Impacts of Ciprofloxacin on Plant Growth and Soil Microbial Biomass

By

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Certificate

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Dedication

To my parents, for allowing me to follow my ambitions and supporting me through it all, for helping me be the person I am today; for helping me out every step of the way. I will always treasure your unconditional support.

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List of Abbreviations

AAU	Agricultural Antibiotic Use
AMK	Amika
ARGs	Antibiotic-Resistance Genes
ATC	Anatomic Therapeutic Chemical
CAZ	Ceftazidime
CIP	Ciprofloxacin
CTC	Chlorotetracycline
DDD/1000	Defined Daily Doses per 1000 people
E. coli	*Escherichia coli*
ENR	Enrofloxicain
EMEA	European Agency for Evaluation of Medicinal Products
HPA	Health Protection Agency
FQs	Fluoroquinolones
IPM	Imipenem
OTC	Oxytetracycline
P. aeruginosa	*Pseudomonas aeruginosa*
STPs	Sewage Treatment Plants
SMZ	Sulfamethoxazole
TC	Tetracycline
WWTP	Wastewater Treatment Plant

Abstract

Antibiotics are finding their way into different compartments of environment and causing damage to non-target organisms. In present study, the effects of ciprofloxacin (CIP) stress at various concentrations on plant growth and soil microbial biomass were investigated. For ***plant growth test***, pot experiment was performed with CIP as an amendment and the effect was evaluated by harvesting the plants after 28 days of sowing and measuring their lengths and biomass. Soil microbial biomass, organic matter and microbial growth were determined using chloroform-fumigation extraction (CFE), Walkey-Black procedure and optical density measurements, respectively. In CFE, the increase in extractable ***microbial carbon*** following fumigation of soil with chloroform is determined. For Walkley-Black chromic acid wet oxidation method, ***organic matter*** in the soil is oxidised by chromic acid solution. To test ***microbial growth***, change in optical density was measured using spectrophotometer to determine the concentration of bacteria in suspension. The results showed that CIP impacted shoot length significantly only at the highest concentration of 200 mg L^{-1} whereas no effect was observed on root length. The fresh biomass of roots suffered a decline of 9.6 and 8.9% at 100 and 200 mg L^{-1} while the decline of 35 and 44.5% was observed for fresh shoot biomass. The negative impacts of CIP were much more prominent in case of soil microbial biomass. Using chloroform-fumigation extraction, the decline observed in the microbial biomass after 15 day's incubation period was 0.089, 7.96, 21.1, 30.9 and 48.1% at 50 (C1), 75 (C2), 100 (C3), 150 (C4) and 200 mg L^{-1} (C5), respectively. Organic matter also observed a declining trend with increase in CIP concentrations from C1 to C3 after which it stabilized. The soil microbial population in terms of optical density over 3 days, exposure showed an increased potency of the drug at high concentrations. Zone of inhibition test reaffirmed inhibitory effects of CIP. The zone increased from 12.6 to 24.9 cm at CIP concentrations from 2.5-200 mg L^{-1} for *E. coli*. While for *P. aeruginosa*, the inhibition zone observed was from 11.5 to 28 starting from CIP concentration of 5 mg L^{-1}

Chapter 1

INTRODUCTION

1.1. Background

Antibiotics can be defined as naturally produced compounds that target certain features of a bacterial physiology by either stunting its growth or destroying the cell altogether. In a wider spectrum, the term antibiotic refers to any agent that hinders the proliferation of microbes including fungi, algae, bacteria or protozoa.

Antibiotics have been in use for hundreds of years but their official discovery was first made in 1928 by a chemist, Alexander Fleming, who observed the inhibitory effect of penicillium on bacterial growth. Penicillin and streptomycin, from genus penicillium of fungus and streptomycetes of bacteria, mark the origin of natural antibiotics. Following the discovery of penicillin, a large number of antimicrobial agents isolated from microbes were discovered. These antibiotics found their way to the market and using these chemicals as test subjects, new generations of antibiotics were developed.

1.2. Classification

Classification of antibiotics can be done on the basis of bacterial spectrum, administration route, bactericidal activity or the origin, whether natural or manufactured. The most widely used and significant classification method however lies in the chemical structure of the drug. The antibiotics in a specific class developed on the basis of structure usually contain similar characteristics including toxic effects, allergic reactions and effectiveness patterns.

1.3. General Use Pattern

Since their discovery in 1900s, antibiotics have been in vigorous use for the protection of public health from microbial infections, disease diagnosis and growth promotion in animals (Fink *et al*. 2012). This growth promotion is attributed to four major mechanisms (Gaskins *et al*., 2002) namely decrease in microbial utilization of nutrients, enhanced nutrient uptake via thinner intestinal wall of antibiotic fed animals,

reduction of infections and a decline in growth inhibiting microbial metabolites. Currently, more than 5000 chemicals are registered for use as medicines for humans and animals (García *et al*., 2013) with a global annual consumption reported as high as 100,000 to 200,000 tons (Jellic *et al*., 2011). Such large consumption has led to significant release of these compounds into the environment. The presence of active compounds of pharmaceutical origin was first reported in 1970 (Gosh *et al*., 2009) but it gained attention as an emerging pollutant in late mid 1990s with the development of analytical technologies (Homem and Santos, 2011).

1.4. Fate of Antibiotics

About 10-90% of the drug consumed is passed into the environment in unadulterated form via gastrointestinal tract (Pereira *et al*., 2012). These drugs end up in the agricultural fields through manure application (Moraru *et al*., 2012) due to its excess in nitrogen and phosphorous nutrition (Bolan *et al*, 2010), irrigation of agricultural land with reclaimed waste water (Gulkowska *et al*., 2008; Kemper, 2008), grazing animals (Ferro *et al*., 2010) and wastewater treatment plants (Senta *et al*., 2013).

1.5. Environmental Threat

The rampant release of drugs into the environment poses a threat that is not yet completely realized (De Graaff *et al*., 2011). On entering the soil, these drugs may damage plant growth (Du *et al*., 2012) when taken up by the roots. Extensive work has not yet been done on the plant uptake of these antibiotics from soil; however several researches show that these drugs are taken up by the plants and cause minor or major damage to their growth and physiology.

Fluoroquinolones (FQs) that find their way into the environment via manure application accumulate owing to their low degradability in soil and strong adsorption to organic matter (Leal *et al*., 2012). Special attention is given to them as manure contaminating antibiotics due to their intensive use for treatment of animal diseases particularly respiratory ailments (ANSES, 2012; European Medicines Agency, 2012). Excessive application of these antibiotics and their metabolites' contaminated manure may result in development of resistance in soil bacteria (Ghosh and LaPara, 2007).

1.6. Scope of study

Antibiotics play a crucial role in the treatment and prevention of a considerable number of infections in humans, animals and plants. However, as the bulk of these drugs find its way into the environment, it can result in serious consequences for the ecosystem by impacting not only the target organisms but also the non-target ones. Soil is one of the most affected environmental compartment exposed to pollutants and as such it is vital to assess the risks associated with these drugs for soil microorganisms and crop systems. In this context, the aim of the current study was to evaluate toxicity of the antibiotic CIP on the growth of plants and assess the damage caused by this pharmaceutical drug to soil microbial biomass.

1.7. Objectives

The specific objectives of present study were;

- To investigate the inhibition potential of different Ciprofloxacin (CIP) concentrations on wheat plant growth and,
- To evaluate the effects of selected CIP concentrations on soil microbial biomass

Chapter 2

LITERATURE REVIEW

Hundreds of pharmaceuticals are used as both veterinary medicine and to treat human infections. More than half of the medicines consumed find their way into the environment in the form of bioactive substances due to their incomplete metabolism. One of the most common sources of antibiotics to terrestrial environment is drug contaminated manure application. In the organic and sustainable farming system, livestock manure is a key ingredient either applied in raw form or in the form of compost as a fertilizer. The concentration of drugs in the soil is dependent on the frequency of applied manure. Sulfonamides for example have been noted to persist in the soil fertilized with manure even after a period of three months (Christian *et al*., 2006). Fluoroquinolones are also known to accumulate in the environment due to their low degradability rates resulting from sorption to organic matter in soil.

3.1. Sources of Antibiotics in Environment

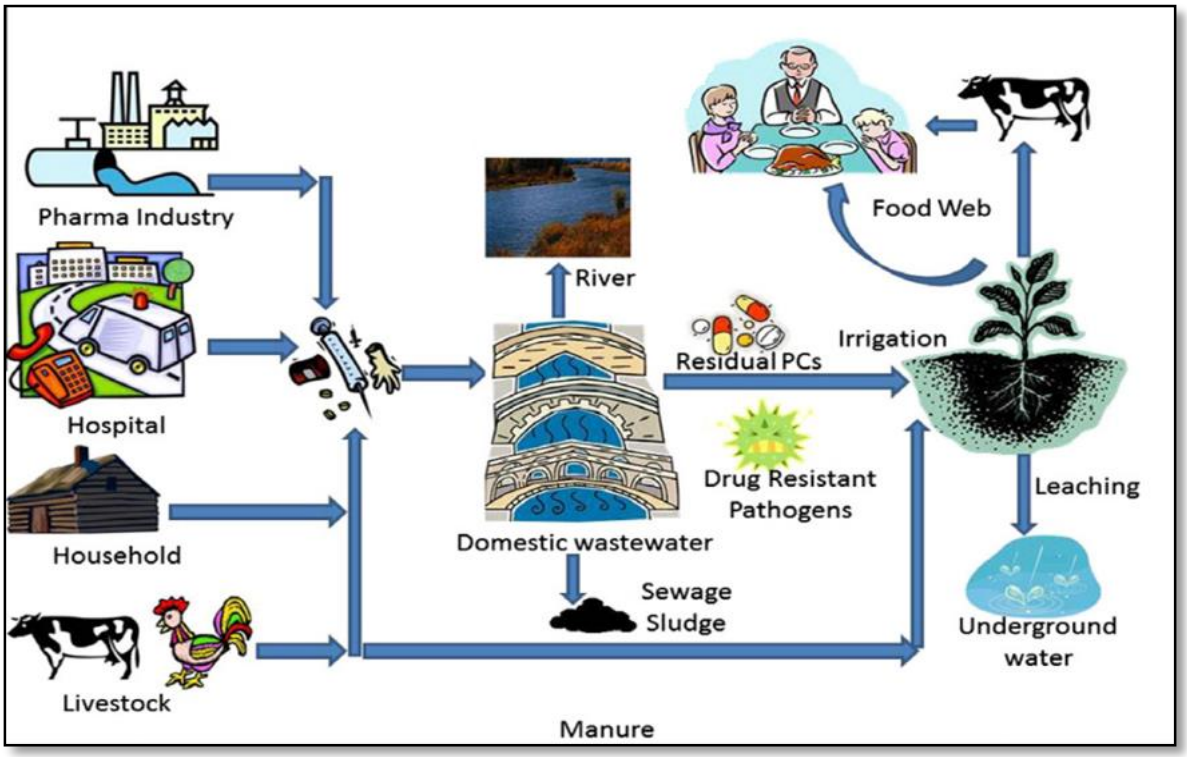


Figure 1: Possible pharmaceuticals routes to environment (Rehman *et al*., 2013)

Some possible routes of antibiotics to different environmental compartments are shown in figure1 (Rehman *et al*., 2013).

3.1.1. Manufacturing Industries/ Human Medicine

Little attention had been paid previously to the pharmaceutical products released from manufacturing plants but in the recent years, concentrations as high as 31 mg L^{-1}, as observed in case of CIP, were found in the effluents released in some of the Asian countries (Larsson *et al*., 2007; Li *et al*., 2008a,b). Even the developed countries can have manufacturing facilities as a significant source of antibiotics for sewage treatment plant (Thomas, 2008).

The per capita usage of human medicine varies markedly across national boundaries (Mölstad *et al*., 2002). Medicines used extensively in one country might be used sparsely or be banned completely in another. The use of prescribed and non-prescribed medicines varies at a great extent in different countries. A large portion of the medicines consumed are excreted out from the body in bioactive form and find their way to the environment.

3.1.2. Animal husbandry

The use of pharmaceutical products in animal husbandry became popular in the early 1950s as feed additives and for prevention and control of various diseases to enhance livestock production. The antibiotics consumed by animals are not completely utilized. Drugs, poorly absorbed in the animal gut, along with the feed additives are excreted (Du *et al.*, 2012) and due to incomplete metabolism, about 25-75% of these are in bio-active form (Zheng *et al*., 2011). The amount of excretion rate depends on the route of antibiotic consumption, its chemistry, amount of time passed after drug administration and the excreting species. For tetracyclines and sulfonamides, the general excretion rate has been observed to range between 40 and 90% (Sorensen, 2001). These drugs and their metabolites are then introduced to the agro-ecosystem via repeated fertilization.

3.1.3. Plants/Agriculture

The use of antibiotics to prevent diseases in economically significant plants, although not extensive, has existed since the mid-20^{th} century. Streptomycin is the most important and heavily used medicine for such purpose with oxytetracycline being used

at a relatively less amount. Most of the use of these medicines is for fruit trees particularly apple and pear applied for prevention and control of fire blight. The total contribution of plant applied antibiotics in USA is less than 0.5% (McManus *et al*., 2002).

3.1.4. Soil

The effect of antibiotics and their efficiency once they enter the environment is dependent on the existing climate, soil type and its physiochemical properties, and various other environmental parameters. In environmental chemistry, antibiotics and their fate in environment has gained special importance, from their consumption to their entry in environment (Jorgensen and Sorensen, 2000). The concentration of antibiotics reported in slurry has been reported at 216 mg L^{-1}, leading to soils contaminated by these pollutants at concentrations as high as 400 g per hectare. The degradation of these contaminants can take up to 150 days at the agricultural sites (Kumar *et al*., 2005).

The antibiotics that enter into the environment are mostly unchanged and form complexes. The structure and properties of these anti-microbial agents determine the chemical and physical behavior of soil. The sorption of antibiotics to soil molecules is largely dependent on the size, shape, molecular structure, water solubility and other physical and chemical properties. The hydrophobicity of a large number of these compounds results in their immobility when they enter the soil which renders them harmless. In most cases, this sorption either completely retards the activity of antibiotics or it reduces their potency (Sengeløv *et al*., 2003). In some cases, however, it has been observed that even if the antibiotics like tylosin and tetracycline are bound to soil, they are still active. They retain their antimicrobial properties and can result in the development of resistant bacteria in the area (Chander *et al*., 2005). Accumulation of antibiotics occurs if the rate of degradation of the compound is slower to the rate with which the drug contaminated manure is applied. This accumulation in the soil is termed as terracumulation (Rooklidge, 2004). These pollutants can carry from terrestrial environment to aquatic via leaching and run-off (Pedersen *et al*., 2003) and are degraded at different rates depending on the compound. Photo-degradation usually plays a minor role due to lack of reach into the soil layers, slurry or sludge (Sengeløv

et al., 2003). Microorganisms play a major role in the degradation of these anti-microbial agents by enzymatic reactions and transformation of compound from parent to daughter molecules (Ahmad *et al*., 1999). Some of the antibiotics on the other hand are not easily degradable. Their adsorption to organic rich feces is strong, as is observed in case of sulphonamides and fluoroquinolones (Marengo *et al*., 1997). High temperatures and aerating the manure has proved ineffective and these pollutants spread into the environment unchanged (Winckler and Grafe, 2001).

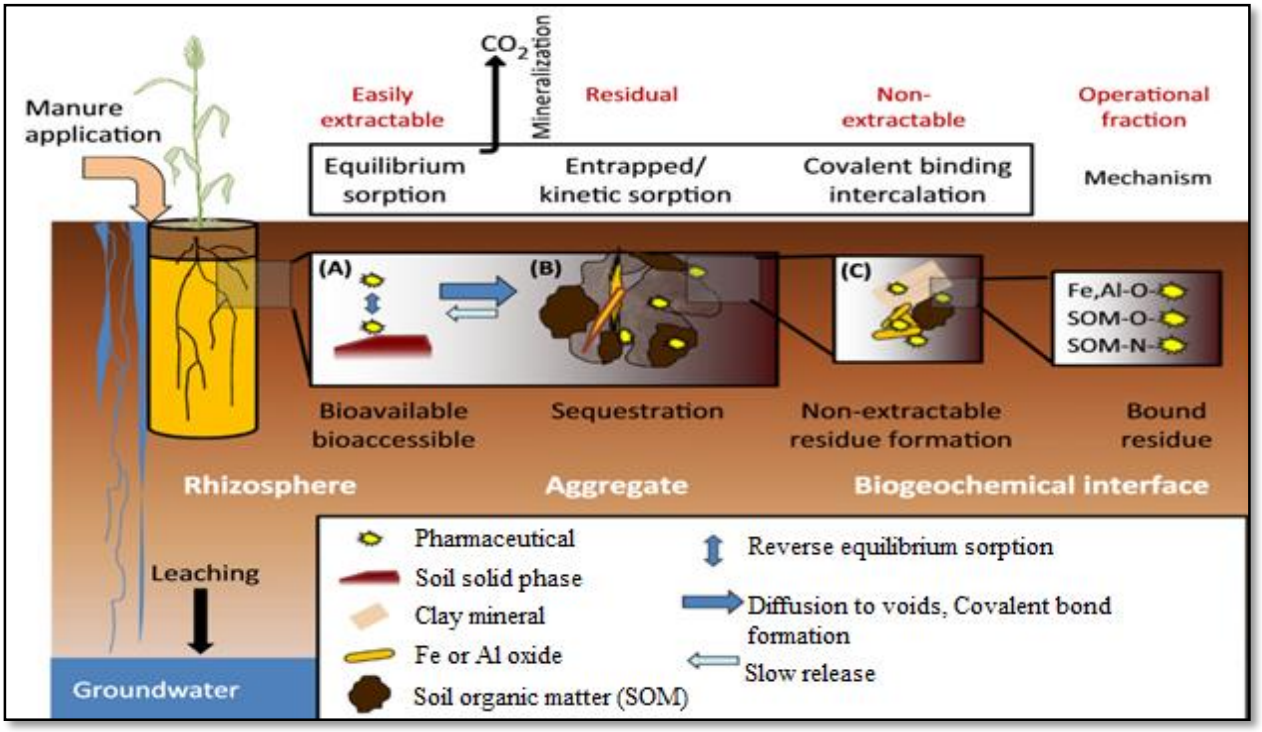


Figure 2. Antibiotic fate in soil and its compartments is indicated by sorption to the solid phase and biotransformation. (A) Bioavailable fraction comprises of reversibly sorbed antibiotics that can be easily removed by simple mechanisms. (B) Sequestration withdraws the agents from biological access and reduces their spreading via kinetic sorption and diffusion into voids of soil organic matter, microaggregates and minerals. Harsh extraction methods such as high pressure and temperature are needed to assess sequestered drugs. Slow release of these residues into bio-accessible forms is possible. (C) Non-extractable residues are formed as a result of covalent bond formation and irreversible intercalation of drugs in nanopores (Jechalke *et al*., 2014).

3.1.5. Aquaculture

Aquaculture is defined by FAO as ‘‘the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants”. The farming of these aquatic organisms requires application of antibiotics for the process of rearing these organisms and increasing their production rate. The major use of antibiotics in aquaculture is as prophylactic agents (Serrano, 2005).

3.1.6. Threshold Limits

Different concentrations of antibiotics in the environment have been reported by researchers over the years. No limit has been set for risk analysis of drug contaminated manure or sludge if it reaches a certain level in the fields. However, the European Agency for Evaluation of Medicinal Products (EMEA) recommends analysis of the potential risk caused by any drug that exceeds the threshold concentration 0.01 mg L^{-1} in surface waters (EMEA, 2006; Grung *et al*., 2008).

3.2. Ecological Risks of Antibiotics

3.2.1. Impact on Plants

Plants play a crucial role in maintaining the integrity of ecosystem. The introduction of toxic substances into the environment causes changes in the structure and function or even genetics of these primary producers (Yi and Si, 2007; Yin *et al*., 2008). The pollutants, once they enter the ecosystem, are taken up by plants. Accumulation of certain veterinary drugs in different parts of plants has been observed in a number of studies. Rice, wheat, lettuce, soybeans and alfalfa have been used extensively as test species to study the accumulation of antibiotics in certain parts of these plants. This accumulation occurs through the water transport system and via passive absorption. When these pollutants enter the primary producers, they can cause serious damage to the physiology of plants and their biochemical activities (Liu *et al*., 2009; Boonsaner and Hawker, 2010; Hillis *et al*., 2011; Li *et al*., 2011; Luo *et al*., 2011).

Accumulation of antibiotics in plants has been reported in a number of studies. In 2005, a study on green onion, cabbage and corn reported the uptake of pharmaceutical products proportional to their presence in the environment. The increasing antibiotic containing manure increased the uptake of antibiotics (Kumar *et al*., 2005). Boxall *et*

al. (2006) observed the uptake of several different medicines in different plants. The study showed that the uptake of antibiotics florfenicol, enrofloxacin, trimethoprim and diazinon by carrot roots while trimethoprim, levamisole, and florfenicol were detected in the lettuce plant. Ciprofloxacin (CIP) has been shown to be absorbed by lettuce and cucumber (Lillenberg *et al.*, 2010) while sulfadimetoxin has been taken up by barley (Brambilla *et al.*, 1996). The uptake of these drugs by plants can lead to impact on human health by microbial infections that cannot be treated by pharmacotherapy (Auerbach *et al.*, 2007; Jia *et al.*, 2008; Du *et al.*, 2012). It can also impact their growth either by direct uptake or by affecting symbiotic relationship with soil bacteria. In lettuce and potato, sulfametazine uptake has been reported by Dolliver *et al*. (2007) while a study by Lillenberg *et al*. (2010) showed uptake of CIP and enrofloxicain (ENR) by lettuce, cucumber and barley.

Various studies have been carried out to ascertain the toxic effects of pollutants on plants. A decrease in the growth of roots and shoots of alfalfa by 85% and 61% was observed on exposure to oxytetracycline (OTC) (Kong *et al*., 2007). Migliore *et al*. (2003) observed toxicity of enrofloxacin at 5mg L^{-1} when taken up by the crop plants. On the other hand, no significant stress resulting from the two sulfonamides was observed by the shoot apparatus. Maize plants were exposed to cholorotetracycline (CTC) at concentrations of 0.05, 0.5, 5 and 50 mg L^{-1} for 10 days and its uptake and effect was observed. Root length, shoot length and fresh biomass of maize were shown to suffer with the highest stress observed by root apparatus (Wen *et al*., 2012). The effect of tetracycline (TC) on wheat at different concentrations (0.5, 1, 5, 10, 25, 50, 100, 150, 200, 250, and 300 mg L^{-1}) was studied by Xie *et al*. (2011) and it was concluded from their study that wheat showed both positive and negative response to TC. At lower TC concentrations, enhanced root growth, seed germination and cell mitosis were observed. At higher concentrations, reverse trend was observed. Another study on wheat highlighted the negative effect of OTC on fresh and dry biomass of root and shoot. The root and shoot biomass decreased by 12 to 90.2% and 21.7 to 88.6% respectively.

3.2.2. Damage to Human and Animal Health

The pharmaceutical industry has encountered a steady incline in the antibiotic resistance rate over the past few decades. Various national and global health agencies including the World Health Organization and US Center for Disease Control have verified the increase in resistance of disease causing microbes which has resulted in the decline in the efficacy of antibiotics. In the US, the reported number of deaths from nosocomical diseases increased several folds from 13,300 in 1992 to 98,000 by 2011 due to the development of resistance in bacteria. Some of the pathogens that have contributed to this death toll include *Enterococci*, *Staphylococcus aureus* and *tuberculosis* which have developed resistance to vancomycin, methicillin and multiple drugs respectively. There is at least one class of antibiotics that is currently resistant to approximately 70% of the nosocomical infections. Antimicrobial susceptibility of 13,993 P. *aeruginosa* isolates from 102 healthcare facilities was tested in 2007 (Miliani *et al*., 2011) for 4 different antibiotics. Of these, 4253 isolates were resistant to CIP, 2967 to imipenem (IPM), 2462 strains were resistant to ceftazidime (CAZ) while amika (AMK) was ineffective against 2691 isolates.

The presence of resistant bacteria in the water systems pose a significant threat to human health by transfer of genes from non-pathogenic to pathogenic bacteria. Streptomycin, tetracycline and ampicillin are a few medicines to name to which resistance genes have been developed and these ARGs are transferable from bacteria to bacteria. Chlorination is not a guaranteed way for complete sanitization of drinking water. It has been observed that there are certain resistant microbes that survive chlorination and thus enter the drinking water system. Studies show that certain strains of the pathogenic bacteria isolated from the chlorinated drinking water developed resistance to approximately all of the antibiotics that were tested.

Although the concentration of pharmaceutical compounds detected in the water system is quite less, their effectiveness at minute concentration for damaging specific proteins makes their presence even at these low concentrations a risk to human and animal health. Diclofenac is an analgesic which caused a remarkable decrease in the vulture population in Pakistan and India by causing renal failure. In India, the oriental white-backed vulture was considered to be the most abundant large bird predator in the late

20th century. Its population observed a severe hit due to the drug, diclofenac. Close to 95% of the vulture population was lost due to this drug by 2003 (Oaks *et al*., 2004). By 2008, this percentage rose to 99.9%, essentially bringing the species to the brink of extinction in the area.

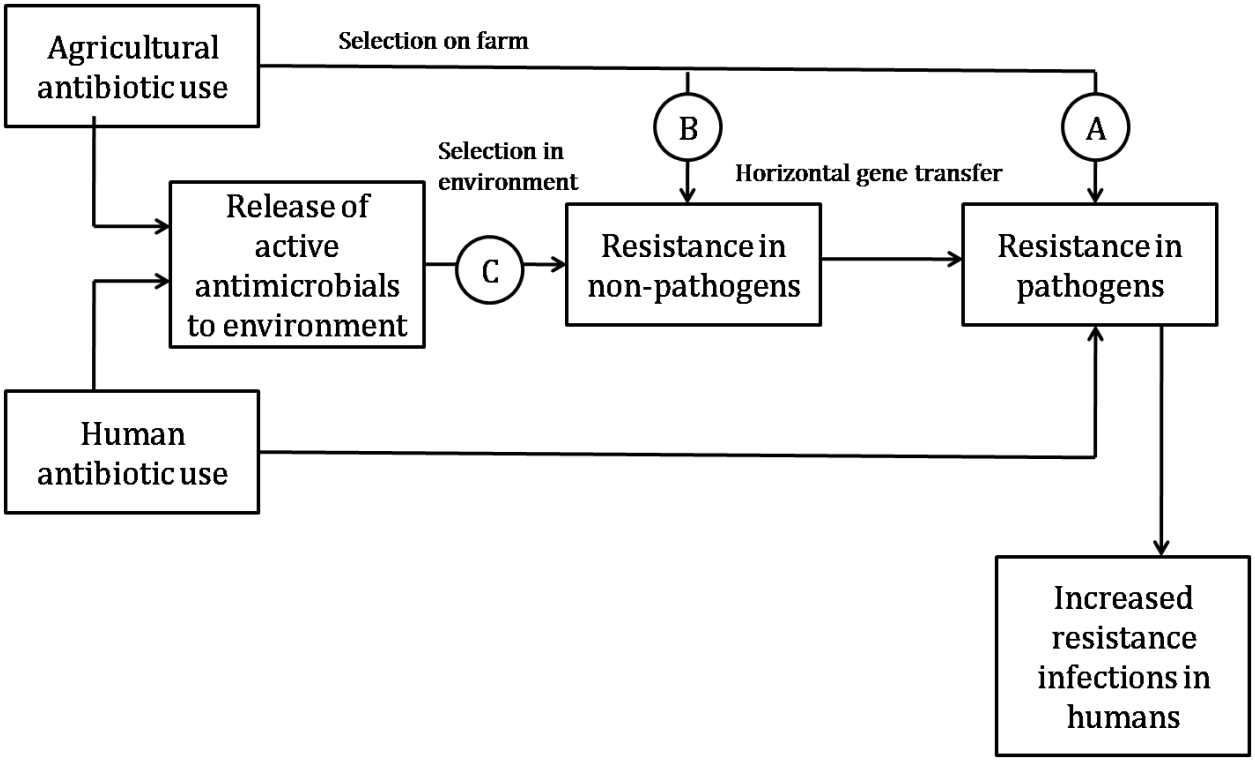


Figure 3: Conceptual model of how agricultural antibiotic use (AAU) can cause increased resistant infections in humans. Scenario A shows the increase in resistance pathogens resulting from AAU and their entry to human food chain or environment. Scenario B highlights the selection of resistance genes in non-pathogenic organisms by AAU and their transference to pathogenic ones, finally leading to more resistant human infections. Scenario C highlights the release of active antimicrobial substances into the environment. Here, the selection procedure follows in the non-pathogens and the transfer of resistance genes to pathogens as is observed in scenario B. Human antibiotic use is given as reference only (Singer *et al*. 2014)

Due to the nature of the danger and the risk posed by the release of pharmaceutical compounds into the environment, a number of studies have been conducted on the human health and the environmental risk assessment of these products. Researchers debate heavily on the level of harm caused by these substances due to their low

concentration. Some of the researchers maintain the view that low amount of antibiotics in the environment will not cause considerable damage to human health while others are of the opinion that even the low quantities can affect life, particularly in aquatic ecosystem. There is a dire need to study the harmful effects of pharmaceutical compounds in the long term and develop an effective and accurate risk assessment procedure using the already available models (Mojica and Aga, 2011). For a better understanding of the risk of antibiotic exposure to agriculture and resistance development resulting from it, a conceptual model was developed (Figure 3) by Singer *et al*. (2014) which provides 3 possible scenarios linking agricultural antibiotic use (AAU) to human health impacts.

3.2.3. Impact on Soil Microbes

Microbial ecosystem can be severely impacted by the antibiotics that find their way to the environment. These compounds can cause serious damage to the structure and physiology of microbial communities. The presence of antibiotics in environment can lead to development of ARGs which can cause the transferance of resistance genes from non-pathogenic to pathogenic bacteria and production of antibiotic resistance of microbes. This can pose a significant threat for the non-target organisms in terrestrial environment (Auerbach *et al.*, 2007; Jia *et al.*, 2008; Du *et al.*, 2012). Studies on the impact of antibiotics on soil microbial communities are inconsistent. In some studies, a positive trend has been observed as reported by a study conducted using the antibiotics oxytetracycline and chlortetracycline. Here, these medicines served as a carbon source to microbes and resulted in enhancing the growth and activity of microbes. In contrast to that, another research showed a major decline in the microbial population of forest soils including fungi, bacteria, nematodes and protozoa on exposure to the antibiotics oxytetracycline and penicillin. Another study showed the negative impact of a combination of medicines including clarithromycin, amoxicillin and erythromycin at concentration of 1000 mg L^{-1} by decreasing the denitrification rate (Mojica and Aga, 2011).

3.3. Antibiotic Resistance

The capacity of life to adapt and modify enabled it to overcome the challenges faced by it since the beginning and come out stronger than ever before. This adaptability is

one of the reasons that microbes, the most ubiquitous of all species can be found even in the harshest of environments, from hot springs to ice sheets, extremely high pressures and at a large pH range. This adaptability of microbes is mostly attributed to their huge population and the ability to transfer desirable genes intra species. This plasticity is also the reason microbes have formed resistance to anthropogenic compounds developed for their destruction (Rojas *et al*., 2013).

The consequences of antibiotics entering the aquatic and terrestrial environment are vast; however the damage they cause to the microbial communities can be considered by far the biggest issue caused by the contamination of environment by these pharmaceutical products. The presence of these antibiotics in environment leads to the selection of antibiotic-resistance genes (ARG) in pressure conditions even at concentrations that are considered harmless in environment and lead to development of resistance in microbes (Mojica and Aga, 2011). The development of antibiotic resistance in microbes can be traced back to the late 20^{th} century where vancomycin, methicillin and fluoroquinolone resistant *Enterococcus, Staphylococcus aureus* and *Pseudomonas aeruginosa* resulted in a rapid increase in infections (Cooper *et al*., 2011). Today, at least 70% of the pathogenic bacteria have developed resistance to the currently available antibiotics causing a rise in the mortality rate to 2 million people each year from microbial infections (Berdy *et al*., 2012). The development of antibiotic resistance coupled with decline in production of new antibiotics is a cause of serious concern for human health.

Development of resistance in bacteria is the major concern for the utilization of human and veterinary medicine which pose a health risk to both humans and animals. The antibiotics used for the poultry are of particular concern as they have greater chance of resulting in the selection of resistant strains to pharmaceuticals used as human medicine. The transference of resistant microbial strains can occur either directly from contact or by ingesting the animal meat. Bacterial strains of certain human pathogens in the environment and in animals have developed resistance to antibiotics and their resistance genes have been isolated (Angulo *et al*., 2004). Xu *et al*. (2014) worked on the abundance of antibiotics and ARGs in sewage treatment plants in China. Quinolones were detected with concentration of 3866 ng L^{-1} and four resistant genes

(gryA, parC, qnrC and qnrD) of this class were determined using quantitative PCR. The process of resistance development is described briefly in Figure 4.

Pathogenic bacteria, both of human and animal origin, are present in the wastewater. Antibiotics contaminating this water cause selective pressure on microbial communities and result in promotion of ARGs (Baquero *et al*., 2008; Farrell, 2009). Presence of antibiotic resistant bacteria has been found in different water sources (Caplin *et al*., 2008; Vanneste *et al*., 2008). Studies have shown that water sources, even the clean ones like drinking water, serve as a major reservoir of not only ARB but also ARGs (Nonaka *et al*., 2007; Hoa *et al*., 2008).

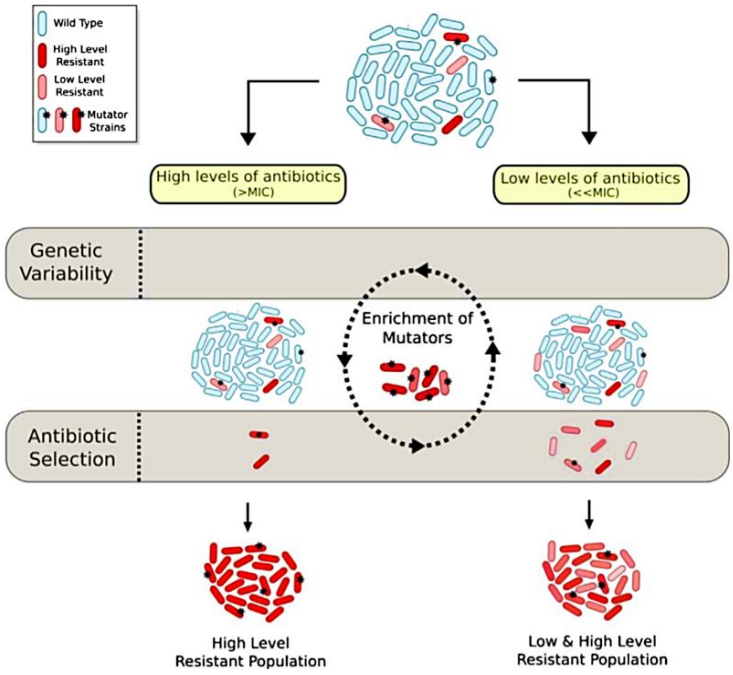


Figure 4: The above figure shows the process of resistance development in bacteria against antimicrobials. High concentration of antibiotics leads to selection of resistant strains already existing and developing high resistant bacterial population. Low antibiotic concentration can lead to increased genetic variability of population, enhanced chances of high and low level antibiotic resistance. Antibiotic resistant selection will increase the overall mutator species enhancing the possibility of resistance to non-related antibiotics (Rojas *et al*., 2013).

3.4. Quinolones

Quinolones are anti-bacterial agents which were developed as non-fluorinated drugs in the early 1960s. This potent class of modern day medicine was initially developed for the treatment of veterinary and human urinary tract infections. First antibiotic to come out was nalidixic acid which, due to its inability to reach most tissues and organs in required concentration, was restricted for use of urinary infections (Nava, 2007). It was then modified to form broad range antimicrobials by the development of first 6-fluorinated derivative in 1980s thereby giving rise to the class fluoroquinolones (Silva, 2004; Stahlmann, 2002). The estimated usage of quinolone in European Union, USA, South Korea and Japan was close to 70 tons in the form of generic quinolones while 50 tons was produced as proprietary products. In China, the estimated quinolones consumption for humans was 1350 tons while that for animals was reported as 470 tons (Sukul and Spiteller, 2007).

3.4.1. Fluoroquinolones

Fluoroquinolones (FQs) class of compounds is composed of a growing group of synthetic antimicrobial agents that contains one fluorine molecule at the 6-position of the quinolone nucleus. In spite of the similarity in the basic structure of these molecules, their pharmacokinetic characteristics, physicochemical properties and microbial activities differ significantly among compounds (Martinez *et al*., 2006).

Due to their excessive use in treatment of human (since 1980s) and animal diseases (ANSES, 2012; European Medicines Agency, 2012; Pico and Andreu, 2007), FQs have become a serious cause of concern in environmental pollution. They form the third largest class of antibiotics with a global market share of about 17% and a net share of 7.1 billion US dollars (Hamad, 2010). The FQs consumed are largely excreted and enter the sewage system. From there, these pseudo-persistent compounds find their way from water bodies to soil (Sturini *et al*., 2012a). Another point of entry of FQs in the soil is through manure application where they accumulate owing to their low degradation rate and strong adsorption to organic matter (Alexy *et al*., 2004).

3.4.1.1. Mode of Action

Fluoroquinolones target bacterial cell by inhibition of two of the enzymes associated with synthesis of bacterial DNA. Fluoroquinolones are specific in their action as they

target the enzymes topoisomerases that are essential to bacterial DNA replication but are absent in human cell. DNA topoisomerases separate the two DNA strands at the time of replication and reseal the parent strands after inserting another DNA strand through the break (Rocha *et al*., 2011). Fluoroquinolones create conformational changes by interaction with enzyme-bound DNA complex and thereby inhibiting normal enzyme activity. The antibiotic-enzyme-DNA complex formed prevents DNA replication by blocking the replication fork and resulting in obstruction of DNA synthesis leading to a quick cell death.

3.4.1.2. *Environmental Presence & Transformation*

The presence of FQs in the environment has been reported frequently due to their extensive use and accumulation. In Switzerland, the concentration of CIP detected in domestic sludge and waste water treatment plant (WWTP) was 249-405 and 45-567 ng L^{-1} respectively while that of norfloxacin (NOR) was 45-120 and 34-367 ng L^{-1} respectively (Golet *et al*., 2002; Fink *et al*., 2012). In US, FQs as high as 0.6-2 µg L^{-1} were detected in wastewaters. From the samples taken from 139 US streams, the concentration of CIP and NOR detected were 0.02 and 0.12 µg L^{-1} respectively. Fink *et al*. (2012) reported the range of 0.7-124.5 µg L^{-1} for CIP in the wastewater of a Swiss hospital.

On entering the environment, FQs are degraded by various processes. Three of the most common ways by which these are transformed in the environment are biodegradation, photolysis and oxidation via mineral oxides. The process of hydrolysis is not involved in the breakdown of FQs (Kummerer *et al*., 2009). Photolysis primarily comes into effect in degradation of FQs in surface water (Babić *et al*., 2013; Ge *et al*., 2010; Sturini *et al*., 2012b). The process is however effective only for the few millimeters of depth of sediment surface making the process slow. The half-life reported for photolysis degraded FQs in surface waters ranges from 6-24d (Lai and Lin, 2009; Xu *et al*., 2009). Oxidation of FQs by mineral oxides is another important process of FQ transformation in environment (Zhang and Huang, 2007). Biodegradation of FQs sorbed to the soil sediments has been observed to be ineffectual (Lai and Lin, 2009). The half-life times of more than 217 days have been reported for FQs in studies where sludge and manure was applied as a fertilizer for plants

(Rosendahl *et al*., 2012). As a result, despite the various processes for the degradation of FQs, the strong sorption of these antibiotics to soil sediments and their unavailability to microbes by their migration to nano-pores in soil matrix makes FQ highly persistent with half-lives 200 times more than the FQs in aqueous sediment matrices (Alexander, 2000).

3.5. Ciprofloxacin

Ciprofloxacin (CIP), belonging to fluoroquinolone class of antibiotics, was patented by Bayer in 1983. It possesses a broad range of use for human and animal diseases against gram positive and negative bacteria (Davis *et al*., 1996). CIP with a half-life of 4 h in human body has been identified by WHO (2007) as a crucial antibacterial human medicine. It is the most frequently prescribed FQ in European countries (Ferech *et al*., 2006) and is used in the treatment of bone and joint infections, sexually transmitted diseases, tuberculosis and typhoid fever to name a few diseases (Rocha *et al*., 2011). It is a proven genotoxic drug and is detected in the natural environment on a regular basis (Kummerer *et al*., 2000). It has been included in an EU project as a crucial substance in eradication of pharmaceutical residues from healthcare sector with its concentration detected in the range up to 0.083 mg L^{-1} (Hartmann *et al*., 1998).

Approximately 45-62% of this drug is excreted by human urine and 15-25% of it is removed from the body in the form of feces (Golet *et al*., 2003). CIP hence finds its way into the environment via sewage systems, water treatment plants or from the drug manufacturing facilities. Other pathways of CIP to environment include application of livestock manure, irrigation of land with contaminated water and by leaching in landfills (Boxall *et al.*, 2003; Topp *et al*., 2008).

3.5.1. Mechanism of Action

CIP shows bactericidal action against a wide range of gram positive and negative bacteria. In gram-positive bacteria, it prevents bacterial multiplication by targeting DNA gyrase (a topoisomerase II) which is involved in super coiling of circular DNA while in Gram-negative bacteria, it targets topoisomerase IV enzyme, that unwinds the super coiled circular DNA (Sharma *et al*., 2009) thus disrupting enzymes necessary for the replication, transcription, repair and recombination of DNA (Bêhal, 2006).

3.5.2. Clinical Particulars

CIP is used for the treatment of infections of respiratory tract (Jones *et al*. 1994), kidney, urinary tract (Mahamat *et al*., 2006), middle ear, genital organs and abdominal cavity. Some other diseases treated by CIP include infections of eyes, skin and soft tissue, bones and joints and diarrhea. It is also used to treat pneumonias caused by bacterial species including *Escherichia coli*, *Enterobacter* spp., *Pseudomonas aeruginosa* and *staphylococci* (Medicine pamphlet).

3.5.3. Side Effects

Detailed results from clinical trials showed certain side effects including nausea (2.5%), diarrhea (1.6%), abnormal liver function tests (1.3%), vomiting (1.0%) and rash (1%) cases (Medicine pamphlet).

3.5.4. Environmental Presence

The oral consumption of CIP has increased up to 30% over the last few years (Batt *et al*., 2007). Most of the data regarding the presence of FQs in soil is on enrofloxacin (ENR) and CIP with CIP ranging from 4 to 40.7% in chicken manure and from 0.65 to 2.1 mg kg^{-1} in poultry manure (Leal *et al*., 2012). Wu *et al*. (2014) reported the ubiquitous presence of four quinolones in the soils of 5 organic vegetable farms in Southern China. The selected antibiotics were detected at a frequency of >97% at concentrations ranging from 0-42 µg kg^{-1}. The level of frequency of drug appearance decreased in the order of ENR>CIP>NOR. The concentrations of CIP detected in different environments is listed in Table 1.

Table 1: Concentrations of CIP in environment

Matrix	Detected Conc.	Regions	References
WWTP effluents	31	India	Larsson *et al*., 2007
Agricultural soils	0.75 *	Austria	Carballo *et al*., 2007
River	$6.7x10^{-3}$ - $1.02x10^{-3}$	Portugal	Pena *et al*., 2007

Sewage treatment plants (STPs)	0.056-0.211	Coimbra	Seifrtova *et al*., 2008
Ground water	$7x10^{-3}$-$14x10^{-3}$	India	Fick *et al*., 2009
Surface water	0.36	US	Sadezki *et al*., 2010
Chicken dung	0.7 to 45.6 *	China	Zhao *et al*., 2010
Production facility wastewater	4.9	Korea	Sim *et al*., 2011
Hospital effluents	0.007-0.1245	Swiss	Fink *et al*., 2012
Poultry manure	0.65-2.1*	Brazil	Leal *et al*., 2012
River	$1.25x10^{-3}$	Pakistan	Ahmad *et al*., 2013

Concentrations are reported as: mgL^{-1}; values with * indicate $mg\ kg^{-1}$

The ecotoxic risk value defined by Veterinary Medicine International Coordination commission for antibiotics is 100 µg kg^{-1}. CIP persists in the environment as shown by Golet *et al*. (2002) who observed their presence on land even 13 months after biosolid application. This finding was later corroborated by Wetzstein *et al*. (2009) and Walters *et al*. (2010).

2.5.5. Bacterial Resistance to CIP

The difference in structure and properties of fluoroquinolones from other medicines and their different mode of action implies that the microbes resistant to the medicines such as tetracyclines, aminoglycosides, macrolides and cephalosporins might be susceptible to CIP. The development of microbial resistance against CIP and other FQs, on the other hand, usually results from reduced outer membrane permeability and mutations in DNA gyrases (Cipro: Retrieved from http://www.rxlist.com/cipro-drug/clinical-pharmacology.htm. retrieved on August 28, 2014).

2.5.6. Degradation

Antibiotics on reaching the aquatic environment are faced with the natural elements which can bring about their degradation. The fate of fluoroquinolones, including CIP, on degradation front can be understood only by contemplating both biotic and abiotic factors. Biotic factors basically involve biodegradation by microorganisms present in

the aquatic environment while photochemical reactions play a vital role in degradation by abiotic factors in the surface layers. The mechanism involved here (Figure 5) is basically the replacement of piperazinyl ring present in the seventh position (Nowara *et al*. 1997).

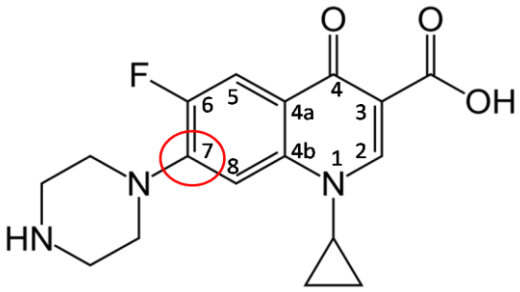


Figure 5: Degradation mechanism of CIP by removal of piperazinyl ring at the seventh position (Nowara et al. 1997).

The biodegradability of CIP is low (Kummerer *et al*., 2000). It usually binds readily with the soil via cation exchange (Pico *et al*., 2007; Vasudevan *et al*., 2009) and hence can stay there for long periods as soil acts its reservoir (Rooklidge *et al*., 2004). In a biodegradation study for FQ degradation, Mougin *et al*. (2013) observed less than 0.01% CIP mineralization after an incubation period of 84 days while Girardi *et al*. (2011) estimated the degradation rate of 0.03% for the first 6 days of experiment after which the rate of degradation slowed down to 0.008% per day. In a closed bottle test by Kummerer *et al*. (2000), no degradation of CIP was observed even after 40 days' experimental period. Walters *et al*. (2010) calculated half-life time of 1-3 years for CIP in a mesocosm soil study showing high resistance to biotic and abiotic degradation. Dalkmann *et al*. (2012) worked on the persistence of CIP in environment by showing CIP sequestered in Mexican soils over 20 years period during long term untreated wastewater irrigation.

In a study conducted on the degradation of CIP in pure and river water (Turiel *et al.*, 2005), it was observed that no degradation occurred in the absence of light attributing the degradation to light. The rate of photo-degradation for the initial two months was particularly low owing to no additional bio-chemical degradation after which it increased rapidly. In the pure samples however, the rate did not increase exceptionally.

This can be explained away by the presence of microbial communities in river water and chemical degradation by various river matrices.

2.6. Wheat

The economic stability of Pakistan is highly dependent on agriculture and contributes 21.4% to GDP providing employment to 45% of working class. Wheat (*Triticum aestivum*) is one of the most widely consumed staple foods of the world especially in Pakistan (Agriculture corner, 2013) with a reported production rate of 24500 kMT and a growth rate of 2.08%. Pakistan is ranked as ninth largest producer and distributer of wheat by the Food and Agriculture Organization.

The presence of pollutants in agricultural field can have negative impacts on the growth of the crop plants. Even if the residual period of these compounds is short, they can cause serious damage to plant growth in a short time. The effects of 9 antibiotics on the physiology and secondary metabolites of wheat at the concentrations of 0.5 and 1.5 mg L^{-1} were analyzed by Opris *et al*. (2013). CIP and cephalosporins caused a stomatal reduction thereby influencing the net assimilation. A reduction in its photosynthetic responses in chlorophyll, pigments and carotids was also observed by application of CIP, tetracycline and erythromycin.

2.7. Soil Microorganisms

Soil microbes are a significant constituent of ecosystem and their properties including biomass, enzymatic activities and soil respiration are commonly exploited as the soil quality indicators (Gao *et al.*, 2013; Kulshrestha *et al*., 2004; Li *et al.*, 2006; Ute *et al.*, 2008; Zielezny, 2006). It is therefore it is important to investigate how exposure to pollutants such as antibiotics can affect their physiology.

A mixture of antibiotics (clarithromycin, enrofloxacin, sulfamethaxozole, tetracycline and trimethoprim) was reported by Ghosh *et al.*, (2009) to have inhibited nitrifying bacteria at concentration of 50 µg L^{-1}. A research conducted by Gutierrez *et al*. (2010) showed that a combination of three sulfonamides (SA) including sulfamethaxozole (SMZ) had an obvious effect on the structure and function of microbial communities. SMZ concentration of 53.6 µg L^{-1} in soil was reported to have a clear short time detrimental effect on readily cultivable bacteria, potential microbial and enzymatic activities (Pinna *et al*., 2012).

2.7.1. Pseudomonas aeruginosa

Pseudomonas aeruginosa (*P. aeruginosa*) is an aerobic, gram-negative, cocobacillus bacterium. It is considered a serious public health threat owing to various nosocomial infections it causes (Master *et al*., 2011). *P. aeruginosa* is a known opportunistic plant pathogen and has been shown to affect lettuce, tomatoes and tobacco plants. Walker *et al*. (2004) reported the pathogenicity of *P. aeruginosa* strains to Arabidopsis and sweet basil *in vitro* and in soil by causing mortality to the plants after 7 day inoculation. Its versatile nature has led to its adaptation in a wide range of environments including rivers, lakes, soil and wastewater (Baleux and Troussellier, 1989; Sherry, 1986; Hall *et al*., 1998; Schwartz *et al*., 2006). Soil is the primary habitat of this versatile bacterium. Excessive use of antibiotics has resulted in development of resistance in *P. aeruginosa* (Master *et al*., 2011).

Antibiotic resistant *P. aeruginosa* have been identified in wastewater (Fuentefria *et al*., 2011), river water (Pirnay *et al*., 2005), ground water (Kaszab *et al*., 2010) and natural water resources (Legnani *et al*., 1999). Humans can be exposed to this bacterium from these sources. Alonso *et al*. (1999) reported greater antibiotic resistance of environmental *P. aeruginosa* isolates taken from soil and water sources compared to clinical isolates. The difference observed in the resistance for CIP and a few other FQs was 2–3 folds.

2.7.2. Escherichia coli

Escherichia coli (E. coli) is a gram-negative, rod-shaped, facultative anaerobic bacteria, most commonly found in the lower intestine of endotherms. The diversity of *E. coli* in nature ranges from pathogenic to commensal strains. The natural habitats of *E. coli* are divided as host and non-host associated habitats (MacFarlane and MacFarlane, 1997; Savageau, 1983). The commensal strains of bacteria are considered harmless, found in the intestines of host, and help in the breakdown of certain carbon compounds (Touchon *et al*., 2009) while the pathogenic strains possess the ability to cause serious damage to the host species (Taylor, 2008). Over the time, different subgroups of *E. coli* have observed a biphasic lifestyle, spending a portion of life in host species and other half in host independent or open phase. The complexity and diverse conditions observed in different phases allowed *E. coli* to develop key

evolutionary adaptations in their genomes (Schubert *et al*., 2009). As a consequence, this bacterium formed a high ability to attain diverse nutrients which aids it in its survival in open/non-host environments (Ihssen *et al*., 2007).

In addition to the ability of absorbing nutrients from open environment, some *E. coli* strains also possess the ability to produce filamentous structures that aid the cell in its attachment to surfaces including those of plants. Consequently, the *E. coli* that are present in soil, water or contaminated seeds can find their way to plants such as radish (Itoh *et al*., 1998) and lettuce (Soloman *et al*., 2002). Additionally, *E. coli* can also access roots and leaves of plants by irrigation or slpashing during rainfall (Natvig *et al*., 2002). If the internal compartments of plants get contaminated, it becomes impossible to remove the bacteria by washing or disinfection (Soloman *et al*., 2002). In this way, the pathogenic strains of *E. coli* contaminate the food chain making them a still underestimated environmental risk.

A report on the 2006 spinach outbreak by Food and Drug Administration (FDA) reported 205 illnesses and 3 deaths. The outbreak was linked to 13 bags of spinach contaminated with *E. coli* O157:H7 isolates. The field to which the isolates were linked included the presence of wild pigs, surface water contaminated with cattle and wild life feces and proximity to irrigation wells as potential environmental risk factors (U.S. Food and Drug Administration, 2007). A total of 66 people infected by Shiga toxin-producing *E. coli* O157:H7 (STEC O157:H7) reported in 9 states of USA were traced back to ready to eat salads and pre-packaged leafy greens in 2011 and 2012 respectively (Centers for Disease Control and Prevention, 2011; Centers for Disease Control and Prevention, 2012). An outbreak of Shiga toxin-producing *E. coli* O121 (STEC O121) strain reported in USA in 2014, infecting 19 persons, was linked to contaminated raw clover sprouts produced by Evergreen Fresh Sprouts, Idaho (Centers for Disease Control and Prevention, 2014). Although the three outbreaks mentioned above are over, however, it should be stressed the *E. coli* served to be a problem for human health and as such, there is a need to develop methods for mitigation of risk resulting from this pathogenic bacteria. With the licensing of FQs for poultry use, a dramatic rise in the blood infections resulting from resistant *E. coli* strains was observed (Figure 6).

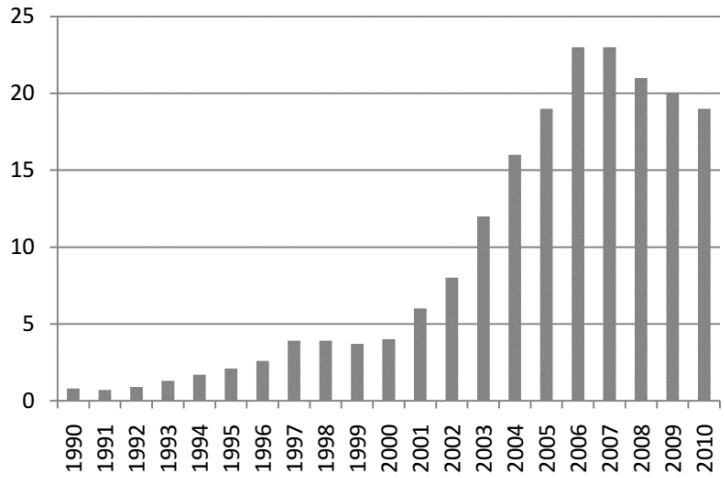


Figure 6: Percentage FQ resistance in human *E. coli* blood infections in England, Wales and Northern Ireland (HPA data).

Chapter 3

MATERIALS AND METHODS

The aim of this study was to quantify the potentials effect of one of the environmentally common anthropogenic antibiotics, CIP, on wheat (*Triticum aestivum*) crop and on the microbes present in soil sampled from the wheat field. Although the concentration of FQs detected in water bodies is in ng and µg L^{-1}, and in soil the range is between µg L^{-1} and mg L^{-1}, however, their continued release into the environment and permanent presence makes them pseudo-persistent contaminants.

The current study was based on antibiotic amended soils and a control soil sample which was incubated with water only. To test the effect of CIP on wheat growth, varying concentrations (0- 200 mg L^{-1}) of antibiotic were applied to the soil and its impacts on plant morphological characteristics and microbial biomass were evaluated.

3.1. Chemicals

CIP (>95% purity), selected for the current study, was obtained from Bayer (Pvt.) Ltd, Pakistan. Analytical grade chemicals used in the investigation included potassium dichromate, analytical grade sulfuric acid (98% purity), ferrous ammonium sulfate, analytical grade hydrochloric acid and di-phenyl amine indicator.

3.2. Seeds

Seeds of *Triticum aestivum* (wheat) were purchased from Faisalabad Seed Bank and kept in dark at room temperature prior to their use. The seeds at the time of experiment were surface sterilized with 3.5% filtered $Ca(OCl)_2$ for 20 min to prevent fungal growth. These were then washed thoroughly with tap water followed by rinsing with distilled water. Seeds were then soaked in water for 24h.

3.3. Soil Treatment- Physical and Chemical Analysis

For the soil experiments, the samples were obtained from the A horizon of a wheat agricultural field in Sakreela (33.76°N; 73.24°E), Pakistan. The samples were air-dried for 24h and passed through a 2 mm sieve. Their physicochemical properties were determined and were stored for later use.

3.3.1. pH

The pH of soil numerically determines the acidity of soil which influences soil conditions and plant growth by affecting the solubility of a number of significant biological elements. The pH of soil was determined with a glass electrode using 1:5 soil-water suspension. The soil suspension prepared was placed on a shaker for 30 min before filtering it using Whattman No. 42 filter paper. Filtrate was then used to test the pH using glass electrode.

3.3.2. Electrical Conductivity

To determine the electrical conductivity of the air dried soil samples, a 1:5 soil-water suspension was prepared. The soil suspension was shaken for one hour to dissolve soluble salts and allowed to settle for 30 min. It was then filtered using Whattman No. 42 filter paper. The filtrate was then analyzed using conductivity meter (Rayment and Lyons, 2011).

3.3.3. Moisture Content

Soil moisture content refers to the amount of water present in a given amount of soil. Rayment and Lyons (2011) described the procedure to evaluate the moisture content of soil. A weighed soil sample is oven-dried for 24 h at 105°C and difference in wet and dry weight is calculated using the formula:

$$\%\, moisture = \frac{wet\ soil - dry\ soil}{dry\ soil}\ x\ 100$$

3.3.4. Water Holding Capacity

The water holding capacity of soil was determined as per methods described by Harding and Ross (1964). A funnel containing filter paper was placed on a measuring cylinder. On the filter paper, 25 g soil was placed and 25 mL water was poured over it gently. The excess water was allowed to filter through for 30 min until the water stopped dripping. The final volume was noted to determine the maximum water holding capacity of soil.

3.4. Solution Preparation

3.4.1. Antibiotic Stock Solution

Different concentrations of CIP used in the current study were prepared from the dilutions of the stock solution. CIP has a solubility of 2 g L^{-1} (Sorensen *et al*., 2000).

CIP stock solution was prepared by dissolving a 250 mg crushed tablet of CIP in 100 mL 0.1 N HCl in a 500 mL volumetric flask. The solution was stirred using magnetic stirrer till complete dissolution of powder and filled up to the mark with distilled water. The CIP solution was then filtered and stored in a bottle wrapped in aluminum foil to prevent photo-degradation until further use. Stock solutions were prepared at least once a month. The dilutions of antibiotic were freshly prepared before the experiment.

3.4.2. Standard Preparation for Spectrophotometric Analysis- Microbial Biomass

Standard solution containing 137.5 mg L^{-1} glucose in a volumetric flask was diluted to prepare the final concentrations of 10, 20, 30, 40 and 50 mg L^{-1}. These values were used as a standard to calculate soil microbial biomass. Using distilled water as blank, absorbance was measured at 350 nm. The calibration curve developed for soil microbial biomass is presented in Figure 7.

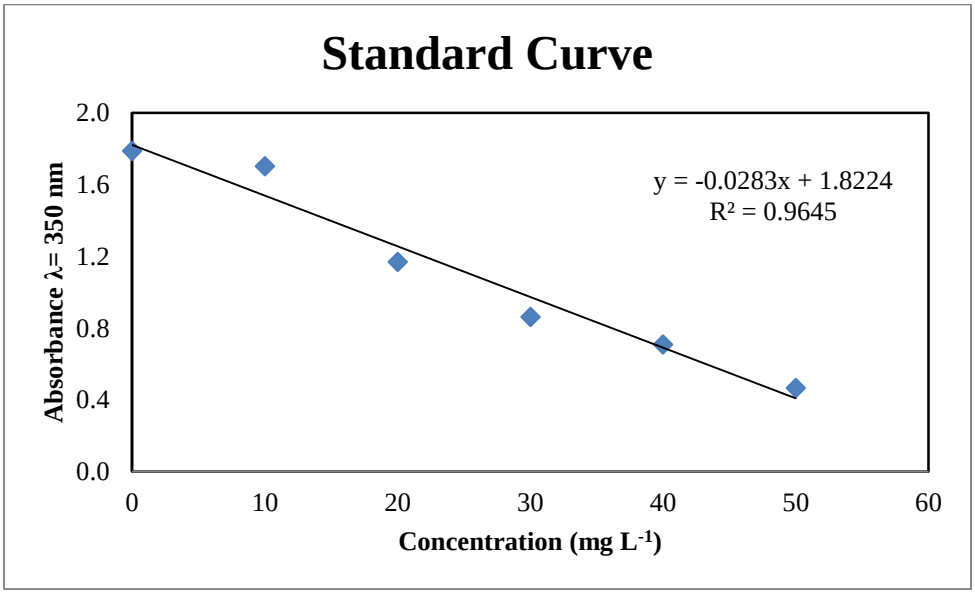


Figure 7: Absorbance and digested glucose standard solution at 350 nm

3.4.3. Oxidant Solution

In 40 mL distilled water, 0.128 g of potassium dichromate ($K_2Cr_2O_7$) was added and mixed well. To this, 200 mL concentrated sulfuric acid was added.

3.4.4. M9 Media

Media was prepared by dissolving 64 g sodium monohydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$), 15 g monopotassium phosphate (KH_2PO_4), 2.5 g

sodium chloride (NaCl) and 5 g of ammonium chloride (NH_4Cl) in 1000 mL distilled water and autoclaved at 121°C for 15 min.

3.4.5. Solutions for Walkey-Black Process

3.4.5.1. *Potassium Dichromate Solution ($K_2Cr_2O_7$), 1 N*

Potassium dichromate was oven dried at 105°C for a period of 2 h followed by cooling in desiccator to remove all traces of moisture. In a 1 L volumetric flask, 49.04 g of potassium dichromate was added, dissolved in distilled water and filled up to the mark.

3.4.5.2. *Ferrous Ammonium Sulfate Solution [$(NH_4)_2 SO_4.FeSO_4.6H_2O$), 0.5 M*

Ferrous ammonium sulfate (196 g) was dissolved in distilled water in a 1 L bottle. To this, 5 mL concentrated sulfuric acid was added, mixed thoroughly and filled to the mark.

3.4.5.3. *Diphenylamine Indicator ($(C_6H_5)_2NH$)*

In 100 mL concentrated sulfuric acid, 1 g of diphenylamine indicator was mixed and stored at room temperature.

3.5. Wheat Cultivation

In the current study, a modified method form the literature (OECD, 1984; Batchlder, 1982; Baguer *et al*, 2000) was used to assess the impact of CIP on growth of plants. For the application of antibiotic, soil was spiked with different volumes of stock solution to achieve desired levels of CIP. In plastic pots, 200 g of test soil was weighed. A total of seven different concentrations including control (0, 1, 5, 25, 50, 100, 200 mg kg^{-1}) of CIP were applied to the soils in each pot. Experiment was performed in triplicates. The soil was thoroughly mixed in order to ensure proper mixing of antibiotic. To each pot, five seeds were added and the pots were watered at regular intervals to maintain moisture level. After one week of seed germination, thinning of plants was done and only one plant was allowed to grow through the full experimental period. After 28 days, the plants were removed from the soil. The root system was initially washed with tap water followed by rinsing with distilled water. Morphological characteristics including root and shoot length and plant biomass were evaluated. To calculate fresh biomass, plants were weighed directly and for dry biomass evaluation, plants were oven-dried at 105°C for 24 h to remove moisture and weighed again.

3.6. Soil Microbial Biomass

From the results of plant growth test, only the concentrations at which growth suppression was observed, a new range was developed containing six different concentrations including control 0 (C0), 50 (C1), 75 (C2), 100 (C3), 150 (C4), 200 (C5) mg kg^{-1}. A rapid chloroform fumigation extraction method (Witt *et al.*, 2000) was used for the estimation of microbial carbon under different antibiotic concentrations over a period of 15 days. The sieved soil samples were adjusted to 40% maximum water holding capacity (MWHC).

For the experiment, soil samples were at each test day split into two portions for fumigation and non-fumigation in screw cap vials. Non-fumigated soil samples taken as control were extracted with 5 mL 0.5 M K_2SO_4 immediately, shaken for 60 min at 35 rpm, and filtered using Whatman No. 42 filter papers. The extracts were frozen until further use. For fumigation, 57 µL chloroform was added to each soil sample, followed by incubation for 24 h in dark at 25°C. After the incubation period, chloroform was allowed to evaporate from the samples by placing them in a fume hood for 30 min. The microbial carbon was then extracted using potassium sulfate as done for non-fumigated samples.

From the extracts, 1.6 mL was pipetted out in screw cap vials and 2.4 mL oxidant solution was added to it. The vials were then placed in the COD reactor at 150°C for 30 min to achieve biomass C oxidation. Spectrophotometric analysis of samples was then measured at 350 nm using a UV-1700 spectrophotometer (PG-Instruments-T60UV, UK) (Cai *et al.*, 2011). Biomass C was calculated using the following formula:

$$\text{Biomass} = E_C/K_{EC}$$

where E_C is the difference of extractable C between the fumigated soil samples and the non-fumigated ones. The extractable part of microbial C (K_{EC}) for the proposed method was given as 0.33.

3.7. Walkey-Black Procedure

To test the effects of the selected antibiotic on soil microbial carbon, rapid dichromate oxidation was done (Ryan *et al.*, 2001). Each 20 g soil sample was amended with CIP by spiking the soil with different concentrations of antibiotic solution. Samples were mixed thoroughly and moisture adjusted to 60%. The soil samples were then sealed

and kept in dark at 25°C. Microbial biomass was calculated at 0, 1, 3, 7, 11 and 15 days.

To detect the soil organic matter, 0.5 g of each oven-dried soil sample was weighed in COD vials to which 2.5 mL of 1 N potassium dichromate solution and 5 mL concentrated sulfuric acid was added. The suspension prepared was swirled to accomplish proper mixing and after digesting at 140°C for 30 min, the mixture was transferred to 250 mL beakers to which 2.5 mL concentrated orthophosphoric acid and 100 mL distilled water were added to it followed by addition of 3-5 drops of diphenylamine indicator. The resulting suspension was mixed well with the help of a magnetic stirrer. It was then titrated against ferrous ammonium sulfate (FAS) solution till the end point where the color changed from violet-blue to green. Two blanks were also prepared as control and to calculate the molarity of FAS.

3.8. Optical Density

Optical density of the microbial cultures was determined at 600 nm to observe the effect of CIP on growth.

3.8.1. Microbial Samples

Microbial samples were obtained from the soil by shaking 10 g soil in 100 mL sterile distilled water in a shaker for 2 h after which the samples were filtered using Wattmann 42. and used for optical density measurement at 600 nm.

3.8.2. Conditions for Growth

Culture bottles were autoclaved along with Erlenmeyer flasks containing M9 media at 120°C for 15 min and stored at 4°C. Microbial cultures from the filtered water (20 mL) were inoculated under sterile conditions and agitated at 120 rev min^{-1} in dark to prevent photo-degradation of CIP. Absorbance (λ= 600 nm) of samples was determined at specific time intervals (0, 3, 6, 21, 24, 48 and 72 h) using 1 mL samples.

3.9. Zone of Inhibition

3.9.1. Culture Selection

Cultures of *E. coli* and *P. aeruginosa* were obtained from microbiology lab, IESE, NUST. The selection of these strains was done on the basis of their documented presence in the environment and their sensitivity and resistance to CIP respectively. The cultures were revived on nutrient agar.

3.9.2. Inhibition Detection

Filter paper discs (2 mm) were autoclaved in a petri plate. Using autoclaved distilled water, several concentrations of CIP were prepared to which the discs were dipped followed by drying in sterile conditions. Agar plates were prepared and spread with microbial dilutions of *P. aeruginosa* and *E. coli*. Discs containing known amount of CIP (0, 50, 75, 100, 150, 200 mg L^{-1}) were placed on agar plates and incubated at 37°C for 24 h. Their inhibition zone diameter was determined using scale.

3.10. Statistical Analysis

Statistical analysis of the plants morphological characteristics and zone of inhibition was done using t-test with significance difference at $p<0.05$. For soil analysis, comparison between control and different concentrations was evaluated using analysis of variance (ANOVA) to ascertain significant differences between organic matter and microbial biomass on application of CIP at varying concentrations.

Chapter 4

Results and Discussion

The agricultural soil used in the study was silty loam. Its physiochemical properties are given in table 2.

Table 2: Physiochemical properties of soil taken from wheat agricultural field.

Parameters	**Values**
pH	7.6
EC	50.1 µS cm^{-1}
WHC	0.6 mL g^{-1}
Total N	0.024%
Total P	7.1 mg kg^{-1}
Total K	70 mg kg^{-1}
Soil Texture (%)	
Sand	32
Silt	50.5
Clay	17.5

4.1. Effects of CIP on plant morphology

The effects of CIP on root and shoot length, fresh and dry biomass of both roots and shoots are depicted in Figure 8. There was no significant effect of CIP on root length (Figure 8a) even at the highest CIP concentration (200 mg kg^{-1}). Shoot growth suffered inhibition in growth at CIP concentration of 200 mg kg^{-1} by 43% (Figure 8b) where the shoot length decreased from 27.43 to 15.67 cm ($p<0.05$). The suppression of plant growth due to antibiotic stress has been reported previously by various studies. A decline in growth of roots as well as shoots of alfalfa by 85 and 61% was observed by Kong *et al*. (2007) on exposure to the antibiotic to oxytetracycline (OTC). In another study, Li *et al*. (2011) exposed OTC-resistant and sensitive wheat cultivars to four different concentrations of 0.01, 0.02, 0.04 and 0.08 $mmol^{-1}$ and noted a decline in

shoot length by 3.3-8.57% while shoot biomass decreased by 5.6-13.75%. Liu *et al*. (2009) studied the toxic effects of six antibiotics on length of roots and shoots of sweet oat, cucumber and rice which indicated the varied susceptibility of plant growth to these antibiotics. But in each case, a hampering in growth was observed.

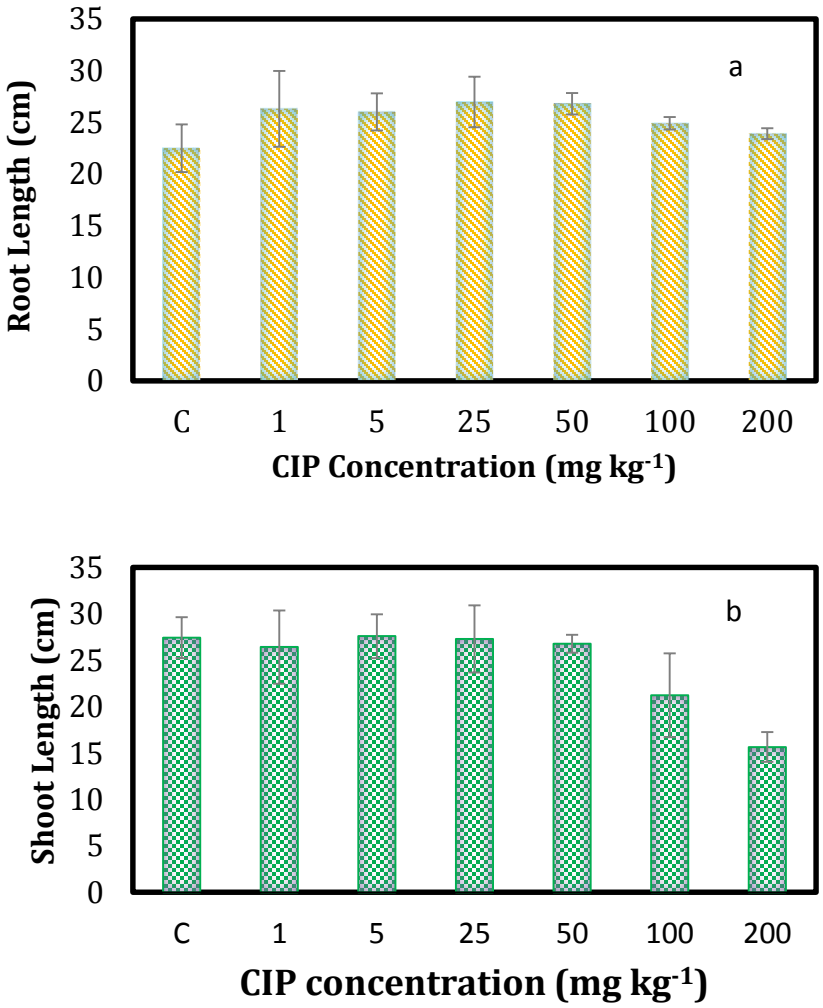
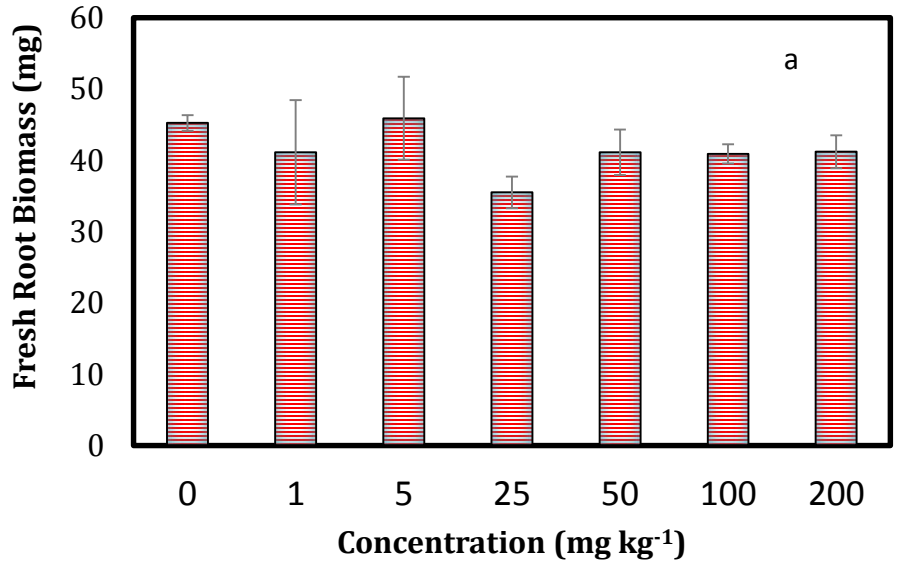
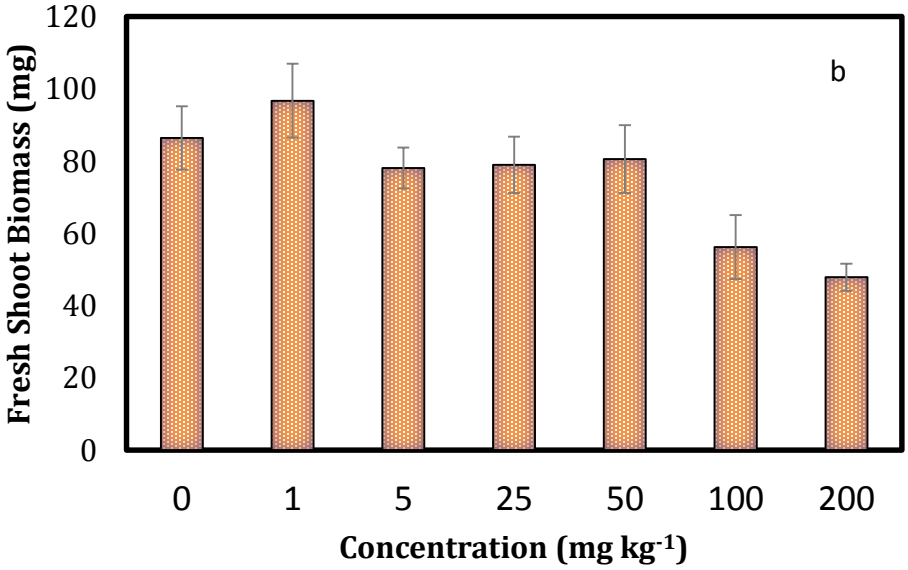
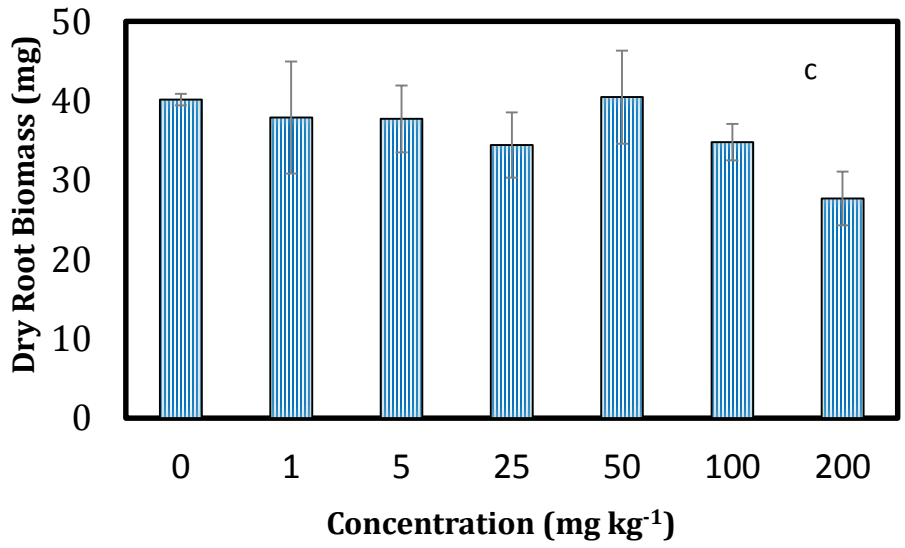


Figure 8: Inhibitory effects of CIP contaminated soil on root and shoot length of wheat over a period of 28 days. Root length (8a) did not suffer a decline whereas a significant decrease ($p<0.05$) was observed at highest applied CIP concentration for shoot length (8b).

In the current study, fresh biomass of roots and shoots did not change significantly at initial concentrations (Figure 9a, 9b) but showed a significant declining trend at higher concentrations ($p<0.05$). CIP affected fresh root biomass at concentrations starting from 25 mg kg^{-1} with maximum decrease at 200 mg kg^{-1}.


Fresh Root Biomass (mg)
60
50
40
30
20
10
0
a
0
1
5
25
50
100
200
Concentration (mg kg-1)


Fresh Shoot Biomass (mg)
120
100
80
60
40
20
0
b
0
1
5
25
50
100
200
Concentration (mg kg-1)



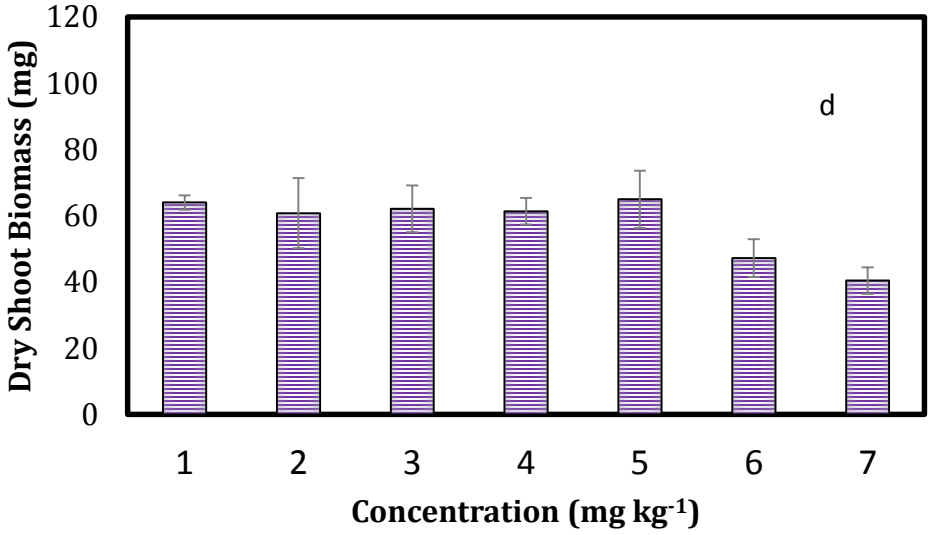


Figure 9: Inhibitory effects of CIP contaminated soil on fresh plant biomass (9 a, b) and dry plant biomass (9c, d); the suppression of mass was significant at the concentrations of 100 and 200 mg kg^{-1}.

The dry root biomass dropped from 40.1 to 27.7 mg (Figure 9c). The percentage decline for dry root biomass was 31.03%. The similar negative trend was observed in

case of shoot biomass (Figure 9d) with respect to CIP concentrations in solution. For the lower concentrations, the biomass showed no statistical difference but a significant decrease of 26 and 36% was observed as the concentration of CIP raised to 100 and 200 mg kg^{-1} respectively. The same negative trend was observed in case of shoot biomass between CIP concentrations in solution and shoot biomass. For the lower concentrations, the biomass showed no statistical difference but a significant decrease ($p<0.05$) of 26 and 36% was observed as the concentration of CIP raised to 100 and 200 mg kg^{-1} respectively.

In an earlier study conducted by Eggen *et al.* (2011) CIP and narasin were applied to carrot and barley and their effects were observed. Both antibiotics had an inhibitory effect on root and leaf in case of carrot and for barley, suppression in growth of seed and leaf was noted as reduced plant biomass. This study corresponds with the present study confirming the negative effect of CIP on plant growth.

The effects of CIP were observed at high concentrations only. This is due to the low bioavailability of CIP which reduces its toxicity against plants. The greater suppression rate in shoot length and biomass compared to roots can be explained by higher concentration of pharmaceuticals in the above ground parts of plants. This has been previously reported in different studies including the uptake of carbamacepine (CZB) by cucumber where the concentration detected in stem and roots was low while about 76-84% accumulated in leaves (Shenker *et al*., 2011). The same case was observed by Wu *et al*. (2010) with higher CZB uptake in aerial parts of soya bean. Winker *et al.* (2010) used ryegrass as model plant and found 34% of the drug in aerial plant parts while only 0.3% in roots when treated with CZB contaminated urine.

4.2. Effects on soil microbial biomass

Time and dose related effects of CIP against microbial biomass in comparison to control are depicted in Figure 10. The CFE tests showed negative impacts of CIP on microbial biomass with the inhibition rate increasing in a concentration dependent manner. The effects of antibiotic on biomass C are significant at $p<0.05$ at all the concentration tested.

A declining trend was observed compared to the respective controls over time for all the treatments with C1 (50 mg kg^{-1}) showing the minimal effect. After 2 day

incubation, the microbial decline rates were 1.4, 2.24, 13.3, 20.5 and 22.3% at C1 to C5 respectively. The degradation rate was fastest at days 3 to 7 than at any other stage. During this interval, the decline rates were observed at 3.59 and 11.4% for C1 and C2. The maximum rate was however observed at the higher concentrations C3, C4 and C5 by 23, 32.5 and 48.4% respectively. The microbial biomass at the end of the experiment for treatments C1 to C5 was 279, 251.8, 215, 189 and 142 mg kg^{-1} respectively and the rate of biomass decline from C2 to C5 was 0.089, 7.96, 21.13, 30.9 and 48% respectively. Using ANOVA, the order of biomass C decline rate for CIP treatments was determined as C5>C4>C3>C2>C1.

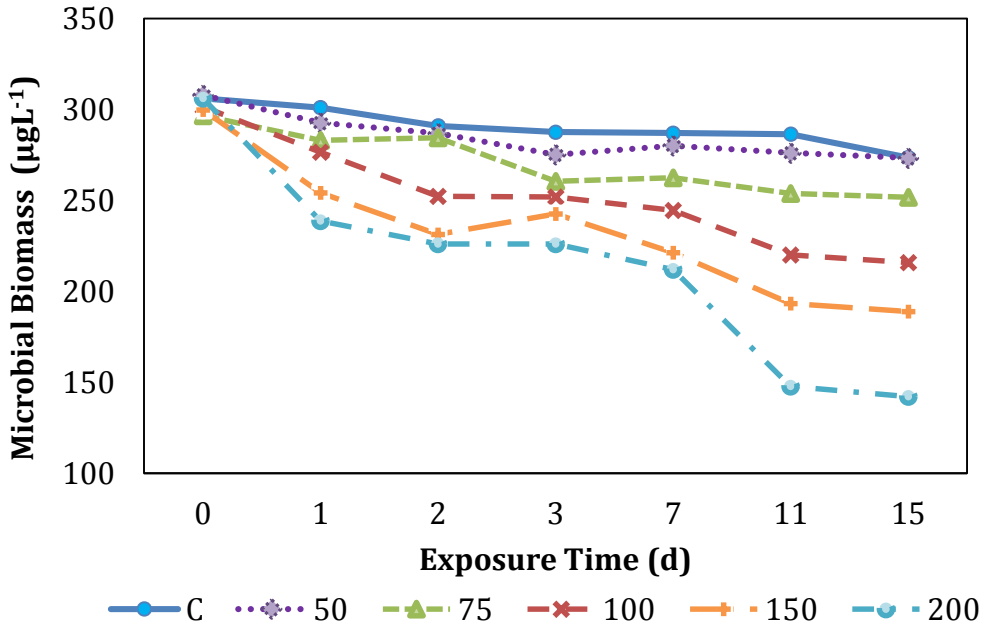


Figure 10: Time and concentration dependent decline in the CIP exposed microbial biomass. Among the treatments, the maximum inhibition was observed at the highest CIP concentration ($p<0.05$).

These results are validated by a previous study by Wunder *et al*. (2013) which reported the potency of CIP even at concentration as low as 0.33 and 3.33 µg L^{-1} against microbial bio-films. At high concentration, a shift in the structure of microbial community was also observed highlighting the selection of resistant bacterial strains. Inhibitory effects of CIP in anoxic and anaerobic conditions were tested by Liu *et al*. (2013b). The results showed a reduction in the sulfate reducing cultures at a range of

10-80 mg L^{-1} CIP concentration. The cultures however recovered after long periods of incubation. At a higher range of 80-100 mg L^{-1}, methanogenesis suffered owing to the inhibition of mixed culture. No significant CIP degradation was observed in the presence of both cultures. In yet another study, Thiele-Bruhn *et al*. (2005) observed a selective pressure of sulfonamide and tetracycline on microbial biomass over a 14-d incubation period. The combined effects of dissolved organic matter (DOM) and chlorotetracycline (CTC) at 0, 10 and 100 mg kg^{-1} on soil enzymatic activities was observed by Liu *et al*. (2014). The study concluded that DOM enhanced the CTC activity against the enzymes by making CTC bio-available to the microbial population. Detrimental effects of the antibiotic sulfamethazine (SMZ) were reported by Pinna *et al*. (2012) on soil microbes where a 63% decline in bacteria/fungi ratio was observed on SMZ treated soil at a concentration of 53.6 mg kg^{-1} after one day incubation period. Martinez *et al*. (2014) studied the effects of CIP presence on partial nitration biofilter used for treatment of synthetic wastewater at the concentrations of 0, 100 and 350 ng L^{-1}. The effects observed at 100 ng L^{-1} for biomass, partial nitration and bacterial structure indicated adaptation to the conditions in the reactor while at the concentration of 350 ng L^{-1}, a different trend was observed. At this high concentration, biomass observed an increase in growth trend while partial nitration process decreased. For the bacterial communities, a shift towards CIP resistant *Commamonas* sp. was observed and the ammonium oxidation bacteria suffered an evident decline in population. Reichel *et al*. (2014) reported a 20% decline in microbial biomass in earthworm burrow, soil macroaggregate microhabitats and rhizosphere on exposure to 10 mg kg^{-1} SDZ in laboratory and field conditions. Structural shift in the bacterial community was also observed.

4.3. Antibiotic effect on Optical Density of soil microbes

Optical density is an indirect mechanism of evaluating microbial biomass. In order to calculate the influence of antibiotic stress on soil microbes, absorbance was measured at 280 nm. The impact of CIP on optical density of soil microbes was distinct. Biomass decreased significantly ($p<0.05$) over the period of 72 h highlighting antagonistic effect of CIP. Absorbance was higher at initial concentrations indicating higher microbial biomass. After the first 6 h, the absorbance was observed to have dropped

from 0.329 at control (C0) to 0.122 at the highest concentration (C5). Absorbance at 24 h for C1 to C5 was measured at 0.246, 0.25, 0.14, 0.17 and 0.13 compared to C0 at 0.331. Exposure time of 72 h caused a decline in absorbance value from 0.285 at control to 0.231, 0.22, 0.129, 0.13 and 0.113 at C1 to C5 (Figure 11).

The negative impact of CIP at high doses reflects increased potency of the drug at high concentrations. Nutrient media without CIP solution served as control for samples and the decreasing trend observed conforms to the results of microbial biomass C decline resulting from CIP stress.

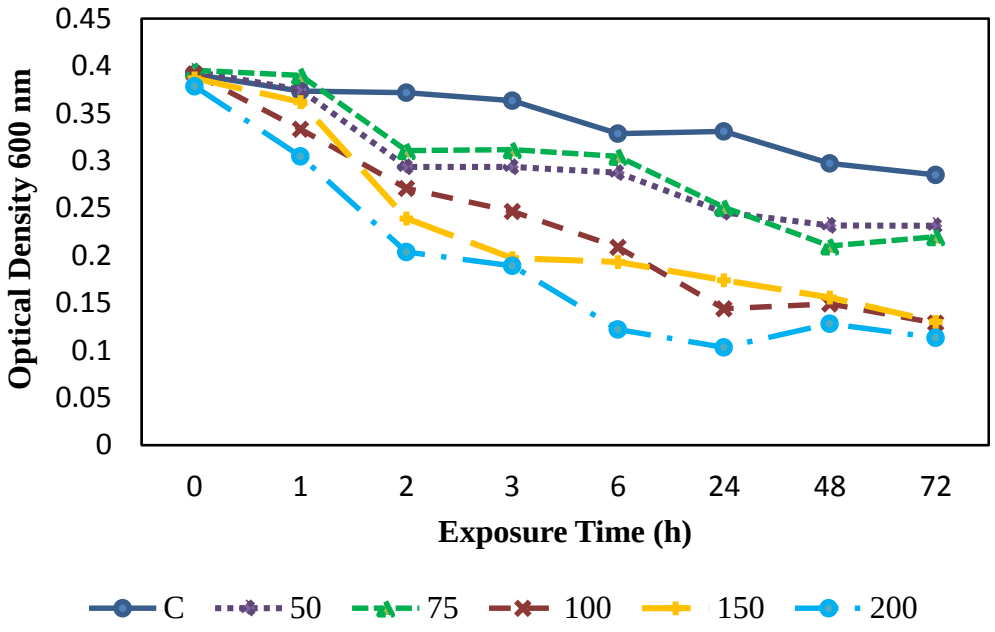
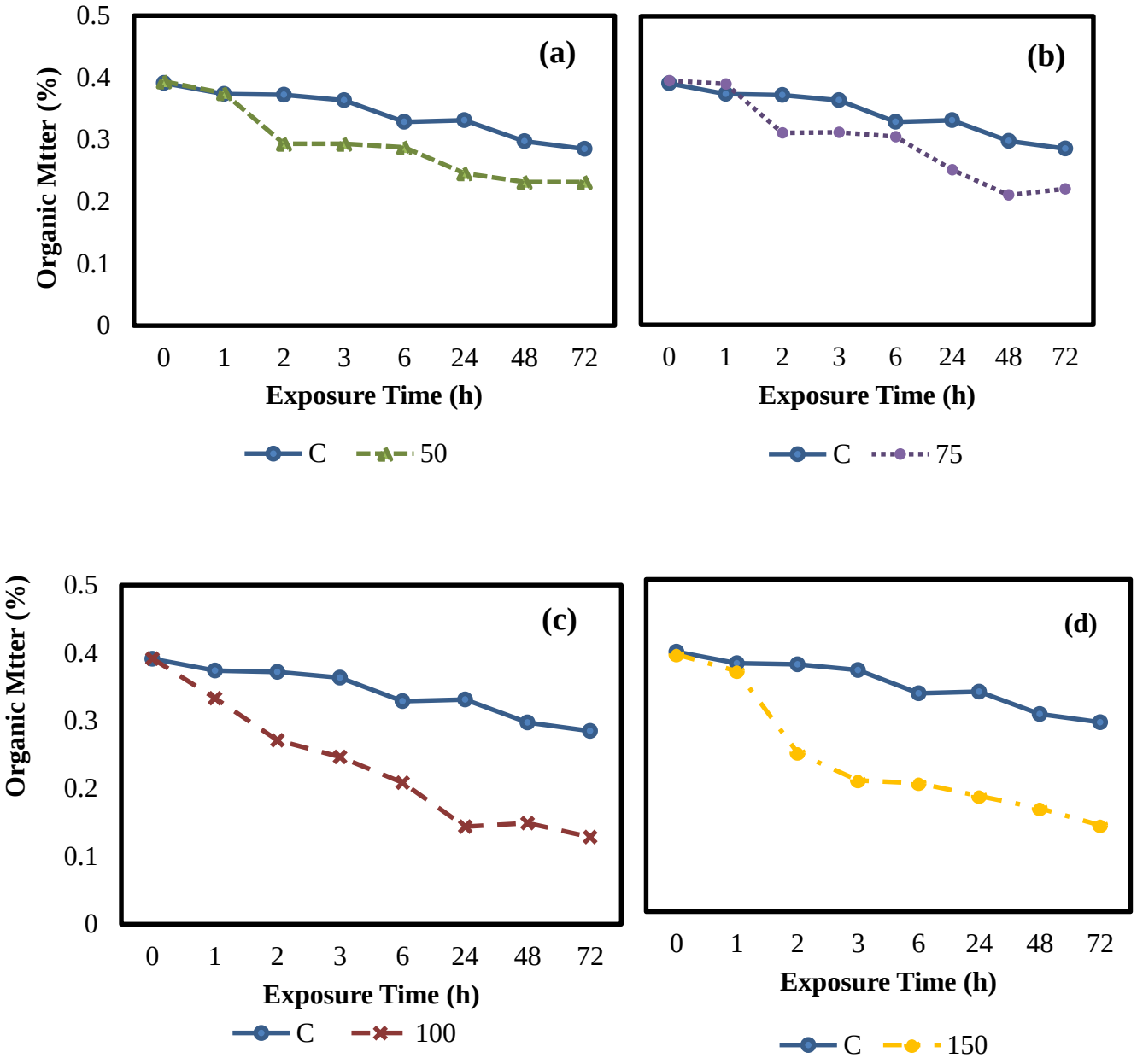


Figure 11: Dose-response curve of CIP for soil microbial growth over 3 day exposure period.

4.4. CIP stress on soil organic matter

Understanding the effects of pollutants on soil organic matter is vital for the improvement of soil

quality and crop yield. For this purpose, the effects CIP had on the soil organic matter were studied for a period of 15 days by varying the concentrations and % decline in organic matter was observed.


(a)
(b)
(c)
(d)
Organic Mtter (%)
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 6 24 48 72
Exposure Time (h)
C
50
75
100
150

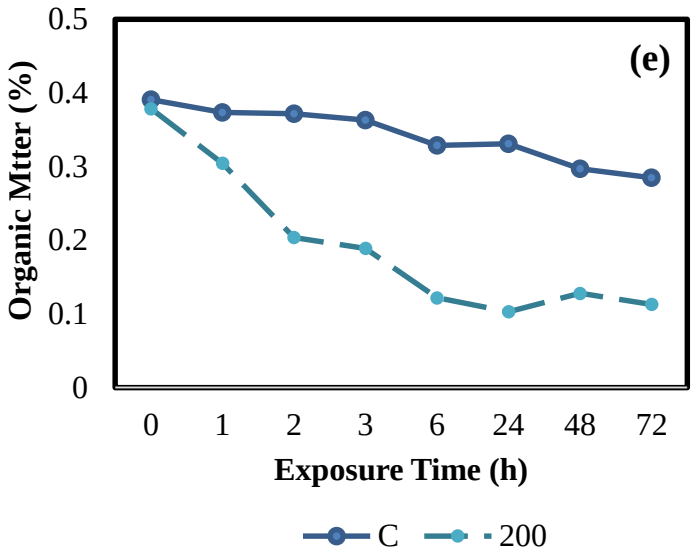


Figure 12: Changes in organic matter compared to control at 50 (a), 75 (b), 100 (c), 150 (d) and 200 mg L^{-1} (e) over 3 days' time period.

The declining trend in organic matter with increasing concentration and passage of time is clearly demonstrated in the graphs (Figure 12). Previous research by Girardi *et al*. (2011) on the effects of 0.2, 2 and 20 mg kg^{-1} CIP on microbial activity by testing acetate mineralization rate and soil respiration showed a 75% decline in mineralization of acetate at the end of 29 days experiment. The soil respiration suffered greatly after 2 days incubation by approximately 70% and by the end of experiment, the decline dropped to only 35%. The maximum effect was observed in the first month of CIP exposure after which the effect was same as for the control. This result agrees with the current study where soil microbes, observed as organic matter, decreased significantly throughout the experiment (Table 3). The % decline was greater at higher concentrations compared to that at the lower concentrations. The order of decline in increasing values was C1>C2>C3>C4>C5 and it corroborates the earlier finding of the detrimental effects CIP has on soil microbes.

Table 3: Decline in soil organic matter ($p<0.05$) on exposure to five different CIP concentrations over a 15 day incubation period

	% Decline in Organic Matter (mg kg^{-1})					
Days	**0**	**50**	**75**	**100**	**150**	**200**
1	0.72	11.16	11.01	10.26	14.95	16.16
3	2.94	18.54	15.00	9.16	14.46	21.72
7	5.73	17.32	17.84	19.64	21.26	20.05
11	5.45	14.82	14.96	18.68	21.16	21.57
15	6.33	16.29	21.62	26.16	26.97	28.50

4.5. Effect of CIP on zone of inhibition

The bacterial population was inhibited significantly as the treatments increased to the concentrations of 2.5 mg L^{-1} and 5 mg L^{-1} for *E. coli* and *P. aeruginosa* respectively. A steady incline was observed in the inhibition zone at varying CIP concentrations with increasing exposure times ($p<0.05$). The increasing antibiotic concentration led to a pronounced decrease in bacterial population. Zone of inhibition ranged from 12.6 to 24.9 mm at CIP concentrations from 2.5-200 mg L^{-1} for *E. coli*. (Figure 14). For *P. aeruginosa*, the range observed was 11.5 to 28 (Figure 13) starting from CIP concentration of 5 mg L^{-1}. The results of this test are complementary to CFE test and OD test and reaffirm the previous results confirming the inhibitory effects of CIP on soil microbial biomass.

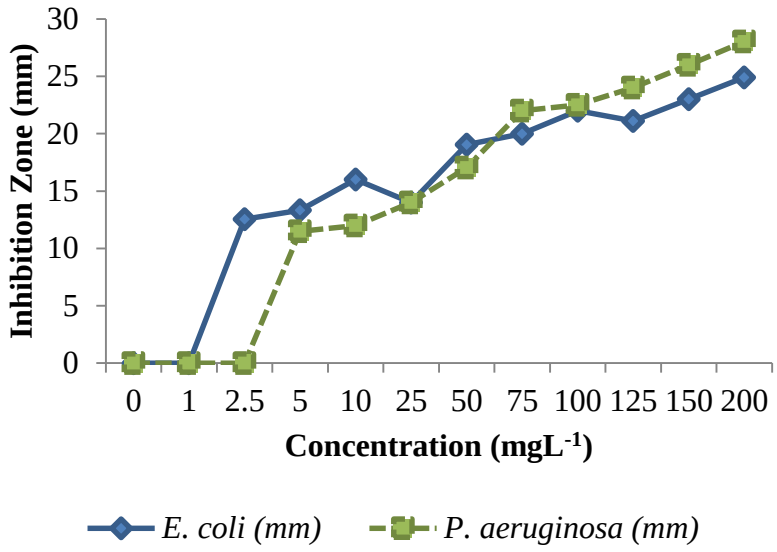


Figure 13: Inhibition zone incline indicating the toxicity of CIP against the two bacterial cultures *E. coli* and *P. aeruginosa* with a significance level of $p<0.05$

In a study on CIP degradation via ozonation, De Witte *et al.* (2010), observed the residual antimicrobial activity by inhibition zone diameter. The CIP degradation resulted in reduced inhibition diameter of *E. coli* from initial 9mm zone created on exposure to 15 mg L^{-1}, to 2 mm, an hour after ozonation started for CIP degradation.

Chapter 5

Conclusion and Recommendations

The results of this study highlight the short-term risks of CIP exposure to crop system and soil microflora. The current study indicates that the presence of CIP had clear detrimental effects ($p<0.05$) on the growth of wheat plants observed as reduced biomass and total length at high (100 and 200 mg kg^{-1}) CIP concentrations. CIP caused shoot length to drop from 27.3 to 15.7 cm while no apparent effect was observed on root length. At the same time, a decline in biomass of both root and shoot was observed at the highest CIP concentration (200 mg kg^{-1}) by 31 and 36%, respectively.

Multiple procedures were employed to estimate the response of varied CIP concentrations on soil microbial biomass. CIP amended soil suppressed microbial biomass depending on the concentration and incubation time. The biomass decreased significantly with increasing CIP concentrations indicating antagonistic effects with a 48% decline observed at 200 mg kg^{-1} after 15 days incubation. The organic matter also suffered a 6-28 % decline with CIP presence.

Recommendations

With increasing consumption and release of FQs, the environmental pollution caused by these drugs and their ensuing detrimental effects will gain even more attention in the coming years. Some of the recommendations for future research are:

- There is a lack of information on the presence of these drugs in various environmental compartments. So studies should also be done to gather information on their presence in the environment.
- Chronic eco-toxicological effects of pharmaceuticals should be studied.
- Uptake of CIP in plants should be studied to directly link them with its effect on plants.
- In addition, the behavior of CIP and other FQs in soil and their degradation kinetics should also be studied.

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