the bulbs. Similarly to previous results, the difference was also greatly observed on radishes at harvest (Figure 38). Hillis et al. (2011) findings assert our results where roots of lettuce, carrot and alfalfa administered with oxytetracycline concentrations of 1 and 10 mg/L endured in a decreasing order notable reduction in length: this decrease was significant. Also, Zhao-Jun, Xiao-Yu, ZHANG, and LIANG (2011) findings where that the shoot length and biomass of wheat decreased significantly when oxytetracycline was available; a decrease of 5.61% in dry biomass and 13.75% in shoot length. Although oxytetracycline significantly accumulated in bulbs in both tested levels (Figure 24), this did not significantly affect the bulb's weight. Consequently, in radishes, oxytetracycline reduces the roots and leaves weight, but not the bulb's weight. F. Liu et al. (2009) statement contradicts our results where tetracyclines increased radish yields in soils.

Additionally, the absence of significance between both oxytetracycline levels implies that as the concentration of oxytetracycline increased from 5 to 10 mg/g in the media, its effect on radish did not increase. Contrarily, Zhao-Jun et al. (2011) study contradicts this finding whereby the decrease in biomass and shoot length was more prominent as the concentration of oxytetracycline increased from 0.01 to 0.08 mmol/L.

Figure 38. Comparison of radish crops at harvest in all three treatments of oxytetracycline (a) control (b) 5 mg/kg (c) 10 mg/kg

d. Comparison of enrofloxacin, tylosin and oxytetracycline on radish growth

Figure 39 compares between the effect of enrofloxacin, tylosin and oxytetracycline on radish weight in its different plant parts: roots, bulbs and leaves. The average weight of the three levels in each antibiotic is tabulated on the graph below.

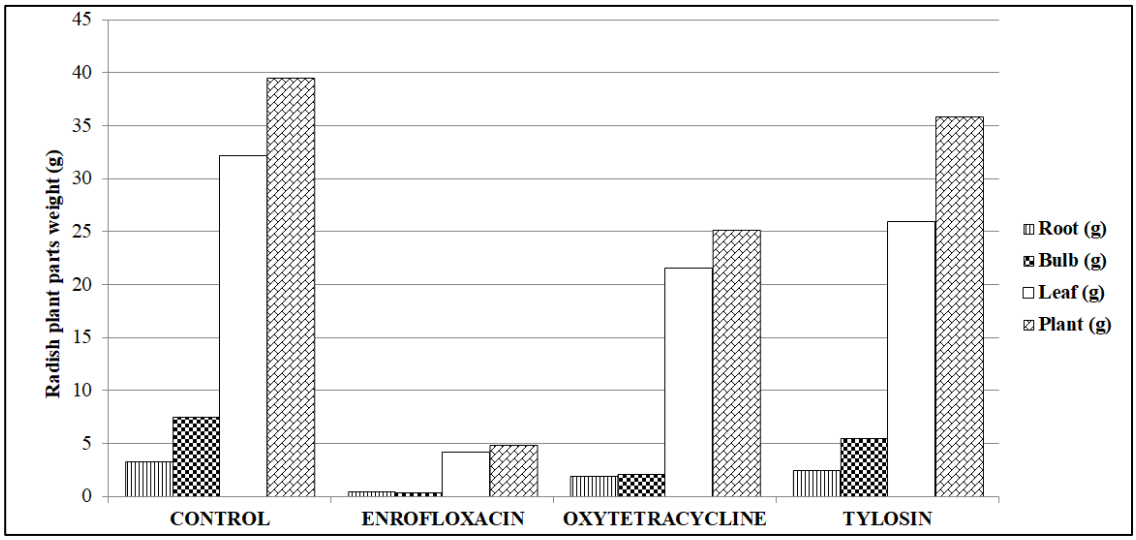


Figure 39. Comparison of enrofloxacin, oxytetracycline and tylosin effect on radish weight grown hydroponically

As mentioned earlier, tylosin caused a slight non-significant decrease in radish weight, followed by oxytetracycline and enrofloxacin caused a drastic effect on radish growth. In Figure 39, compared to the average plant weight control, radishes grown in tylosin, oxytetracycline and enrofloxacin decreased by 9.29%, 36.56% and 87.91% respectively. Moreover, the bulb and the root of the radish were the most affected parts in all three antibiotics (a decrease of 92.49%, 57.46% and 26.1% with enrofloxacin, oxytetracycline and tylosin respectively).

Nevertheless, leaves were the least affected parts. Compared to the control, tylosin and oxytetracycline hold a greater weight (least affected) than the ones with enrofloxacin; 19.2%, 33.03% and 87.04% decrease respectively. Consequently, the decreasing order in which these antibiotics affect radish weight negatively is: enrofloxacin>oxytetracycline>tylosin.

As for toxicity, it was observed that enrofloxacin depicted the greatest toxicity on both lettuces and radishes. F. Liu et al. (2009) informs that antibiotics detain different toxic consequences because of their different performance in soil: adsorption,

degradation and chelation with metals play a main role for tylosin and tetracyclines toxicity effects.

E. Antibiotic persistence in soil

In this experiment, the persistence of enrofloxacin, tylosin and oxytetracycline in soil was tested and a sample was extracted every week for a period of 2 months. The extraction was performed using water. An initial concentration of 5 mg/kg of each antibiotic was added into each pot (5 kg soil/pot), which was replicated three times.

1. Enrofloxacin persistence in soil

Figure 40 below shows the decrease in the concentration of the extracted enrofloxacin using water (Table 33 in appendix).

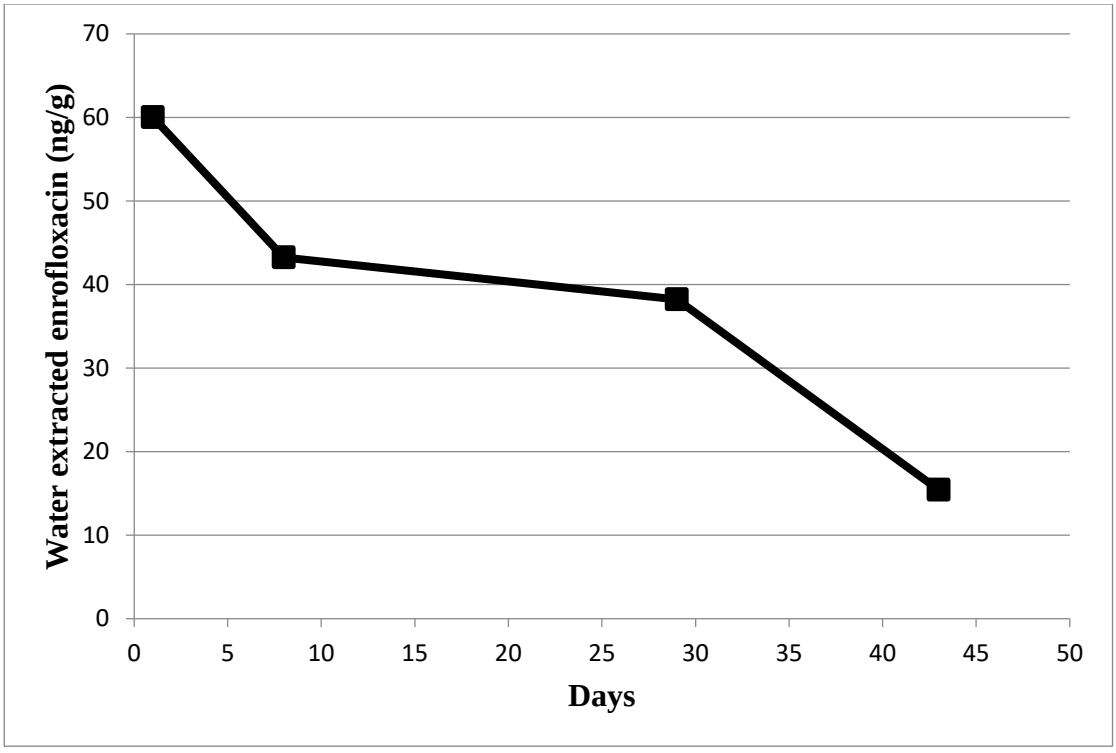


Figure 40. Concentration of extracted enrofloxacin using water

The results obtained and graphed in Figure 40 reveal that on day 1, water extracted 60 ng/g enrofloxacin and on day 43, 2.83 ng/kg. This denotes that 43 days after the application of enrofloxacin in soil, 75% degraded. The results speculate that enrofloxacin persists in the environment for more than 43 days and its soil half-life can be estimated to be around 25 days. This is not in accordance with van der Marel (2013) who states that fluoroquinolone half-life is greater than 50 days and Trouchon and Lefebvre (2016) who states that ciprofloxacin half-life vary between 1,155 and 3,466 days in soil. Lillenberg et al. (2010) affirms that the lower the fluoroquinolone concentration, the greater the adsorption rate to the soil; hence the lower the degradation. The adsorption rate was estimated to be close to 100% for both enrofloxacin and ciprofloxacin and due to synergism, the antimicrobial activity of enrofloxacin is enhanced in the presence of ciprofloxacin (Lillenberg et al., 2010).

2. Tylosin persistence in soil

Figure 41 below displays the degradation of tylosin through its extracted concentration over 2 months using water (Table 34 in appendix).

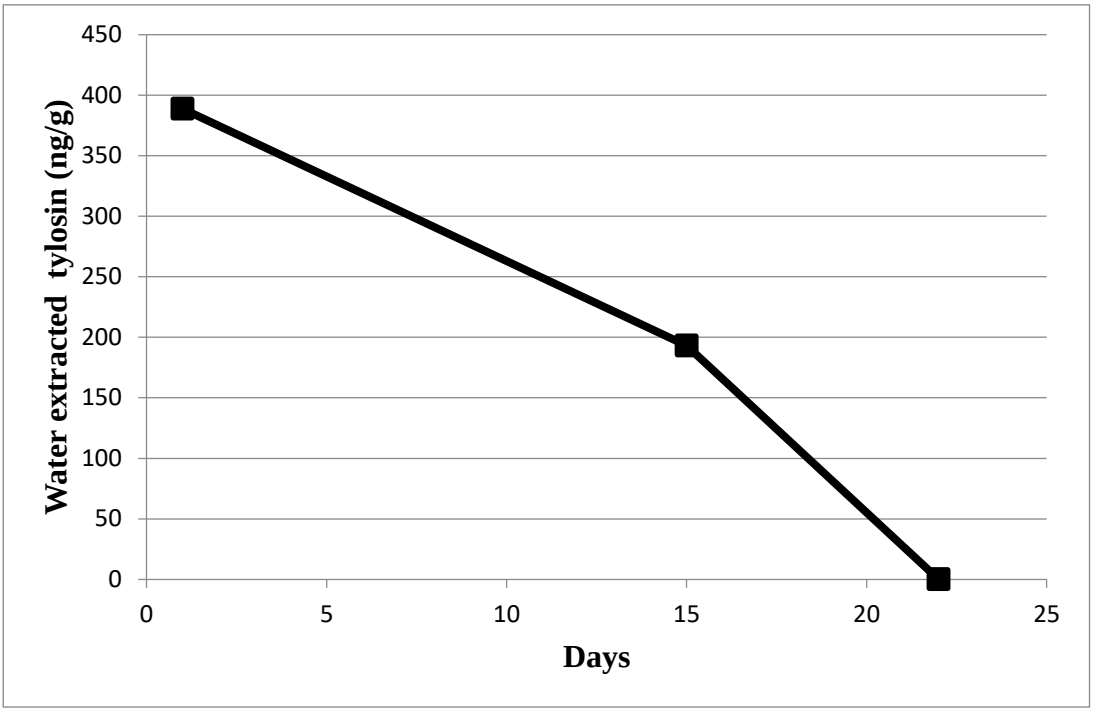


Figure 41. Concentration of extracted tylosin using water

The results obtained and graphed in Figure 41 indicate that on day 1, water extracted 388.7 ng/g of tylosin and on day 22, no amount of tylosin was extracted; tylosin degraded completely. Consequently, tylosin does not persist in the environment for more than 22 days. D. Hu and Coats (2007) proclaim that the two main factors that influence tylosin loss in the environment are abiotic degradation and sorption. Their findings are in accordance with our results, whereby tylosin A and tylosin D have a half-life in soil of 7 and 8 days respectively. Nevertheless, D. Hu and Coats (2007) also reveal that the half-life of tylosin in water is of 200 days.

3. Oxytetracycline persistence in soil

Figure 42 below shows the decrease in the concentration of the extracted oxytetracycline using water (Table 35 in appendix).

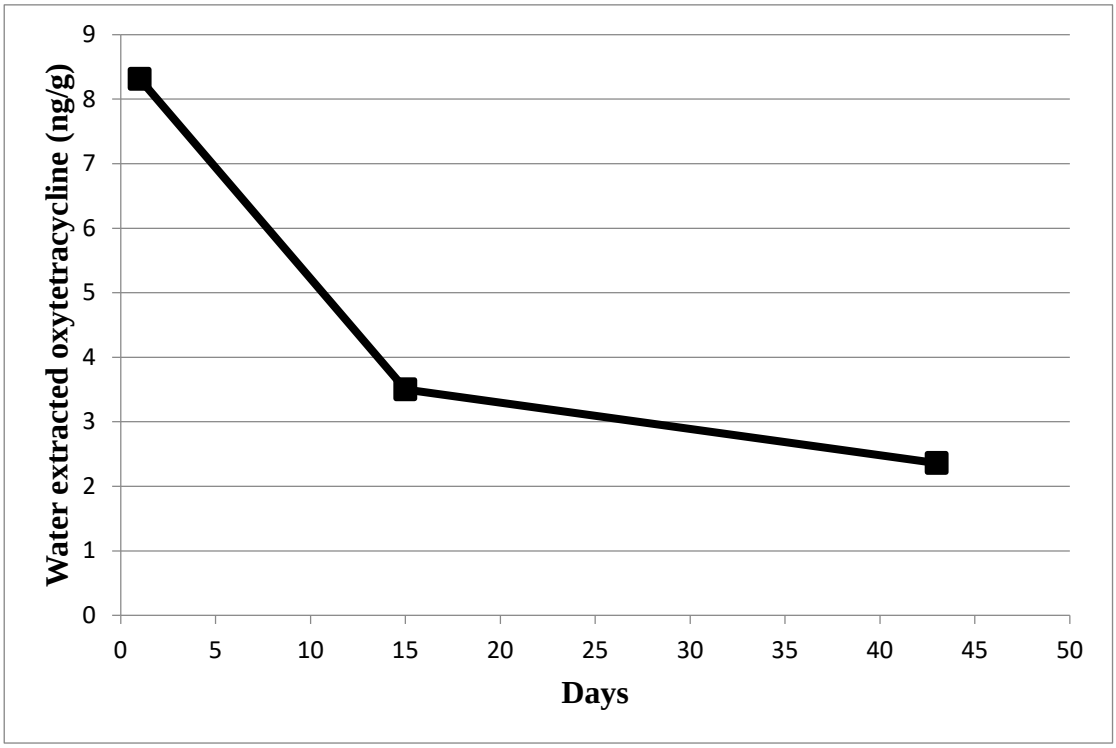


Figure 42. Concentration of extracted oxytetracycline using water with respect to time

The results obtained and graphed in Figure 42 reveal that on day 1, water extracted 8.31 ng/g oxytetracycline and on day 43, the extracted amount of oxytetracycline decreased gradually till 2.36 ng/g. This shows a 70% degradation of oxytetracycline in soil after 43 days of application. Therefore, oxytetracycline persists in the environment for more than 43 days and its half-life in soil can be estimated to be around 24 days. Halling-Sørensen, Sengeløv, and Tjørnelund (2002) asserted that tetracyclines chelate with both divalent and trivalent metal ions like Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+}, and Fe^{3+}. This suggests that in the soil, the levels of metals will disturb the degradation and effectiveness of the antibiotic. Our results are contradicted by van der Marel (2013) who announced that the half-life of oxytetracycline is greater than 100 days and by Wei, Wu, Nie, Yediler, and Wong (2009) who indicated that around 30% of tetracycline was degraded in a period of 60 days. Pan and Chu (2016a) experiment

showed that among 5 different antibiotics, tetracycline had the highest level of adsorption, lowest grade of degradation and highest phytotoxicity.

4. Comparison of enrofloxacin, tylosin and oxytetracycline persistence in soil

Figure 43 below compares between degradation of enrofloxacin, tylosin and oxytetracycline and their persistence in the environment.

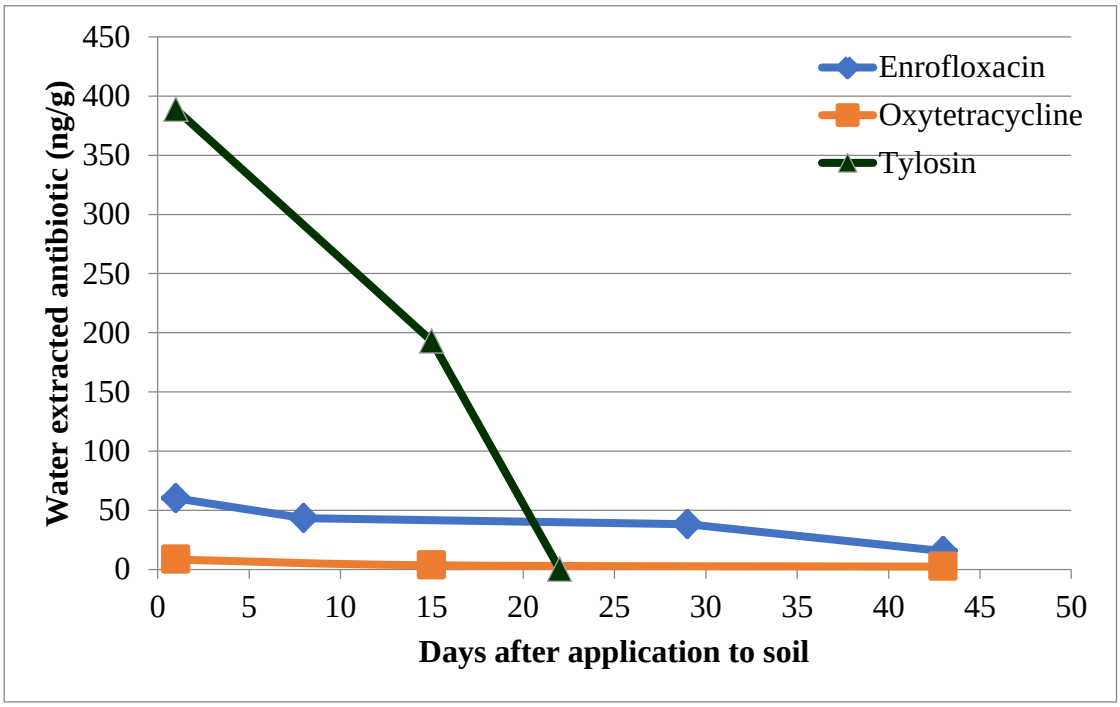


Figure 43. Comparison of extracted enrofloxacin, oxytetracycline and tylosin

Comparing the three antibiotics to each other, it is observed that from day 1 till day 43, enrofloxacin and oxytetracycline decreased gradually until around 25% of each remained in the soil, whereas tylosin degraded completely on day 22. Accordingly, tylosin does not persist in the environment for more than 22 days, whereas enrofloxacin

and oxytetracycline do for more than 43 days. The persistence of oxytetracycline can be explained by its positive charge which allows it to greatly adsorb to the negatively charged soil particles, hence it is harder to be free in the soil and be subjected to degradation. Jia et al. (2008) justify oxytetracycline high adsorption ability in soil to be due to CEC, soil texture, oxides and organic matter. The persistence of enrofloxacin could be explained by its high adsorption to soil due to cation exchange (Leal, Alleoni, Tornisielo, & Regitano, 2013). D. Hu and Coats (2007) clarify that soil detaining different properties, adsorb almost 100% of fluoroquinolones; desorption is low. They also attribute this behavior with clays abilities to adsorb plane anionic substrates with the polarity of fluoroquinolones compounds, thus suggesting that hydrophobicity independent mechanisms are involved (cation bridging, cation exchange, hydrogen bonding, surface complexation) (D. Hu & Coats, 2007). Additionally, Leal et al. (2013) report that the sorption of fluoroquinolones decreases at higher levels although it is concentration dependent.

SUMMARY, CONCLUSION AND RECOMMENDATIONS

In recent decades, worldwide as well as in Lebanon antibiotics have been extensively used in agriculture and animals as a mean to treat and prevent illnesses, to promote growth (animal fattening) and to increase feed efficiency. Antibiotics enter the environment through wastewater irrigation, biosolids and animal manure used to fertilize agricultural land, thus finding their way into the food chain; some antibiotics remain persistent in the soil from days to months. The antibiotics extensive usages have raised questions on their limits, sources and fate in the environment as well as for their hazards to humans. Therefore the objectives of this research are to evaluate the uptake and accumulation site of enrofloxacin, tylosin, oxytetracycline and gentamicin in water and soil cultures by lettuce, cucumber and radish crops and investigate their effects on the crop growth as well as study the persistence of enrofloxacin, tylosin and oxytetracycline in soil.

A pot experiment where lettuce and cucumber crops were grown in soil cultures mixed without and with 5% manure was conducted to study the uptake and accumulation site of enrofloxacin and gentamicin as well as the effect of manure on the absorption of antibiotics by crops (lettuce and cucumber).

For enrofloxacin,

- A higher enrofloxacin level in the soil led to a greater accumulation in lettuce leaves, but not in lettuce roots
- Manured soil increased the absorption and accumulation of enrofloxacin in both lettuce and cucumber by around 25%

- As the level of enrofloxacin increased in the pot, its accumulation in cucumber leaves and fruits increased significantly, but in roots it was not significant
- Enrofloxacin was absorbed and accumulated in cucumber roots at an average of 4.29 ng/g and in fruits and leaves at an average of 3.23 and 10.66 ng/g respectively
- The accumulation of enrofloxacin in cucumbers in a decreasing order is: leaf>root>fruit
- The accumulation of enrofloxacin in the cucumber fruit and lettuce leaves did not exceed the MRL range

For gentamicin,

- Manured soil increased the accumulation of gentamicin in the cucumber fruit: there was an 80% increase
- Gentamicin was absorbed and accumulated in roots at an average of 3.36 ng/g and translocated to leaves and fruits at an average of 5.36 ng/g and 9.78 ng/g respectively
- The accumulation of gentamicin in cucumbers in a decreasing order is fruit>leaf>root
- In presence of manure, gentamicin accumulated in lettuce leaves at a level greater than the MRL limit whereas in the absence of manure, its accumulation level was acceptable
- In cucumber fruits, gentamicin accumulated at a level that falls within the MRL level

To study the major accumulation sites of enrofloxacin, tylosin and oxytetracycline, and the effect of antibiotics charge on their uptake by crops, lettuce and radish were grown hydroponically in a greenhouse, administered with nutrient solution and the antibiotic separately

For enrofloxacin,

- In both lettuce and radish parts, a higher antibiotic level did not lead to a higher antibiotic accumulation
- Although enrofloxacin was distributed all over the plant parts roots:bulbs:leaves at 1:1:1 ratios (uniform concentrations), it was stored the highest in the bulb at an average of 67.68 ng/g
- Enrofloxacin and oxytetracycline are the two antibiotics that accumulated the least in the radish tissues (less than 67.68 ng/g) compared to tylosin (more than 220 ng/g)
- In radish edible parts (bulb and leaves), enrofloxacin accumulated at all levels at a concentration greater than the MRL range

For tylosin,

- Tylosin accumulated the most in lettuce leaves at an average of 343.83 ng/g and in roots at an average of 218.94 ng/g
- All parts of the radish accumulated tylosin at an average of 222.98 ng/g for roots, 384.26 ng/g for leaves and 407.45 ng/g for bulbs
- In both radish and lettuce, as the concentration of tylosin increased, its accumulation did not significantly increase
- In both radish and lettuce, the sequence of distribution in a decreasing order is: tylosin>enrofloxacin>oxytetracycline

- At all tylosin levels, accumulation of the antibiotic in radish and lettuce edible parts (bulb and leaves) was 4 times greater than the MRL acceptable upper limit

For oxytetracycline,

- In lettuce, roots accumulated oxytetracycline to a certain limit (around 20.43 ng/g) and then the excess was translocated to the leaves at an average of 6.83 ng/g
- In radish, the edible parts accumulated oxytetracycline at an average of 30.98 ng/g in the bulb and 22.76 ng/g in the leaf
- In water, oxytetracycline was absorbed by both lettuce and radish, thus implying that its charge is the reason why it was not absorbed by crops grown in soil
- Oxytetracycline accumulated the least in lettuce leaves. Among enrofloxacin and tylosin at an average of 6.83 ng/g compared to 59.39 and 343.83 ng/g respectively
- The sequence of accumulation of oxytetracycline in radish crops in a decreasing order goes as follows: roots>bulbs≥leaves
- In radish, as the level of oxytetracycline increased in the nutrient solution, its accumulation in bulbs and leaves increased as well. In roots it did not increase significantly
- In lettuce and radish edible parts (bulb and leaves), at all oxytetracycline levels, it accumulated within the acceptable MRL range

To study the effect enrofloxacin, tylosin and oxytetracycline on plant growth, lettuce and radish were grown hydroponically in a greenhouse, administered with nutrient solution and the antibiotic separately.

For enrofloxacin,

- Enrofloxacin reduced lettuce crop weight by 71.36%
- Enrofloxacin reduced the overall radish crop weight by 87.91% and radish roots and bulbs weight by 92.49%
- A higher enrofloxacin level in the nutrient solution did not lead to a significant change on both lettuce and radish growth
- Visually, as the level of enrofloxacin increased, phytotoxicity was more pronounced on both crops

For tylosin,

- Lettuce plant weight decreased in the presence of tylosin by 17.89%; however, the decrease was mild and not significant
- Tylosin had only a negative impact on lettuce roots: 37.29% decrease
- In radishes, tylosin had no significant impact on the crop weight; a 9.29% decrease

For oxytetracycline,

- Overall weight of lettuce decreased by 65.97% and that of radish by 36.56%
- Lettuce and radish plant roots weight decreased by 72.03% and 42.78% respectively
- Radish bulb weight decreased by 72.14%

- In radishes, a higher oxytetracycline level did not cause a significant impact on radish weight, but in lettuce it was only significant on leaves weight (64.81% decrease in lettuce leaves weight)

To investigate the persistence of enrofloxacin, tylosin and oxytetracycline in soil, a pot experiment was conducted in a greenhouse, and sampling with extraction using water was done weekly. The results of the experiment can be summarized in the following points:

- 75% of enrofloxacin degraded in 43 days
- Enrofloxacin half-life in sandy loam soil can be estimated to be around 25 days
- 100% of tylosin degraded in 22 days or less
- 70% of oxytetracycline degraded in 43 days
- Oxytetracycline half-life in sandy loam soil can be estimated to be around 24 days; the persistence of oxytetracycline can be explained by its positive charge which allows it to greatly adsorb to the negatively charged soil particles, hence it is harder to be free in the soil and be subjected to degradation

Our results have clarified one of the reasons why oxytetracycline was not absorbed and accumulated by crops grown in soil as well as it demonstrated the fate of some of the most used veterinary antibiotics and their effect on plant growth. Although the manifestation, effects and fate of antibiotics have been scientifically studied, it is often challenging to have a valid explicit comparison due to the different experimental schemes and laboratory conditions the studies are performed under as well as due to the

contradictory results obtained. Regarding the actual hazard it plays on human health and the environment, data still lacks. Nevertheless, some recommendations are:

1. Reduce the preventive use of antibiotics in animal feed and apply a monitoring system
2. Educate consumers, veterinarians as well as farmers on the risks of antibiotic resistance
3. Several studies are necessary to tackle different issues such as field studies, sediment/soil sorption and degradation studies

APPENDIX

Table 17. List of different modes of action of antibiotics and some examples

Mode of action	Comment	Examples
Inhibitors of cell wall synthesis	Selectively inhibit or kill bacteria by targeting the cell wall	Penicillins, vancomycin, cephalosporins, and bacitracin
Inhibitors if cell membrane function	The selection of this class of antibiotics is poorly selective as the cell membrane is found in both prokaryotes and eukaryotes. The disruption of the cell membrane could result in the leakage of essential solutes for the survival of the cell.	Colistin and polymixin B
Inhibitors of protein synthesis	Protein synthesis is crucial for the survival and multiplication of bacterial cells. This class' antibiotics bind to the intracellular ribosomes 30S and 50S subunits, thus leading to the disruption of the bacteria normal cellular metabolism.	**Aminoglycosides,** lincosamides, **macrolides, tetracyclines,** streptogramins, and chloramphenicol
Inhibitors of nucleic acid synthesis	This class works by binding to parts involved in the synthesis of RNA and DNA which alters normal cellular processes thus compromising bacterial proliferation and survival.	Metronidazole, quinolones, **fluoroquinolones,** and rifampin
Folic acid synthesis inhibitors	The folic acid pathway is essential for the production of precursors essential for DNA synthesis.	Trimethoprim and sulfonamides

Table 18. Concentrations of enrofloxacin in lettuce roots and leaves in soil

Manure (mg/kg)	Enrofloxacin Concentration (ng/g)	
	Root	**Leaf**
0	8.6^{b}	8.47^{b}
5	11.24^{a}	10.28^{a}
SEM*	**0.394**	**0.486**
Enrofloxacin level (mg/kg)	**Enrofloxacin Concentration (ng/g)**	
0	0^{c}	0^{d}
5	9.58^{b}	7.66^{c}
10	14.46^{a}	13.23^{b}
20	15.63^{a}	16.62^{a}
SEM*	**0.557**	**0.687**
Probabilities		
Manure	0.0002	0.018
Drug Concentration Level	0.0001	0.0001
Treatment x Drug Concentration Level	0.0408	0.3263

*SEM: Standard Error Mean

For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 19. Concentrations of enrofloxacin in cucumber roots, leaves and fruits in soil

Manure	Enrofloxacin Concentration (ng/g)		
	Root	**Leaf**	**Fruit**
0	2.2	6.48^{b}	1.7^{b}
5	3.75	9.55^{a}	3.15^{a}
SEM*	**0.616**	**0.393**	**0.407**
Enrofloxacin level (mg/kg)	**Enrofloxacin Concentration (ng/g)**		
0	0^{b}	0^{d}	0^{c}
5	3.11^{a}	7.16^{c}	0.24^{c}
10	5.86^{a}	11.45^{b}	3.02^{b}
20	3.90^{a}	13.37^{a}	6.44^{a}
SEM*	**0.869**	**0.556**	**0.575**
Probabilities			
Manure	0.0186	0.0001	0.0225
Drug Concentration Level	0.0027	0.0001	0.0001
Treatment x Drug Concentration Level	0.003	0.0043	0.1982

*SEM: Standard Error Mean

For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 20. Concentrations of gentamicin in cucumber roots, leaves and fruits in soil

Manure (mg/kg)	Enrofloxacin Concentration (ng/g)		
	Root	**Leaf**	**Fruit**
0	3.93^{a}	7.52	1.40^{a}
5	0.99^{b}	7.15	6.63^{b}
SEM*	**0.402**	**0.703**	**0.197**
Gentamicin level (mg/kg)	**Gentamicin Concentration (ng/g)**		
0	0^{c}	0^{d}	0^{c}
5	2.13^{b}	3.11^{c}	2.19^{b}
10	$0.79^{b,c}$	6.77^{b}	7.19^{a}
20	7.16^{a}	19.47^{a}	6.69^{a}
SEM*	**0.568**	**0.994**	**0.350**
Probabilities			
Manure	0.0001	0.711	0.0013
Drug Concentration Level	0.0001	0.0001	0.0173
Treatment x Drug Concentration Level	0.0001	0.1499	0.0111

*SEM: Standard Error Mean

For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 21. Concentration of enrofloxacin in lettuce roots and leaves grown in water

Enrofloxacin level (mg/kg)	Enrofloxacin concentration (ng/g)	
	Root	**Leaf**
0	0^{b}	0^{b}
5	50.80^{a}	48.99^{a}
10	54.20^{a}	69.78^{a}
SEM*	**5.142**	**7.419**
Probabilities		
Drug Concentration Level	0.0022	0.0031

*SEM: Standard Error Mean

For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 22. Concentration of tylosin in lettuce roots and leaves grown in water

Tylosin level (mg/Kg)	Tylosin concentration (ng/g)	
	Root	**Leaf**
0	0^{b}	0^{b}
5	0^{b}	374.47^{a}
10	437.87^{a}	313.19^{a}
SEM*	**20.098**	**50.955**

Probabilities		
Drug Concentration Level	0.0002	0.0042

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 23. Concentration of oxytetracycline in lettuce roots and leaves grown in water

Oxytetracycline level (mg/kg)	Oxytetracycline concentration (ng/g)	
	Root	**Leaf**
0	0^{b}	0^{b}
5	20.44^{a}	4.06^{b}
10	20.42^{a}	9.59^{a}
SEM*	**1.992**	**1.529**
Probabilities		
Drug Concentration Level	0.001	0.0125

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 24. Concentration of enrofloxacin in radish root, bulb and leaf grown in water

Enrofloxacin level (mg/kg)	Enrofloxacin concentration (ng/g)		
	Root	**Leaf**	**Bulb**
0	0^{b}	0^{b}	0^{b}
5	53.64^{a}	55.18^{a}	63.04^{a}
10	55.94^{a}	49.16^{a}	72.31^{a}
SEM*	**2.533**	**4.692**	**11.118**
Probabilities			
Drug Concentration Level	0.0001	0.0011	0.0145

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 25. Concentration of tylosin in radish root, bulb and leaf grown in water

Tylosin level (mg/kg)	Tylosin concentration (ng/g)		
	Root	**Leaf**	**Bulb**
0	0^{b}	0^{b}	0^{b}
5	201.70^{a}	410.64^{a}	386.51^{a}
10	244.25^{a}	357.87^{a}	428.38^{a}
SEM*	**43.016**	**20.437**	**25.243**
Probabilities			

Drug Concentration Level	0.0181	0.0001	0.0001

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 26. Concentration of oxytetracycline in radish root, bulb and leaf grown in water

Oxytetracycline level (mg/kg)	Oxytetracycline concentration (ng/g)		
	Root	**Leaf**	**Bulb**
0	0^{b}	0^{c}	0^{c}
5	45.22^{a}	19.75^{b}	23.03^{b}
10	49.48^{a}	25.77^{a}	38.92^{a}
SEM*	**5.217**	**1.025**	**2.296**
Probabilities			
Drug Concentration Level	0.0019	0.0001	0.0001

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 27. Average total, root and leaf weight of lettuce grown in water spiked with enrofloxacin

Enrofloxacin level (mg/kg)	Average lettuce weight (g)		
	Plant weight	**Root Weight**	**Leaf weight**
0	64.45^{a}	6.22^{a}	58.11^{a}
5	19.11^{b}	0.94^{b}	18.11^{b}
10	16.89^{b}	0.84^{b}	16.83^{b}
SEM*	**3.164**	**0.409**	**3.275**
Probabilities			
Drug Concentration Level	0.0001	0.0003	0.0004

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 28. Average total, root and leaf weight of lettuce grown in water spiked with tylosin

Tylosin level (mg/kg)	Average lettuce weight (g)		
	Plant weight	**Root Weight**	**Leaf weight**
0	68.11	6.22^{a}	61.78
5	50.33	3.78^{b}	46.67
10	52.89	3.44^{b}	48.89
SEM*	**5.439**	**0.416**	**5.213**
Probabilities			

Drug Concentration Level	0.1177	0.0062	0.1669

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 29. Average total, root and leaf weight of lettuce grown in water spiked with oxytetracycline

Oxytetracycline level (mg/kg)	Average lettuce weight (g)		
	Plant weight	**Root Weight**	**Leaf weight**
0	56.00^{a}	4.83^{a}	47.33^{a}
5	25.45^{b}	1.67^{b}	23.45^{b}
10	17.33^{b}	1.55^{b}	15.78^{c}
SEM*	**2.565**	**0.354**	**1.756**
Probabilities			
Drug Concentration Level	0.0004	0.003	0.0002

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 30. Average of total, root, bulb and leaf weight of radish grown in water spiked with enrofloxacin

Enrofloxacin level (mg/kg)	Average radish weight (g)			
	Plant weight	**Root Weight**	**Leaf weight**	**Bulb Weight**
0	32.84^{a}	3.40^{a}	25.11^{a}	4.29^{a}
5	4.22^{b}	0.31^{b}	3.78^{b}	0.30^{b}
10	5.33^{b}	0.41^{b}	4.55^{b}	0.26^{b}
SEM*	**1.394**	**0.513**	**1.437**	**0.646**
Probabilities				
Drug Concentration Level	0.0001	0.0085	0.0001	0.0097

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 31. Average total, root, bulb and leaf weight of radish grown in water spiked with tylosin

Tylosin level (mg/kg)	Average radish weight (g)			
	Plant weight	**Root Weight**	**Leaf weight**	**Bulb Weight**
0	35.89	1.78	31.00	12.89
5	38.11	2.33	25.17	4.33
10	33.56	2.45	26.78	6.5
SEM*	**6.841**	**0.843**	**4.919**	**4.848**
Probabilities				
Drug Concentration Level	0.8968	0.8388	0.7257	0.4565

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 32. Average of total, root, bulb and leaf weight of radish grown in water spiked with oxytetracycline

Oxytetracycline level (mg/kg)	Average radish weight (g)			
	Plant weight	**Root Weight**	**Leaf weight**	**Bulb Weight**
0	49.78^{a}	4.44^{a}	40.33^{a}	5.00
5	25.34^{b}	2.00^{b}	21.50^{b}	2.45
10	24.78^{b}	1.67^{b}	21.56^{b}	1.67
SEM*	**4.457**	**0.623**	**3.252**	**1.020**
Probabilities				
Drug Concentration Level	0.0154	0.038	0.0131	0.13

*SEM: Standard Error Mean
For each factor a, b in a column with different superscript differ significantly (P<0.05)

Table 33. Concentration of extracted enrofloxacin from soil over 43 days

Enrofloxacin Persistence extracted with water	
Day of extraction	**Concentration (ng/g)**
1	60
8	43.21
29	38.22
43	15.41

Table 34. Concentration of extracted tylosin from soil over 43 days

Tylosin Persistence extracted with water	
Day of extraction	**Concentration (ng/g)**
1	388.7
15	193.04
22	0

Table 35. Concentration of extracted oxytetracycline from soil over 43 days

Oxytetracycline Persistence extracted with water	
Day of extraction	**Concentration (ng/g)**
1	8.31
15	3.5
43	2.36

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