Bioremediation of Aminoglycoside Antibiotic (Streptomycin) in water by White Rot Fungi (*Ceriporia lacerata* and *Trametes versicolor*)

by

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ABSTRACT

Bioremediation of Aminoglycoside Antibiotic by White Rot Fungi (*Trametes Versicolor* and *Ceriporia Lacerata*)

Karimot Afolabi

Water pollution is an issue of great concern worldwide as it has led to various diseases and fatalities. A large portion of antibiotics consumed ends up in wastewater where they are slowly degraded. Taking antibiotics with this contaminated water can induce drug resistance to disease treatment. Antibiotics have been detected in wastewater treatment plant effluents, surface water, ground water and drinking water in several countries due to poor treatment and disposal methods, insufficient policy regulations and lack of public awareness. Bioremediation of antibiotics from aqueous solutions is gaining considerable attention because wastewater treatment plants are not specially designed to remove them. The present study used laboratory-scale experiments to examine the potential of white rot fungi to bio-remediate aminoglycoside antibiotic (streptomycin) in aqueous solution. White rot fungi produce ligninolytic enzymes which enable them to degrade a wide range of organo-pollutants with one or more lignin in their substrates. The results showed that WRF can uptake streptomycin at the various concentrations (100-400ppm) investigated and can use it as a carbon source. Maximum bioremediation (79-88%) was observed at the lowest concentration of streptomycin (100 ppm), indicating that white rot fungi can efficiently remove streptomycin even at low concentration. Bioremediation using white rot fungi shows potential for future development due to its efficiency, environmental compatibility and possible cost-effectiveness. This information provides insights in the development of biological remediation systems to remove antibiotics from wastewater before they are discharged into lakes, streams and rivers.

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CHAPTER ONE

1.0 Introduction

Antibiotics are chemotherapeutic compounds used in animal husbandry and for human health to prevent or treat infections, as growth promoters and sometimes as food preservatives (Tortella et al., 2015). Partial or incomplete metabolism and inefficient removal of antibiotics during wastewater treatment have led to easier introduction of antibiotics into all parts of the environment including water, sediment, soil, etc. through wastewater discharges and agricultural runoff. Since 1982, several widely used antibiotics including macrolides, tetracyclines and sulphonamides, have been confirmed in the environment (Singh et al. 2017). Selective pressure due to widespread overuse of antibiotics has resulted in the emergence and spread of antibiotic-resistant pathogens. Some bacteria are resistant to more than one antibiotic and are termed multiple antibiotic-resistant (MAR) bacteria. The increase usage of antibiotics and consequent development of MAR bacteria pose serious risks to human and veterinary health; thus, antibiotics have become a major group of micropollutants that are of growing concern (Prieto et al. 2011).

Moreover, the presence of antibiotics in the environment can affect natural microbial communities. Natural microbes play a key role in fundamental ecological processes, most importantly the maintenance of soil and water quality. Their large reservoir of genetic diversity and metabolic capability made biogeochemical cycling and organic contaminant degradation possible (Coelho et al., 2015). The presence of antibiotics in the environment, can hamper microbial community structure and functioning in different ways and have both direct (short-term) and indirect (long-term) effects on microbial communities. The direct effects are bactericide (capable of killing bacteria) and bacteriostatic (inhibit the growth of bacteria) actions.

These can lead to disappearance of some microbial populations and their ecological functioning. An indirect impact includes the development of antibiotic resistant bacteria (Jureczko et al. 018).

Antibiotics like aminoglycosides, macrolides and fluoroquinolones have been detected in wastewater treatment plant (WWTP) effluents, surface waters, seawater, groundwater and even drinking water in several countries (Dzomba et al., 2015). Antibiotics are slowly degradable compounds under normal operating conditions in wastewater treatment plant (WWTP) as WWTP are not specially designed to remove them during treatment process (Martins et al., 2018). Consequently, they enter the environment after the treated water has been discharged into surface water. Insufficient policy regulations, lack of public awareness and the constant exposure of the environment to antibiotic substances are contributors to this major environmental problem (Sengupta, 2014). This is a great concern for environmental scientists and doctors because taking antibiotics therapeutically with this contaminated water can induce drug resistance to disease treatment.

Furthermore, water contaminated with antibiotics can lead to development of allergic conditions, discoloration of secondary teeth in children and even fatality in humans, animals and plants. This is due to accumulation of these drugs in the food web and disturbance of important ecological systems such as nutrient recycling (Sengupta, 2014).

These problems from water contaminated with antibiotics challenge the initial motive for producing these drugs which was to treat diseases. Therefore, it is essential to develop a cost effective approach to efficiently remove these pollutants from wastewaters. Scientists have approached the removal of antibiotics in water using several physicochemical processes such as electrochemical treatment, chemical oxidation, ozonation, photo degradation and biological treatment with activated sludge (Sires et al., 2007; Cruz-Morato et al., 2013; Coelho et al., 2015).

Chemical and physical treatment methods are potentially expensive and are largely ineffective (as they can complicate risks) because they end up adding more pollutants into the environment (Coelho et al., 2015). Chemical oxidation processes usually achieve lower decomposition of target compounds and the reaction products of this treatment are usually more toxic than their parent compounds (Negron_Encarnacian & Arce., 2007; Cruz-Morato et al., 2013). Biological treatment with activated sludge (this involves treating wastewater by using aeration and recycled activated sludge), a widely and universally used technology in WWTP, can also lead to additional production of antibiotic resistant bacteria in the final effluents. The fates of antibiotics in sewage are adsorption and biodegradation by active sludge. Increase in concentration of antibiotics in WWTP due to industrial and domestic activities reduce the bio-degradation efficiency of microbes in the sludge. Another problem with the removal of antibiotics using the activated sludge process is the issue of desorption of the antibiotics after it has been originally adsorbed by the activated sludge (Cruz-Morato et al., 2013). The above biological treatment, regardless of its efficiency, leads to the production of bacteria that are resistant to antibiotics degradation in the final effluents (Yu et al., 2017). Treatment with fungi are unlikely to face similar issues because fungi metabolize antibiotics by using it as a nutrient and energy source.

In recent years, the number of research studies on potentially efficient processes to clean up and minimize the pollution of water bodies has been increasing. In this context, the use of bioremediation processes for the removal of antibiotics from aqueous solutions is gaining considerable attention. An alternative lies in the use of living microorganisms for remediation of these antibiotics. Bioremediation involves the use of microbes to remove or breakdown complex hazardous substances into simpler, less toxic or nontoxic substances. The process is generally

60–70% less costly than other technologies (Laxminarayan et al. 2013). Fungi such as Pleurotus ostreatus (Singh et al., 2017), Gloeophyllum striatum (Wetzstein et al. 1999), Phanerochaete chrysosporium (Guo et al. 2014; Martens et al. 1996) and Trametes versicolor (Rodrı´guez-Rodrı´guez et al. 2012) have been reported for their use in bioremediation of antibiotics. Bioremediation can be defined as the ability of certain biomolecules or types of biomass to bind, concentrate and transform or disable selected ions or other molecules present in aqueous solutions. Bioremediation using microorganisms shows great potential for future development due to its environmental compatibility and possible cost-effectiveness. A wide range of microorganisms, including bacteria, fungi, yeasts, and algae, can act as biologically active remediators, which are able to at least modify toxic species (Coelho et al., 2015). The process of bioremediation of antibiotics involves the adsorption and biodegradation of antibiotics in wastewater. The adsorption process involves the accumulation of the antibiotics onto the surface of the microorganisms, while the biodegradation process makes it possible to completely remove the antibiotics from the environment once it is decomposed.

However, there is inadequate research on the bioremediation of aminoglycosides (streptomycin, gentamycin, and neomycin) in wastewater (Sengupta, 2014). Living organisms may provide an alternative for remediation of these antibiotics. Bioremediation with white rot fungi is an emerging, efficient, economic and effective method that can use naturally occurring fungi to detoxify man-made antibiotics such as penicillin, tetracycline, aminoglycosides, and fluoroquinolones (Cruz-Morato et al., 2013; Rodarte-Morales et al., 2011 & Liu et al., 2016). White rot fungi are heterotrophs with rigid cell wall capable of excreting extracellular enzymes to break down complex polymers and utilize organic substrates as energy and nutrient source.

My research aims to discover the potential of white rot fungi (*Ceriporia lacerata* and *Trametes versicolor*) to bio-remediate aminoglycoside (streptomycin) in water. White-rot fungi (WRF) have been shown to eliminate a wide range of pharmaceuticals (such as antibiotics: ciprofloxacin, norfloxacin, orfloxacin) even at very low concentrations (Jureczko & Pryzystas, 2018; Al et al., 2011; Cvancarova et al., 2015). The potential of WRF to be useful biocatalysts (substance that increase the rate of a reaction) is due to their broad specificity to attack substrates (substance on which an organism grows), through the action of intracellular (i.e. cytochrome P450 system) and extracellular (i.e. laccases and peroxidases) enzymes (Marco-Urrea et al., 2009; Prieto et al., 2011; Rodriguez-Rodriguez et al., 2012). The main reactions involved in the transformation of antibiotics by WRF include hydroxylation, formylation, deamination and dehalogenation (Cruz-Morato et al., 2013; Harms et al., 2011). This versatility in degrading a wide variety of xenobiotics make these microorganisms potentially useful in bioremediation applications. To date, however, there is little information on the ability of WRF to remove streptomycin in wastewater.

I will analyze the potential of WRF to bioremediate streptomycin using high performance liquid chromatography (HPLC) technique to determine the concentration of aminoglycoside antibiotic removed, adsorbed and degraded in laboratory tests. Also, I will study the changes in the radial growth and biomass weight of the fungi to examine their response and capability to remove aminoglycoside antibiotic at different concentration (streptomycin) investigated. The result of my analysis can be modeled for bioremediation of surface water that has been contaminated with wastewater. This wastewater could have been discharged from pharmaceutical industries, agricultural runoffs where antibiotics have been used as growth stimulators for animals; and sewage containing improperly disposed antibiotics. The application

of my research to bioremediation of surface water is necessary especially because aquatics can bioaccumulate these antibiotics and circulate it in the food web. This research will draw water managers' attention to the possibility and benefits of using white rot fungi to bioremediate antibiotics in surface water like rivers, streams and lakes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Physicochemical properties of antibiotics (aminoglycosides)

Antibiotics specifically treat infections caused by bacteria, such as staphylococcus, streptococcus, or Escherichia coli. They can either kill the bacteria or keep them from reproducing and growing (Anderson, 2016). A subset of antibiotics called aminoglycosides treats gram-negative infections such as pneumonia, peritonitis (inflammation of the membrane that lines the abdominal cavity), urinary tract infections, blood stream infections, wound or surgical site infections, and meningitis. Aminoglycoside antibiotics have been in use for more than 60 years to combat severe bacterial infections (Romanowska, 2012). The term aminoglycoside was derived from the chemical structure of these compounds, are made up of amino groups (—NH_2) attached to glycosides (derivatives of sugar: 2-deoxystreptamine, or 2- DOS). They include streptomycin (used to inhibit the growth of a variety of bacterial organisms, including the organism that causes tuberculosis); neomycin, gentamicin, tobramycin, netilmicin and amikacin. Almost all aminoglycosides have a common core, neamine as shown in Figure 1 below. The drugs are electrically neutral as they are made up of positively (NH_3^+) and negatively (OH^-) charged ions. The positive charge on the antibiotics results from the presence of many NH_2 groups, which later become NH^{3+} (Jana & Deb, 2006; Kaul et al., 2003).

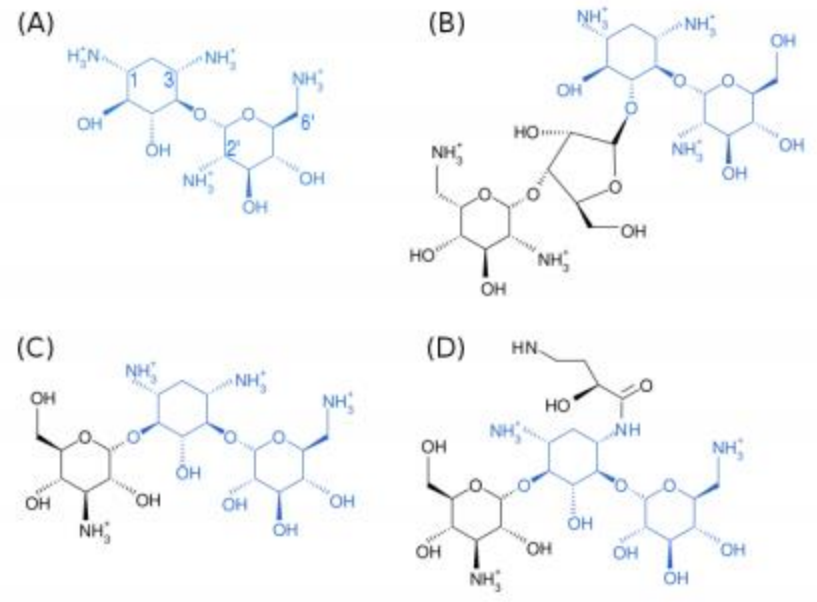

Figure 1: Chemical structures of exemplary aminoglycosides: (A) neamine, the common core of most aminoglycosides; the positions of the amine groups are numbered; (B) paromomycin (4, 5- di-substituted 2-DOS); (C) kanamycin and (D) amikacin (both are 4,6-disubstituted 2-DOS) (Romanowska, 2012).

Veterinary antibiotics are made of organic compounds that have a wide variety of functional groups which affect their chemical properties. Most have octanol-water partition coefficient (log K_{ow}) values less than five, indicating they are relatively non-hydrophobic (Tolls, 2001). Additionally, the water solubility for many antibiotics exceeds 1 g/L suggesting hydrophilic properties Beausse et al., 2004; Boxall et al., 2004; Tolls et al., 2001). For instance, streptomycin has a solubility of 20g/L.

Aminoglycoside antibiotics primarily bind to bacterial ribosomes: organelles fundamental to protein synthesis. This can inhibit protein synthesis and result in the death of bacterial cell (Romanowska, 2012; Legget, 2017). The prevalence of aminoglycoside resistance has remained low and emergence of bacterial resistance during therapy has been unusual (Romanowska, 2012). All aminoglycosides share the potential for nephrotoxicity (poisonous effect on the kidneys), ototoxicity (damage to the inner ear, resulting from drug exposure) and, rarely,

neuromuscular blockade (blockage of neuromuscular transmission), but allergic reactions remain rare (Legget, 2017; Romanowska, 2012). The cost of many aminoglycosides falls below other agents, making it a relatively inexpensive alternative (Legget, 2017).

Streptomycin is an aminoglycoside antibacterial and antimycobacterial that acts by inhibiting the initiation and elongation processes during protein synthesis. It works by inhibiting the ability of 30S ribosomal subunits to make proteins which results in bacterial cell death (Wanwright, 1991). It has a molecular formula of $C_{12}H_{39}N_7O_{12}$ and a molecular weight of 581.58g/mol as illustrated in Figure 2 below (Larranaga et al., 2016). It was discovered in 1943 as it is produced naturally by the soil actinomycete: *Streptomyces griseus* (O'Neil, 2013; Torok et al., 2009; Rennebers et al., 2008). It's on the World Health Organization's list of essential medicines which list the most essential and safe medicines needed in the health system. Its other chemical names include agrimycin, Strepcen, agrept, vetstrep, gerox and so on. It is a highly polar organic base with IUPAC name of 2-[(1R,2R,3S,4R,5R,6S)-3-(diaminomethylideneamino)-4-[(2R,3R,4R,5S)-3-[(2S,3S,4S,5R,6S)-4,5-dihydroxy-6-(hydroxymethyl)-3-(methylamino)oxan-2-yl]oxy-4-formyl-4-hydroxy-5-methyloxolan-2-yl]oxy-2,5,6-trihydroxycyclohexyl]guanidine (Goodman, 1975). Streptomycin is a solid, hygroscopic powder (tends to absorb moisture from air) which is odorless and has a slightly bitter taste (Lewis, 2004; O'Neil, 2013). It is very soluble and miscible with water at 25°C (USEPA, 2012). It has a vapor pressure of 5.82×10^{-28} mmHg at 25°C. It's quite stable but very hygroscopic when heated to decompose, thus it emits toxic fumes of nitrogen oxides (Goodman, 1975).

Streptomycin is a broad-spectrum antibiotic, typically used in combination with isoniazid, rifampicin and pyrazinamide for treatment of active tuberculosis (Wanwright, 1991). It is also used to treat several bacterial infections like brucellosis, plague, tularemia, rat bite fever and

endocarditis (Rennebers et al., 2008); Torok et al., 2008). Common side effects associated with its use include hypotension, headache, neurotoxicity, numbness of the face (cardiovascular), dermatitis, skin rash, angioderma (dermatitis), nausea, vomiting (gastrointestinal), tremor, ototoxicity (auditory) (USEPA, 2012). Its use during pregnancy may result in permanent deafness in the developing fetus.

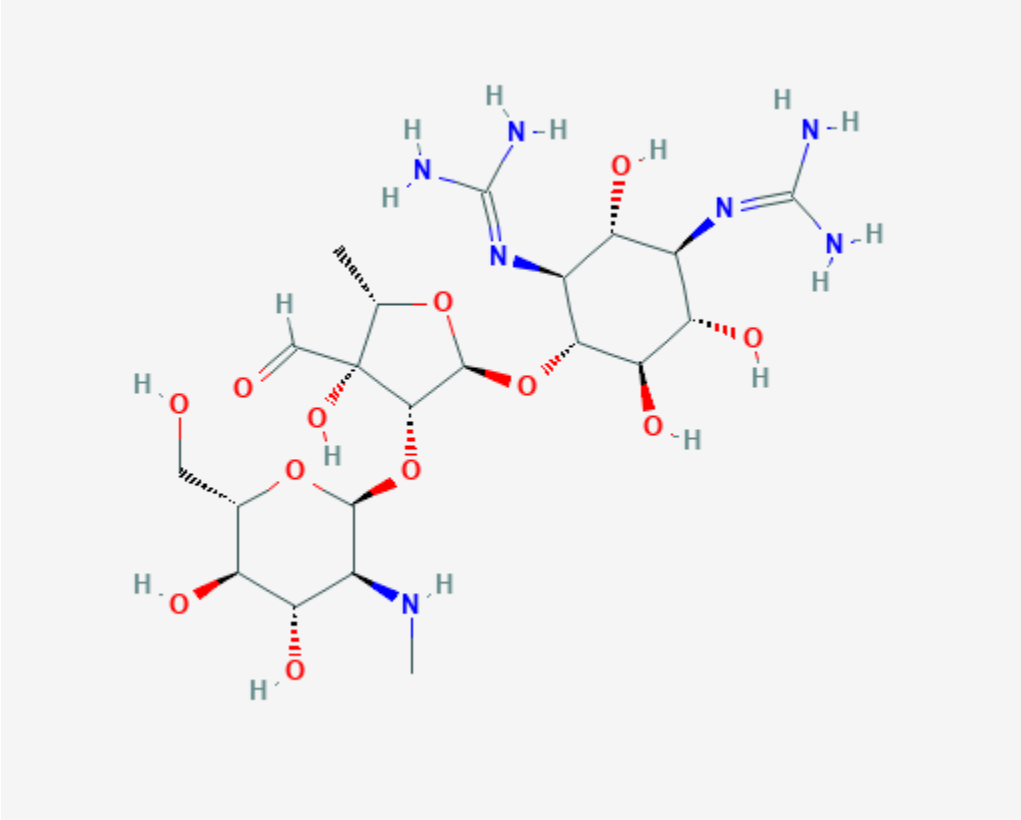

Figure 2: Structure of streptomycin (Larranaga et al., 2016).

2.2 Fate of antibiotics (aminoglycosides) in the environment

Many in the livestock industry routinely use antibiotics to prevent and treat diseases (like pneumonia, coccidiosis), to promote growth, animal feed and/or drinking water commonly contain sub therapeutic concentrations of antibiotics. Such additions have been a regular part of swine production since the early 1950s (Cromwell, 2001). When used in this manner, antibiotics

can select for resistant bacteria in the gastrointestinal tract of animals, providing a potential reservoir for dissemination of drug resistant bacteria into other animals, humans, and the environment (Andremont, 2003). Federal law on these antibiotic uses in livestock became more restrictive a few years ago, in response to concerns about antibiotic resistance.

Antibiotics used in animal agriculture can enter the environment via several routes, including the drug manufacturing process, disposal of unused drugs and containers, and through the use and application of waste material containing the drugs. Other pathways of antibiotic entry into the environment include the excretion of waste products by grazing animals, atmospheric dispersal of feed and manure dust containing antibiotics, and the incidental release of products from spills or discharges. Since the animal gut cannot completely absorb many antibiotics, the parent compound and associated metabolites can be released in dung (Feinman & Matheson, 1978; Halling-Sørensen et al., 1998; Boxall et al., 2004). Elmund et al. (1971) estimated that as much as 75% of the antibiotics administered to feedlot animals could be excreted into the environment. Feinman & Matheson (1978) discovered that animals can excrete about 25% of the oral dose of the tetracycline antibiotic in feces; another 50-60% in unchanged or as an active metabolite in urine. Oral administration of the tylosin resulted in a maximum of 67% of the antibiotic excreted, mainly in the feces. The high levels of antibiotics passed out of livestock can then enter waterways near the feedlots and farms.

The practice of land application of livestock manure can introduce antibiotics into the environment on a large scale (Chee-Sanford et al., 2009). Once released into the environment, antibiotics can be transported either in a dissolved phase or adsorbed to colloids (Colloids are mixtures in which one or more substances are dispersed as relatively large solid particles or liquid droplets throughout a solid, liquid, or gaseous medium) or soil particles which then

migrate into surface water and groundwater (Campagnolo et al., 2002; Kolpin et al., 2002; Yang and Carlson, 2003; Krapac et al., 2004). Manure and waste slurries potentially contain significant amounts of antibiotics which can persist in soil after land application (Donohoe, 1984; Gavalchin and Katz, 1994).

Table 1: Survey of the most commonly used antibiotics in animal production (AHI, 2001)

Antibiotic class	Amount
	metric tonnes
Ionophores/Arsenicals	3520
Tetracyclines	3239
Other antibiotics-includes macrolides, lincosamides, polypeptides, streptogramins, cephalosporins	1937
Penicillins	821
Sulfonamides	269
Aminoglycosides	117
Fluoroquinolones	16

Table 2. Antibiotics commonly used in swine, poultry, and beef cattle production industries (USGAO, 1999; USDA, 2007).

Antibiotic class	Industry
Aminoglycosides	Swine, poultry, beef cattle
β-Lactams	Swine, poultry, beef cattle
Chloramphenicol	Beef cattle
Ionophores	Poultry, beef cattle
Lincosamides	Swine, poultry
Macrolides	Swine, poultry, beef cattle
Polypeptides	Swine, poultry
Quinolones (and Fluoroquiniolones)	Poultry, beef cattle
Streptogramins	Swine, poultry, beef cattle
Sulfonamides	Swine, poultry, beef cattle
Tetracyclines	Swine, poultry, beef cattle
Others:	
Glycolipids (Bambermycin)	Swine, poultry, beef cattle
Carbadox	Swine
Aminocoumarins (Novobiocin)	Poultry
Aminocyclitols (Spectinomycin)	Swine, poultry

Putting soil adsorption into context, studies have shown that under a broad range of environmental conditions, tetracyclines (tetracycline, chlortetracycline and oxytetracycline) can adsorb strongly to clays (Pinck et al., 1961a, 1961b; Sithole and Guy, 1987a, 1987b; Allaire et al., 2006), soil (Krapac et al., 2004) and sediments (Rabolle and Spliid, 2000). Sorption of

chlortetracycline also occurs rapidly in sandy loam soil (Allaire et al., 2006). Macrolides such as tylosin have a weaker tendency to adsorb to soil materials (Rabolle and Spliid, 2000), although a sorption kinetic study showed that 95% of tylosin can be adsorbed within three hours in both sandy loam and clay soils (Allaire et al., 2006). Sulphonamides exhibit weak sorption to soil and are probably the most mobile of the antibiotics (Tolls, 2001). Pinck et al. (1962) determined that two macrolide antibiotics (carbomycin and erythromycin) adsorbed significantly (231-263 mg/g) to montmorillonite and to a much lesser extent (0-39 mg/g) to vermiculite, illite and kaolinite. In a review on the fate of antibiotics in the environment, Huang et al. (2001) concluded that there was little information on the sorption of aminoglycoside and beta-lactam antibiotics. Because aminoglycosides can be protonated under acidic conditions, they could adsorb onto clay minerals under certain conditions, while β-lactams are highly polar compounds and would not be expected to adsorb readily to soil components. Tetracycline and macrolide antibiotics have strong adsorption, their mobility in the environment may be facilitated by transport with manure and soil colloidal material (Kolz et al., 2005a). Interestingly, although most antibiotics do not require metal ion coordination to exert biological action, other compounds such as bacitracin, streptonigrin, bleomycin and tetracycline require the presence of metals ions like Ca^{2+} and Mg^{2+} to function properly (Ming, 2003). Sorption of these drug compounds in clays, where intercalation of metal complexes occurs, may provide suitable conditions for the drug to exert a biological effect on bacterial growth. The presence of these antibiotics in soil can lead to uptake of these drugs by plants and bioaccumulation of antibiotics in animals that wander through.

Plant uptake and bioaccumulation of antibiotics have received considerable interest due to issues of food safety and human health. Several studies have shown that uptake of antibiotics occurs with a variety of plant species (Dolliver et al., 2007; Boxall et al., 2006; Kumar et al.,

2005). Scientists have detected antibiotics in plants that have been irrigated with wastewater and reclaimed water (Wu et al., 2015; Kinney et al., 2006; Ternes et al., 2007; Pan et al., 2014). For instance, erythromycin was found to accumulate over five months in soil irrigated with reclaimed water (Kinney et al., 2006), while six tetracycline, 4-epi-anhydro tetracycline, doxycycline, and six quinolones accumulated in soil during a one-month period of reclaimed water irrigation (Wang et al., 2014). Plant detoxification mechanisms (an established process of removing toxic substances from plants) can be explored to ensure the biotransformation (alteration or chemical modification) of these compounds (Park and Choung, 2007; Sandermann, 1992). Most commonly, farmers place swine and feedlot cattle waste effluent in lagoons or storage before applying it to the land. Treatment with liquid manure can produce crop yields equal to those obtained with chemical fertilizers (Schmitt et al., 1995; Sarmah et al., 2006). To use and dispose of the manure effluent, CAFO (Confined Animal Feeding Operators) operators often contract with neighboring growers to apply effluent to their farm lands or apply it to land surrounding their facilities (Chee-Sanford et al., 2009).

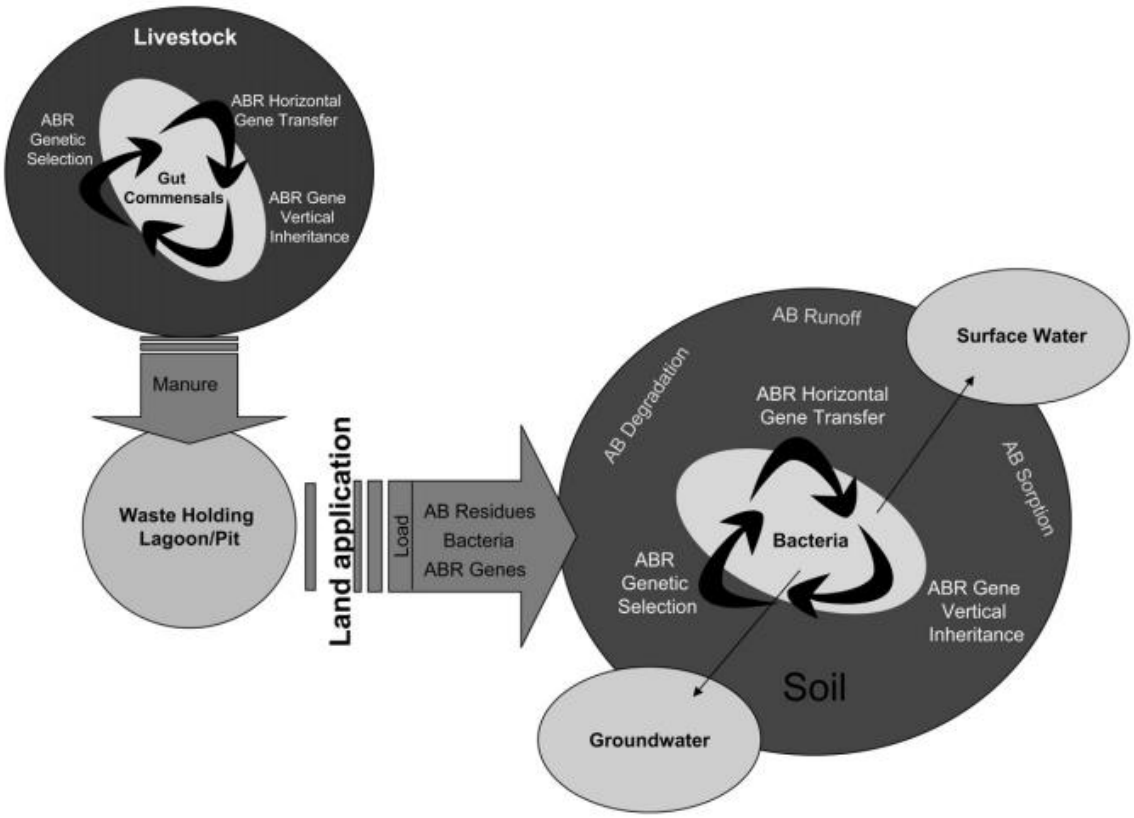

Fig. 3: Conceptualized view showing the possible fates of antibiotic residues and mechanisms of antibiotic resistance gene acquisition and dissemination by bacteria, beginning with land application of animal waste as the source of entry of drugs, bacteria, and resistance genes into the soil environment. AB = antibiotic, ABR = antibiotic resistance (Chee-Sanford et al., 2009).

2.3 Antibiotics (aminoglycosides) in wastewater

The popularity of antibiotics in veterinary care, medicine, and farming (Baquero *et al.* 2008; Bergeron *et al.* 2015) has increased the presence of those substances in surface and ground water, wastewater, municipal sewage, soil and in the influents and effluents of wastewater treatment plants (WWTP) (Kümmerer, 2009).

Among a wide variety of pharmaceutical compounds detected in water bodies and waste streams, antibiotics assume special significance because of (i) the extensive use in human therapy, veterinary medicine and as husbandry growth promoters (more than 50,000,000 lbs.

produced annually in the Unites States) (Karthikeyan & Meyer, 2006); (ii) contributions from numerous sources (WWTP, confined animal feeding operations (CAFOs)); (iii) their ability to alter microbial community structure facilitating the development of antibiotic-resistant human pathogens (Meyer et al., 2000) and (iv) their potential to serve as indicators for the presence of resistant pathogens. Most antibiotics are poorly absorbed by humans and animals after intake, with about 25% to 75% of added compounds leaving the organisms unaltered via feces or urine (Chee-Sanford et al., 2001).

Studies have shown that microorganisms become resistant to antibiotics through mutation or gene transfer (Karthikeyan & Meyer, 2006; Łebkowska 2009; Rzewuska, 2009). According to Karthikeyan & Meyer (2006), fluoroquinolones use in poultry husbandry has promoted the evolution of a fluoroquinolone-resistant pathogen (*Campylobacter jejuni*). The authors explained that exposure to fluoroquinolones can result in a high fluoroquinolone minimal inhibitory concentration. This increase in the minimum concentration of fluoroquinolone that prevents the visible growth of *Campylobacter jejuni* is mostly due to frequent use of the drug. Karthikeyan & Meyer (2006), stated that development of resistance to fluoroquinolones typically occurs within two years of their widespread application in poultry production. Microorganisms have also developed resistance to β-lactam antibiotics. This group of antibiotics includes several types, based on similar chemical structure: carbapenems, penicillin's, monobactams, cephalosporins and β-lactamase inhibitors. The antibiotic resistance of some bacteria against these antibiotics has been related to the presence and activity of β-lactamases. These enzymes can break down the active ingredients in antibiotics to make them ineffective (Huizen, 2017). Rzewuska, 2009 discovered that gram-negative bacteria capable of producing extended-spectrum β-lactamases (ESBL) and metallo-β-lactamases (MBL) are responsible for numerous infections.

Antibiotics have been found in ground and surface waters, landfill leachate and liquid waste near animal operations (Gao et al., 2012). Data have shown that conventional wastewater treatment does not eliminate antibiotics or their metabolites. In 2012, Gao et al., reported that the concentrations of tetracycline and sulfonamide in raw wastewater in China measured 1,129.2 ng/L and 1,535.9 ng/L, (considerably high) respectively, and the decrease in their concentration during the wastewater treatment was 42.2% for tetracycline and 83% for sulfonamide. Thus, more than 50% of the tetracycline remains in the water. In other investigations related to the presence of antibiotics worldwide, sulfonamides have been detected in leachate from a Danish landfill (Holm et al., 1995); in Berlin drinking water wells, for which 80% of the groundwater was bank-filtered surface water (Hartig and Jekel, 2001); and in groundwater in Germany (Sacher et al., 2001). Oxy-tetracycline high concentrations ranging from 0.1–11µg/g have been reported in sediments under a marine salmon farm (Coyne et al., 1994). Studies in the United States have identified antibiotics (sulfonamides and trimethoprim) in groundwater down-gradient from a landfill containing hospital waste (Eckel et al., 1993), in water supply wells in a Nebraska bank filtration site (Heberer et al., 2001), and in ground-water from Washington (Lindsey et al., 2001). In addition, tetracyline have been detected in ground water samples collected near waste and wastewater lagoons (>1µg/L, Thurman and Hostetler, 1999) and liquid hog lagoon samples (5 to 700µg/L, Meyer et al., 2000). A screening study, using radioimmunoassay and immunoassay tests, conducted for different classes of antibiotics in liquid waste from CAFOs reported the following order in terms of frequency of detection: tetracycline > sulfonamides > beta-lactams > macrolides (Meyer et al., 1999).

Antibiotics have been found to persist in reclaimed water due to their slow rate of degradation. Reclaimed water (treated municipal wastewater) has emerged as a potential source

for toilet flushing, as a way of replenishing ground water and as a potential irrigation solution to freshwater shortages. The U.S Environmental Protection Agency (EPA) projects that the use of reclaimed water for landscape and agricultural irrigation will rise (EPA, 2012). However, we do not yet know how much of that water will contain antibiotics. Previous research has focused predominantly on the presence of microbial pathogens, heavy metals and organics (Kulkarni et al., 2017; Sheikh et al., 1990; EPA, 2012). Limited data exists on the occurrence of antibiotics in reclaimed water used for irrigation (Wu et al., 2014; Wu et al., 2015; Kinney et al., 2006). Although the concentrations of antibiotics in wastewater effluent are relatively low, the combination of antibiotics, nutrients, and bacteria in soil and plants subsequently irrigated with this reclaimed water could result into antibiotic resistance among bacterial populations present in these environments (Negreanu et al., 2012; Fahrenfeld et al., 2013).

Professionals in the United States use antibiotics extensively as therapeutic drugs to treat humans, and as therapeutic, prophylactic, and non-therapeutic drugs for livestock animals (Kim & Aga, 2007; CVM, 2014). Consequently, most antibiotic residues enter wastewater due to incomplete metabolism or incorrect disposal (Kummerer, 2001). As stated earlier, conventional wastewater treatment plants (WWTPs) in the United States were not designed to remove pharmaceuticals (Pruden et al., 2013) resulting in the frequent detection of multiple antibiotics in municipal wastewater and treatment plant effluents (USGS, 2016; Zhang & Li, 2011). This problem requires an effective treatment method that is environmentally friendly. Bioremediation of antibiotics in water seems to be a more efficient way of removing these harmful contaminants from water.

2.4 Bioremediation of wastewater with microbes

In 2016, Liu and his colleagues used laboratory-scale experiments to develop methods for gentamicin removal from the environment. They discovered a fungus strain Aspergillus terreus FZC3 that could remove gentamicin in submerged fermentation. Liu et al had isolated Aspergillus terreus FZC3 from solid waste and the sewage water from a gentamicin production factory. They explained that the gentamicin removal efficiency exceeded 95% by day 7 under optimized culture conditions. The authors also showed that the bioremediation process involve both biosorption and biodegradation. They speculated that the fungus, Aspergillus terreus FZC3, absorbed gentamicin and subsequently degraded it. They also found that Aspergillus terreus FZC3 survived and maintained high bioremediation efficiency over a wide pH range, indicating a potential for future use in the large-scale bioremediation of gentamicin.

In 2011, Randhawa and his colleagues stated that Periconiella species of fungus isolated from cow dung could be an excellent degrader of biomedical waste. They analyzed the bioremediation process of pesticides--chlorpyrifos, cypermethrin, fenvalerate and trichlopyr butoxyethyl ester--using fungus isolated from cow dung. They concluded that the higher nutrient availability and larger microbial population of the cow dung slurry and soil-pesticide mix was found to positively influence the bioremediation of pesticides under controlled environmental conditions (pH and temperature). However, the use of fungi isolated from cow dung for bioremediation of xenobiotics has not been adequately researched.

In 2014, Sengupta aimed to remediate tetracycline (TC) and its transformation products Epi-tetracycline (ETC) and anhydro-tetracycline (ATC) from water sources using vetiver grass and tetracycline-tolerant bacteria associated with roots of vetiver grass. The author recovered vetiver root-associated bacteria (Burkholderia cepacia and Serratia marcescens) from a

hydroponic TC remediation system. He explained that these bacteria can tolerate and grow in the presence of TC concentrations as high as 1000 ppm. Sengupta discovered that these bacteria can completely remove both TC and its potential harmful isomers ETC and ATC within 15 days of treatment. He also stated that the TC-tolerant bacteria, B. cepacia and S. marcescens, have a low optimum temperature range of 28-30°C and could potentially be used in controlled settings to remediate TC.

2.5 Mechanism of action of white rot fungi

Among the biological processes used for degradation of antibiotics, myco-remediation is potentially the most efficient, eco-friendly and cheapest because fungi show high capacities of degrading a wide range of toxic xenobiotics. Compared to bacteria, fungal bioremediation is a promising technology that uses their metabolic potential to remove high concentrations of pollutants (Ellouse & Sayadi, 2016). This explains why they have been extensively investigated since the mid-1980s for their bioremediation capacities. Recently, there has been a great interest in white-rot fungi due to their capability to degrade a wide range of persistent environment pollutants even the insoluble chemicals (Cameroon et al., 2000, Ellouse & Sayadi, 2016).

White rot fungi (WRF) are basidiomycetes capable of degrading the lignin component of lignocellulose substrates, leaving behind cellulose and a distinctive white color in wood. They particularly produce three principal ligninolytic enzymes including lignin peroxidases, manganese peroxidases and laccases (Zahmatesh et al., 2018; Hataka et al., 2001; Novotny et al., 2004). These enzymes are highly non-specific about their substrates and these give them the capability to degrade a wide range of highly recalcitrant organo-pollutants with molecular structure like lignin (Novotny et al., 2004; Pointing, 2001) which is utilized as an energy and nutrient source (Osono, 2007). WRF generally act via the extracellular ligninolytic system

showing good potential applications in pharmaceutical, chemical, agro-food, paper, textile, and cosmetic industries (Ellouse & Sayadi, 2016). The physiological and morphological study of WRF may be utilized to transform wastewater treatment into a robust and reliable waste treatment process. The importance of high extracellular levels of these enzymes to enable the efficient degradation of recalcitrant compounds under in vivo conditions relates to the sorption and complexation of enzymes and the probable loss of their activity once externalized.

Research showed that white-rot fungi can degrade lignin through the mycelia of the organisms that penetrate the cell cavity and release ligninolytic enzymes to decompose materials to a white sponge-like mass (Gao et al., 2010). The lignin degradation system consists of peroxidases, H_2O_2-producing enzymes, veratryl alcohol (3, 4-dimethoxybenzyl alcohol), oxalate, and manganese (Zahmatesh et al., 2018). These enzymes are glycosylated heme proteins (This is a form of hemoglobin protein that is covalently bond to glucose) that couple the reduction of hydrogen peroxide to water with the oxidation of a variety of substrates (Hammel et al., 2008, Ellouse & Sayadi., 2016). *Phanerochaete. chrysosporium* has been shown to degrade many xenobiotics and recalcitrant compounds, both in soil and in liquid cultures, suggesting the attractive use of such fungus in bioremediation (Glen et al., 1983). The redox potentials of lignin peroxidases and manganese peroxidases in are higher than for other peroxidases (Cameron et al., 2000). This explains why they have been shown to oxidize chemicals that are not easy to be oxidized by other microorganisms. These chemicals include Polycyclic aromatic hydrocarbons (PAH), phenol and its derivatives, cyanide, TNT (Ellouse & Sayadi., 2016). Lignin peroxidases (LiPs) belong to the family of oxidoreductases (Hammel et al., 2008; Martinez et al., 2005) and were first described in the basidiomycete *P. chrysosporium* in 1983 (Glen et al., 1983). This enzyme has been recorded for several species of white-rot basidiomycetes (Pointing et al., 2005;

Ellouse & Sayadi., 2016). LiP is dependent of H_2O_2, with an unusually high redox potential and low optimum pH (Piontek et al., 2001; Erden et al., 2009). This enzyme can oxidize a variety of substrates including polymeric ones (Oyadomari et al., 2003) and it's consequently a great potential for application in various industrial treatment processes (Erden et al., 2009). Likewise, Manganese peroxidases (MnPs) belong to the family of oxidoreductases (Hammel et al., 2008). Following the discovery of LiP in *P. chrysosporium*, MnP secreted from the same fungus was found as another lignin-degrading enzyme (Paszczynski et al., 1985) and was secreted by almost all white-rot fungi. MnP catalyzes the oxidation of phenolic structures to phenoxyl radicals (Hofrichter, 2002) and produce manganese ion Mn^{3+}, which are highly reactive and can form complexes with chelating organic acids, such as oxalate, lactate, or malonate. Laccases are blue multicopper oxidases, that can catalyze the monoelectronic oxidation of a large spectrum of substrates, for example, ortho- and para-diphenols, polyphenols, aminophenols, and aromatic or aliphatic amines, coupled with a full, four electron reduction of O_2 to H_2O (Cabana et al., 2006; Levin et al., 2003; D'Annibale et al., 2005; Blanques et al., 2008). Laccases act on both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants. This explains their potential use for xenobiotic degradation, and bioremediation purposes (Cameron et al., 2000). Furthermore, the common presence of one or more substructures in the lignin molecule and in xenobiotics explains the ability of white-rot fungi to degrade such a wide range of environmental organic pollutants, even at high levels (Gadd, 2001; Khadrani, 1999). It has been shown that laccase metabolizes these compounds without any net energy gain (Han et al., 2004). Indeed, the oxidation of lignin by WRF is performed to access wood polysaccharides, being their main energy source (Radtke et al., 2004). This implies that the

presence of lignin-cellulosic substrates is required to ensure the degradation of xenobiotic compounds (Baldrian et al., 2004).

2.6 Determination of concentration of aminoglycosides in water

Liu et al. (2016) examined the removal of gentamicin in submerged fermentation using the novel fungal strain Aspergillus terreus FZC3 isolated from gentamicin production wastes. They optimized some parameters to improve the ability of the Aspergillus terreus FZC3 to remove gentamicin and develop its potential for use in large-scale applications. The parameters optimized include the fermentation parameters, including the liquid potato dextrose (LPD) medium concentration, the gentamicin concentration, the shaking frequency, the inoculum size, the temperature and the initial pH. The author explained that the LPD medium concentration had a large impact on gentamicin removal by the FZC3 fungus. They stated that gentamicin removal rate decreased from 91% to 40% when the medium was diluted from 1/1 to 1/20. They speculated that the medium concentration most likely affected gentamicin removal via effects on fungal biomass production whereby a higher LPD concentration produced greater fungal biomass, resulting in a higher gentamicin removal rate. They noted that more biomass produces a larger adsorption surface and more catabolic enzymes because if a medium is diluted with deionized water, its buffering capacity might be weakened compared with the original medium. They succinctly explained how active fungi growth usually lowers the pH and in turn lowers the gentamicin removal. The researchers stated that it is essential to adjust the PH of the reaction solution to efficiently remove gentamicin. They also noted that at lower gentamicin concentration (50mg/L to 200mg/L) more gentamicin was removed (93 to 88%), while at higher concentration (400mg/L) less gentamicin was removed (44%). Their findings were compared with that of Lin et al. (2014) where cephalexin bioremediation efficiency was not significantly

affected by an increase in the antibiotic concentration. This article helped to me decide the working pH and concentration of antibiotics to be remediated by the white rot fungi.

Mode of action of gentamicin removal used by Liu et al. (2016) is described below:

They collected FZC3 thalli at different times ranging from 24 to 168 h and separated it from the LPD medium by vacuum filtration using a 0.45-μ m membrane filter. They transferred 1.5-mL aliquot of the separated liquid into a 2-mL microcentrifuge tube and centrifuged for 15 min. They injected the supernatant into an autosampler vial through a 0.22-μ m membrane filter and determined the gentamicin concentration in the supernatant by high performance liquid chromatography – evaporative light scattering detector (HPLC-ELSD). They assessed the amount of gentamicin absorbed by FZC3 using the following procedure. They treated the separated fungal thalli with 100 mL of 20 mM trifluoric acetic acid (TFA) in a 250-mL Erlenmeyer flask wrapped in tinfoil and shaken on an orbital shaker at 150 rpm. After 2 hours, they determined the gentamicin concentration in this desorption solution as described above. The removal (R), adsorption (A), and degradation (D) were calculated using the following equations:

$$R = \frac{[(C1-C2)-(C1-C3)]}{C1*100\%} = \frac{(C2-C3)}{C1*100\%}$$

$$A = \frac{C4}{(C2-C3)} * 100\%$$

$$D = R - A$$

where: c_1 is the initial concentration of gentamicin in the fermentation broth; c_2 is the concentration of gentamicin in the control at the end of the experiment; c_3 is the concentration of gentamicin in the spent medium at the end of the experiment; and c_4 is the concentration of gentamicin in the desorption solution (Liu et al., 2016).

2.7 High-performance liquid chromatography technique

High-performance liquid chromatography (HPLC) is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. It basically uses a highly improved form of column Chromatography technique in which a solvent is forced through a column under high pressures of up to 400 atmospheres (Clark, 2017). The column is packed with very small silica particle size (stationary phase) to give greater surface area for interactions between the stationary phase and the molecules flowing (mobile phase) past it. This will also enhance better separation of the components of the mixture. Liquid (mobile phase) is passed through the packed column, dissolved sample (in a liquid) is injected into the flow path of the "sample band" mobile phase. Each component in the sample interacts slightly differently with the stationary phase, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column (Snyder & Dolan, 2006). The sample band separates into individual analyte bands as it passes through the HPLC column. Analytes bands are detected, chromatogram is generated, and analyte bands are represented as "peaks" which are quantitated. The peak height or area is related to the injected analyte concentration using a response curve obtained under the same separation conditions (Sirad, 2012).

HPLC has been used to analyze pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples and industrial chemicals. HPLC has also been widely used for the analysis of antibiotics as it is superior to conventional microbiological assays in terms of specificity, sensitivity and analysis time. Aminoglycosides are analyzed by reversed phase HPLC (Gerber et al., 2004; Clark, 2016, Snyder et al., 2006).

The two most common types of HPLC are the normal-phase HPLC and reversed-phase HPLC. For the normal-phase HPLC, the column is filled with tiny silica particles (polar adsorbent), and a non-polar solvent, like hexane or chloroform. Normal-phase HPLC separates analytes based on their affinity for a polar stationary surface. It works effectively for separating analytes readily soluble in non-polar solvents. A typical column has an internal diameter of 4.6 mm or smaller and a length of 150 to 250 mm (Gerber et al., 2004). Non-polar compounds in the mixture will pass more quickly through the column, as polar compounds will stick longer to the polar silica than non-polar compounds.

However, the reversed-phase HPLC has a non-polar stationary phase and an aqueous moderately polar mobile phase. The column which is the stationary phase is filled with silica particles which are modified to make them non-polar. This is done by attaching long hydrocarbon chains (8–18 C atoms) to its surface (Iler, 1979; Gerber et al., 2004). For the mobile phase, a polar solvent such as a mixture of water and methanol or acetonitrile. Methanol or acetonitrile which are less polar solvent can be added to water to reduce the surface tension of water as well as the retention time. Polar compounds in the mixture will pass more quickly through the column because a strong attraction occurs between the polar solvent and the polar molecules in the mixture (Gerber et al., 2004).

2.7.1 Mechanism of Operation of HPLC

The sample mixture to be separated and analyzed is introduced, in a discrete small volume (typically microliters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are a function of specific physical interactions with the adsorbent (also called stationary phase). The velocity of

each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase (Gerber et al., 2004). The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time is measured under specific conditions to indicate identifying characteristics of a given analyte.

Many different types of columns are available, filled with adsorbents varying in particle size and surface. The use of smaller particle size packing materials requires the use of higher operational pressure and typically improves chromatographic resolution (the degree of peak separation between consecutive analytes emerging from the column) (Gidddings, 1965).

Common mobile phases used include hexane or chloroform (for normal-phase HPLC) or any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol) (Karger, 1997). The aqueous component of the mobile phase may contain acids (such as formic, phosphoric or trifluoroacetic acid) or salts to assist in the separation of the sample components (Gerber et al., 2004).

The chosen composition of the mobile phase (also called eluent) depends on the intensity of interactions between various sample components ("analytes") and stationary phase (*e.g.*, hydrophobic interactions in reversed phase HPLC). Analytes partition between the stationary and mobile phase during the separation process taking place in the column, based on their affinity for either phases (Gerber et al., 2004; Clark, 2016). The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depends on the nature of the column and sample components (Clark, 2016).

The reservoir holds the solvent, which is referred to as the mobile phase because it moves as shown in Figure 4. There is usually a minimum of two reservoirs in a system, with each holding up to 1000 cubic centimeters of solvent and usually fitted with a gas diffuser through which

helium can be bubbled (Snyder, 2006). A pump is used to generate a specified flow of the mobile phase. Although manual injection of samples is still possible, most HPLCs are now fully automated and controlled by computer. The injector, or auto sampler, introduces the solvent into a phase stream that carries the sample into the high pressure (up to 400 bar) column, which contains specific packing material needed to effect separation (Gerber et al., 2004). The packing material is referred to as the stationary phase because it is held in place by the column hardware. A detector is needed to see the separated compound (analyte) bands as they elute (to remove adsorbed material from an adsorbent by means of a solvent) from the high-pressure column. The detector reads the strength of the signal as the analyte moves through the mobile phase. The information is sent from the detector to a computer which generates the chromatogram. The mobile phase exits the detector and is either sent to a waste, or collected, as desired.

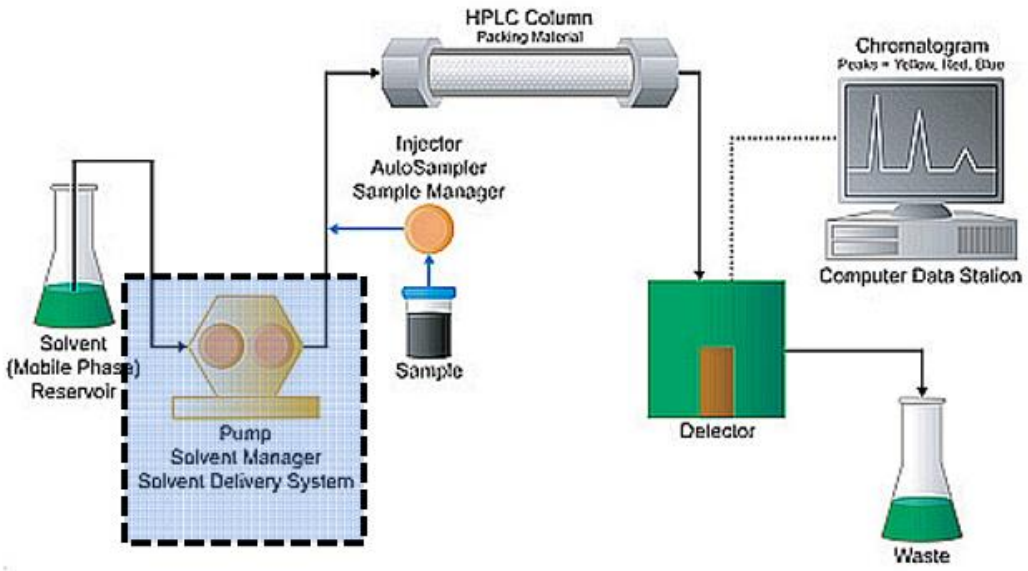

Figure 4: Schematic diagram of HPLC (Retrieved from www.chemguide.co.uk)

2.7.2 Identifying and Quantitating Compounds

A compound can be identified or quantified by comparing its peak's (highest) retention time with that of injected reference standard (substance with known concentration) in the same chromatographic system (i.e same mobile and stationary phase). This reference standard (1000 ppm streptomycin) is measured by determining the elapsed time between the moment of injection (time zero) and the time when it elutes maximally represented by its maximum peak (Gerber et al., 2004). For instance, a reference standard: acrylamide was found to separate and elute from (a specific) column at 2.85 minutes (retention time) as shown in Figure 8 below. If an analyte containing acrylamide was injected into the HPLC system under the same conditions, it is expected that its peak elution would be at 2.85 minutes. Once the analyte is established, the concentration of each compound present in it can be determined. The detector basically responds to the concentration of the compound as it moves through (passes) the mobile phase. A more concentrated compound is represented by a strong signal with a peak height above the baseline. The area under a peak is a measure of the concentration of the compound analyzed. This area value is integrated and calculated automatically by the computer data station.

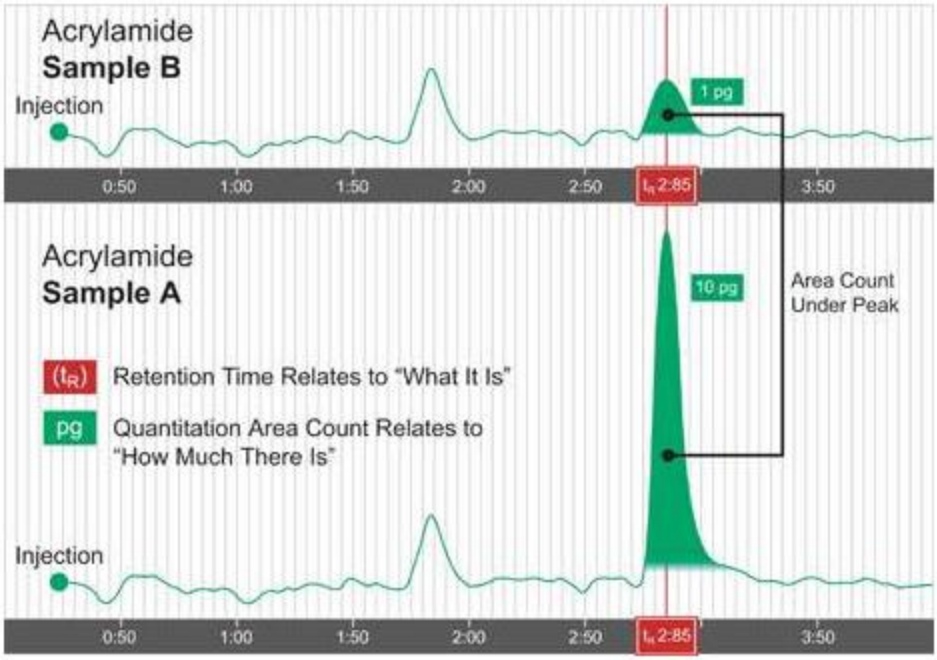

Figure 5: Stacked chromatogram of Sample A and B containing acrylamide (Retrieved from www.waters.com)

CHAPTER THREE

3.0 Introductory discussion

White rot fungi (Ceriporia lacerata and Trametes versicolor) was selected for the purpose of this bioremediation research. Ceriporia lacerata is a bioactive flavonoid[1] that can be isolated from Roxb: Cleistocalyx operculatus (Wang et al., 2013) while Trametes versicolor is a medicinal mushroom that has good antioxidant properties. White rot fungi (WRF) contains polysaccharides and protein-bound glucans. It grows on the trunk of old Pistacia chinesis tree of the family (Anacardiaceae) (Badshah & Muhammad, 2018). The mycelium of the white rot fungi investigated was obtained from the mycology laboratory of The Evergreen State College. The fungi were inoculated in malt extract broth prepared by dissolving 17 g of malt extract powder and 3 g of mycological peptone in 1-liter de-ionized water. A desired pH of 5 was achieved by adjusting the malt extract broth with drops of lactic acid and sodium hydroxide. 100 to 400 microliter of streptomycin stock solution were added to 50 mL MEB using micropipettes to obtain a concentration of 100 to 400 ppm streptomycin respectively. The streptomycin stock solution was prepared using aseptic technique to prevent introduction of contaminants by dissolving 1.5 g of streptomycin salt in 30 ml of deionized water to make 50000 ppm (50mg/ml) of streptomycin. It was later filtered using sterilized 0.22 µm membrane filter and syringe into a sterile falcon tube and stored prior to use at 4°C in the refrigerator.

[1] Flavonoid is one of the most ubiquitous groups of natural health promoting products present in plants.

3.1 Growth Studies

I maintained the mycelium of white rot fungi (*Ceriporia lacerata* and *Trametes versicolor*) on Malt extract Agar (MEA) medium at 25 ± 2 °C for seven days. The inoculation of the mycelium fungi was carried out under aseptic condition using a laminar flow hood and flame to ensure that bacteria and other microorganisms in the environment do not contaminate or affect the growth of the fungi in the MEA.

I studied the effect of different concentrations of streptomycin (100, 200, 300, 400 ppm) on radial growth of *C. lacerata* and *T. versicolor* mycelium and on biomass production. First, I inoculated mycelium bits (5 mm) into 50 ml of malt extract broth (MEB), respectively.

I used Biotech research grade streptomycin (99% pure; obtained from Thermo Scientific) and deionized water to prepare stock solution of the antibiotics. I prepared working concentrations of the streptomycin (100, 200, 300 and 400 ppm) from the stock solution.

After seven days, I transferred the developing fungi (*Ceriporia lacerata* and *Trametes versicolor*) thalli onto malt extract broth (MEB) in 125 ml Erlenmeyer flasks supplemented with 100, 200, 300 and 400 ppm of streptomycin as a carbon source for the fungi growth. I allowed the fungi to grow on the rotary shaker at 25±2°C and 120 rpm for fourteen days. Thereafter, the fungi thalli were separated from the MEB medium by vacuum filtration using 0.45µm membrane filter. In this study, all the treatments were performed in duplicate. The same media containing 100, 200, 300 and 400 ppm streptomycin without fungi were treated under the same conditions as controls. This is illustrated in Figure 6 below. After fourteen days of incubating the fungi in MEB and 100 to 400 ppm of streptomycin, the radial growth, biomass weight of the fungi was studied.

Figure 6: Laboratory experiment showing WRF in MEB growing on a rotary shaker at different concentrations of streptomycin.

3.2 High performance liquid chromatography analysis

The concentration of streptomycin removed by the white rot fungi was determined using high-performance liquid chromatography technique.

I separated the fungi thalli from the MEB medium by vacuum filtration using a 0.45 µ m membrane filter into a sterile falcon tube. 10 ml of each samples was sent for HPLC analysis at Lab/Cor Materials LLC, Seattle, WA.

The analyst at Lab/Cor filtered each of the samples through 0.2-micron sterile filter and diluted it from 1 to 5 with HPLC grade water. The samples were run on Shimadzu LC-2040 3D

HPLC/PDA (Photometric Diode Array) system. Column: Restek Raptor ARC-18, 100 mm x 2.1 mm. The flow rate of the sample was 0.6mL/min; its particle size is 1.8µm and the mobile phase is made up of aqueous 0.1% formic acid, 5 mM ammonium formate. The High-Performance Liquid Chromatography technique (HPLC) using Photometric Diode Array (PDA) Detector involved separating the sample band into individual analyte bands as it passes through the HPLC column. The analytes bands are detected, and a chromatogram is generated. These analyte bands are represented as "peaks" which are quantitated. The peak height was related to the injected analyte concentration using a response curve obtained under the same separation conditions. The analyst had initially prepared streptomycin standards by dissolving 0.0504 g streptomycin sulfate in 10 mL HPLC grade water. A standard run of pure streptomycin (100- 400 ppm) was performed to generate a standard curve used to comparatively assess and determine the concentration of streptomycin left in the samples analyzed. I calculated and compared the concentration of streptomycin removed by the fungi as well as the percentage removal of streptomycin by both fungi investigated using the equation below:

$$R = \frac{c1-c2}{c1} * 100\%$$

c_1 is the initial concentration of aminoglycosides in the fermentation broth (MEB medium) and c_2 is the concentration of aminoglycosides in the spent medium (MEB medium) at the end of the experiment.

3.3 Quality Control and Assurance Protocol

GLASSWARES AND PLASTICS

All glassware and plastic used were pre-treated to avoid contamination and interferences by washing with a soap solution and rinsed copiously with distilled water. Clean hardware was acid

washed in a 1.5 M hydrochloric acid bath for 24 hours, then triple rinsed with deionized water and allowed to drain in a dust free environment.

pH METER

The pH meter was calibrated to working range of pH 4 and 7 with commercial pH solution. The electrode was washed with deionized water, gently mopped dry with a Kim wipe and the buffer solutions were then used to calibrate the pH meter.

3.4 Statistical Analysis

I used JMP Pro 14 software for statistical analysis of my results. One-Way Analysis of Variance (ANOVA) was used to determine if there is a significant difference in the concentration of streptomycin removed by *Ceriporia lacerata* and *Trametes versicolor*. It was also used to determine if there is a significant difference in the radial growth and biomass weight of the two species of fungi investigated.

For the analysis of streptomycin removed by the fungi, my proposed null hypothesis (Ho) was that there is no significant difference in the concentration of streptomycin removed by both species of fungi investigated. My alternate hypothesis (Ha) was that there is a significant difference in the concentration of streptomycin removed by both species of fungi investigated. For the fungi growth studies, my proposed null hypothesis (Ho) was that there is no significant difference in the radial growth and biomass weight of the fungi investigated. My proposed alternate hypothesis (Ha) was that there is a significant difference in the radial growth and biomass weight of the fungi investigated. Concentration of streptomycin is my continuous/independent variable and fungi species is my categorical/dependent variable. Radial growth and biomass weight of fungi are continuous response variables.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

The two white rot fungi investigated, *Ceriporia lacerata* and *Trametes versicolo*r responded slightly differently to the various concentrations of streptomycin (100 ppm, 200 ppm, 300 ppm and 400 ppm) added to the Malt Extract Broth (MEB) medium.

4.1 Radial Growth Studies

The radial growth studies of *Ceriporia lacerata* and *Trametes versicolo*r at different concentrations of streptomycin revealed that as the concentration of the antibiotic increased from 100 to 400 ppm, there was a subsequent reduction in the radial growth of the fungi. The fungi used had an initial radius of 2.5 mm and were found to grow substantially after fourteen days of incubation. The results presented in Table 3 and 4 below showed that there was an increase in the radial growth of the fungi after being exposed to lower concentrations of streptomycin. The highest increase in radial growth (6.9 mm) was recorded in the fungal mycelium of *Trametes versiocolor* inoculated in 50 ml MEB and 100 ppm streptomycin exhibiting a 174% increase in radial growth (over the initial size of 2.5 mm) as illustrated in Figure 7 below. On the other hand, the fungal mycelium of *Ceriporia lacerata* inoculated in the same solution had a slightly smaller radial size of 6.5 mm exhibiting a 156% increase in radial growth. The radial growth of both fungi was found to reduce as the concentration of streptomycin increased from 100 ppm to 400 ppm as shown in Figure 8 below. For instance, Trametes versicolor had a 54% increase in radial growth while Ceriporia lacerata had a 34% increase in radial growth (over fourteen days), at the higher concentration of antibiotic. I observed that streptomycin inhibits the fungi growth at high concentration.

From my results, *Trametes versicolor* had the highest increase in radial size at all concentrations of streptomycin it was exposed to. The figure shows that both fungi species achieved similar percentage radial growth at 300 ppm, indicating that they respond to increase in streptomycin concentration similarly at this concentration. From the analysis of variance carried out using $\alpha = 0.05$, a p-value of 0.6078 indicates that there is no significant difference in the radial growth of the two species of fungi investigated. Both *Ceriporia lacerata* and *Trametes versicolo*r growth is inhibited at high streptomycin concentration. In 2017, Singh et al reported that there was no inhibitory effect of ciprofloxacin on the growth of the fungus (Pleurotus ostreatus) investigated. On the contrary, the researcher observed a maximum radial growth at an antibiotic concentration of 500 ppm. Singh's results on biodegradation of ciprofloxacin by white rot fungus: *Pleurotus ostreatus* differ significantly from this present study.

Radial and Biomass growth studies of Trametes versicolor

Samples	Concn (ppm)	Radius (mm)	% R.G	Wet Wgt (g)	Dry Wgt (g)
TV11	100	6.8	172	5.532	0.689
TV12	100	6.9	176	5.549	0.678
TV21	200	5.8	132	4.783	0.492
TV22	200	5.8	132	4.795	0.498
TV31	300	4.6	84	3.984	0.389
TV32	300	4.6	84	3.972	0.386
TV41	400	3.9	56	3.356	0.272
TV42	400	3.8	52	3.361	0.278

Table 3: Radial and Biomass growth studies of *Trametes versicolor*. This figure shows the various concentration of streptomycin used (Concn (PPM), percentage radial growth (% R.G) and biomass weight (Both wet and dry weight) of *Trametes versicolor*.

Radial and Biomass growth studies of Ceriporia lacerata

Samples	Concn (PPM)	Radius (mm)	% R.G	Wet Wgt (g)	Dry Wgt (g)
CL11	100	6.5	160	5.327	0.668
CL12	100	6.3	152	5.235	0.643
CL21	200	5.6	124	4.281	0.481
CL22	200	5.7	128	4.302	0.482
CL31	300	4.3	72	3.828	0.343
CL32	300	4.5	80	3.895	0.348
CL41	400	3.4	36	3.093	0.225
CL42	400	3.3	32	3.126	0.227

Table 4: Radial and Biomass growth studies of *Ceriporia lacerata*. This table shows the various concentration of streptomycin used (Concn (ppm), percentage radial growth (% R.G) and biomass weight (Both wet and dry weight) of *Ceriporia lacerata*.

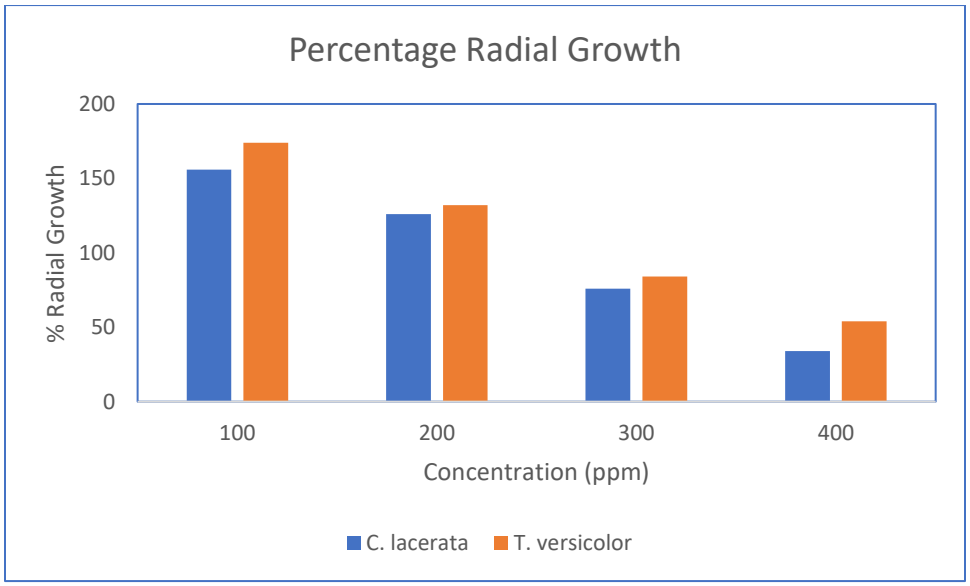

Figure 7: Bar graph comparing percentage radial growth of C. lacerata and T. versicolor. This figure shows the various concentration of streptomycin used (Concn (ppm) and the percentage radial growth (% R.G) observed.

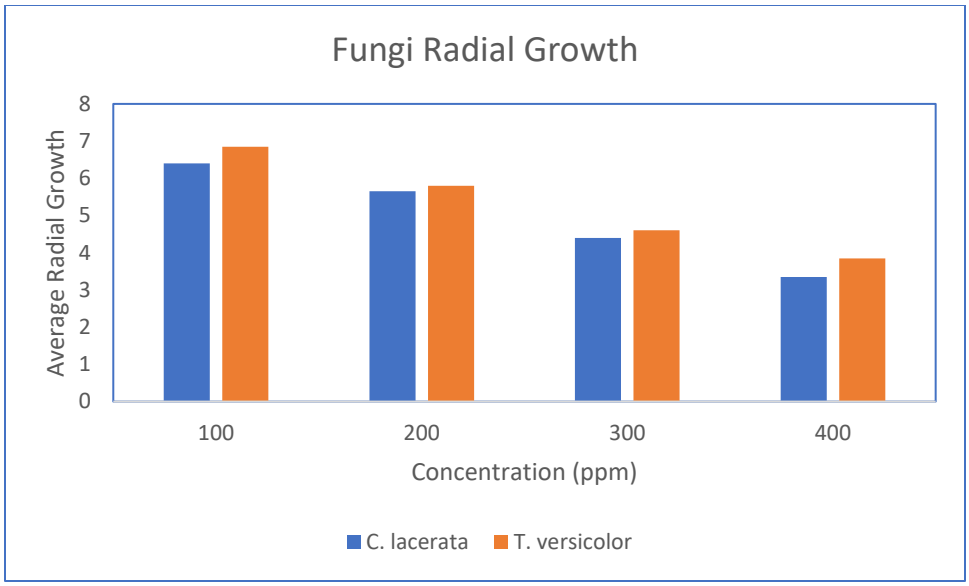

Figure 8: Bar graph comparing average radial Growth of C. lacerata and T. versicolor. This figure shows the average of two duplicates of the fungi radial growth at different concentration of streptomycin used (Concn (ppm).

4.2 Biomass Studies

Biomass studies of WRF also exhibited a pattern like that observed in the radial growth studies. The fungi (*Ceriporia lacerata* and *Trametes versicolo*r) I studied had an initial weight of 0.15 g and the investigation of the changes in their biomass after fourteen days of incubation varies significantly with different concentration of streptomycin (100, 200, 300, 400 ppm) as presented in Figure 12 below. Both species showed an inhibitory effect, with reduced fungi growth in higher concentrations of streptomycin. For *Trametes versicolo*r the maximum growth in biomass (5.549 g) was observed in the medium (50 ml MEB) containing 100 ppm streptomycin, and the least growth in fungi biomass (3.356 g) was recorded in the medium containing 400 ppm streptomycin as presented in Figure 9 below. The largest increase in biomass weight (5.327 g) for *Ceriporia lacerata* was observed in the medium (50 ml MEB) containing 100 ppm streptomycin, and the least growth in fungi biomass (3.093g) was recorded in the medium containing 400 ppm streptomycin as presented in Figure 9 below. From the analysis of variance

carried out (with $\alpha = 0.05$), a p-value of 0.5248 indicates that there is no significant difference in the biomass weight of the two species of fungi investigated. The biomass growth pattern is like the radial growth pattern. From the results presented above, I speculate that streptomycin might be toxic as it inhibits the growth of these fungi at high concentration. Further work needs to be carried out to understand the mechanism behind the inhibitory growth effect of streptomycin on fungi.

A similar result was observed in a research conducted by Liu and his colleagues in 2016 where they attempted to remove gentamicin by the novel fungal strain: *Aspergillus terreus*. Their results showed that the highest fungal biomass growth was observed in medium containing 50 mg/L gentamicin, while the lowest fungal biomass growth was observed in the medium containing 400 mg/L gentamicin.

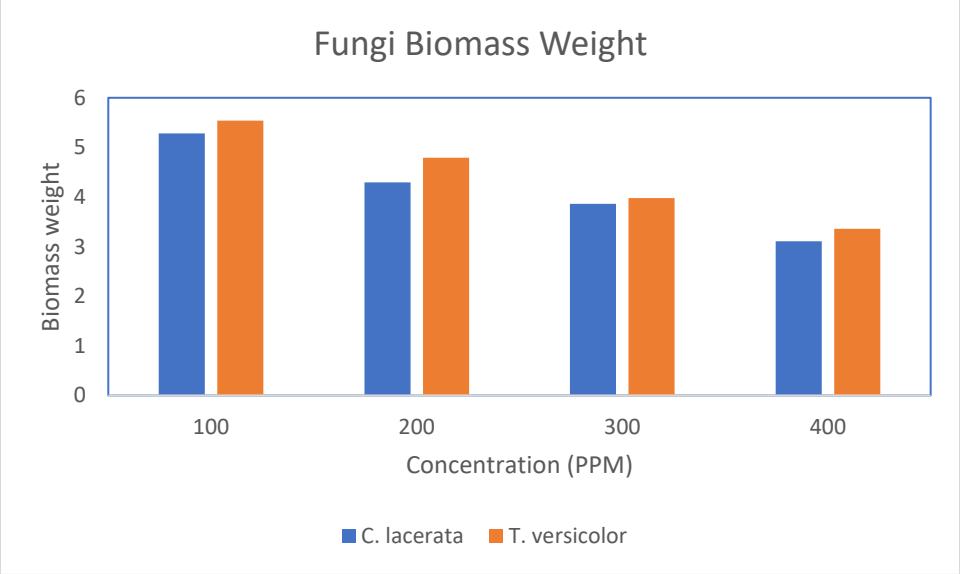

Figure 9: Bar graph comparing Biomass Weight of C. lacerata and T. versicolor at streptomycin concentration of 100 to 400 ppm

4.3 HPLC Analysis of Streptomycin bioremediated by WRF

The ability of both fungi investigated to bioremediate streptomycin is closely linked to the concentration of streptomycin in the MEB medium. It was observed that both fungi remove proportionally more streptomycin at lower concentration than at higher concentration as presented in Table 5 below. For instance, when 100 ppm of streptomycin was added to the MEB medium, T. versicolor removed 88.8% of streptomycin in 100 ppm streptomycin but it decreased sharply to 60.65% when 400 ppm streptomycin was added. Similarly, C. lacerata removed 79.1% of streptomycin in 100 ppm streptomycin and 50.05% streptomycin when the concentration was increased to 400 ppm.

However, Figure 10 revealed that the bioremediation capacity of T. versicolor was higher than that of C. lacerata. Although, from Figure 11 presented below the concentration of streptomycin removed at 300 ppm by both fungi is almost the same, T. versicolor still removed a greater amount compared to C. lacerata. The results presented showed that T. versicolor removed more streptomycin compared to C. lacerata for all the different concentrations of streptomycin studied. The increase in percentage concentration of streptomycin removed by the fungi followed the same trend observed for both fungi biomass weight and radii. The fungi with the highest biomass weight (T. versicolor at 100 ppm) had the highest percentage removal of streptomycin, likewise the fungi with the least biomass weight had the least percentage removal of streptomycin (C. lacerata at 400 ppm). From the analysis of variance carried out at $\alpha = 0.05$, a p-value of 0.5095 indicates that there is no significant difference in the concentration of streptomycin removed by both species of fungi investigated.

These results are in agreement with that of Liu et al., 2016 where the researchers discovered that the fungal strain: Aspergillus terreus FZC3 removed proportionally more gentamicin (93-88%) at

a lower concentration (50 – 200 mg/L) and less gentamicin (44%) at a higher concentration (400 mg/L).

Table 5: Table showing results of HPLC analysis of streptomycin bio-remediated by fungi. The table shows C_1 (ppm): initial concentration of streptomycin added to 50 ml of the MEB medium; C_2 (ppm): average concentration of streptomycin left in the MEB at the end of the experiment; C_3 (ppm) is the concentration of streptomycin removed by the fungi and % CONCN: percentage concentration of streptomycin removed by the fungi

HPLC analysis of streptomycin bio-remediated by fungi

Samples	C1 (ppm)	C2 (ppm)	C3 (ppm)	% CONCN
T. versicolor	100	11.20	88.80	88.80
C. lacerata	100	20.90	79.10	79.10
T. versicolor	200	77.90	122.10	61.05
C. lacerata	200	88.30	111.70	55.85
T. versicolor	300	125.80	174.20	58.07
C. lacerata	300	130.80	169.20	56.40
T. versicolor	400	157.40	242.60	60.65
C. lacerata	400	199.80	200.20	50.05

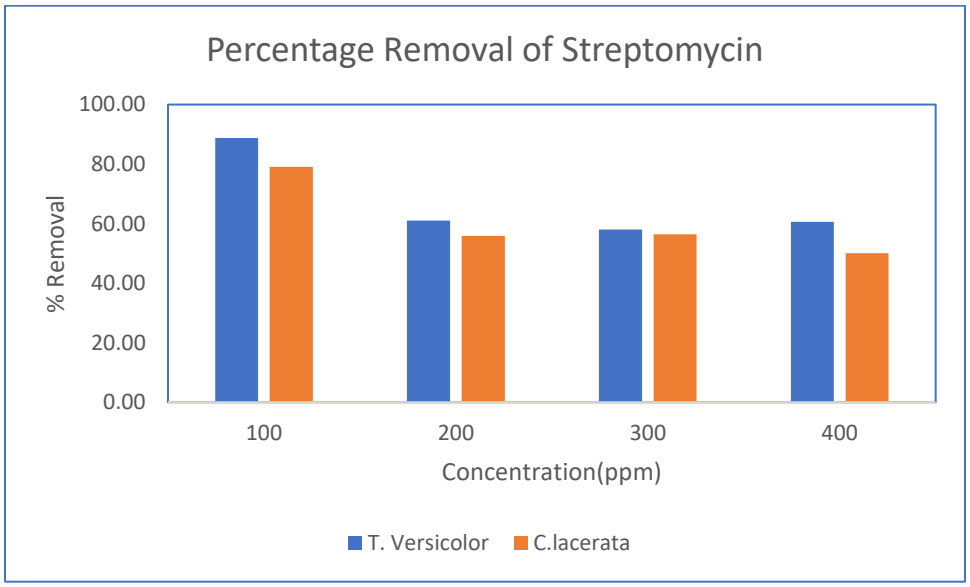

Figure 10: Bar graph comparing Percentage removal of streptomycin by T. versicolor and C. lacerata at streptomycin concentration of 100 to 400 ppm

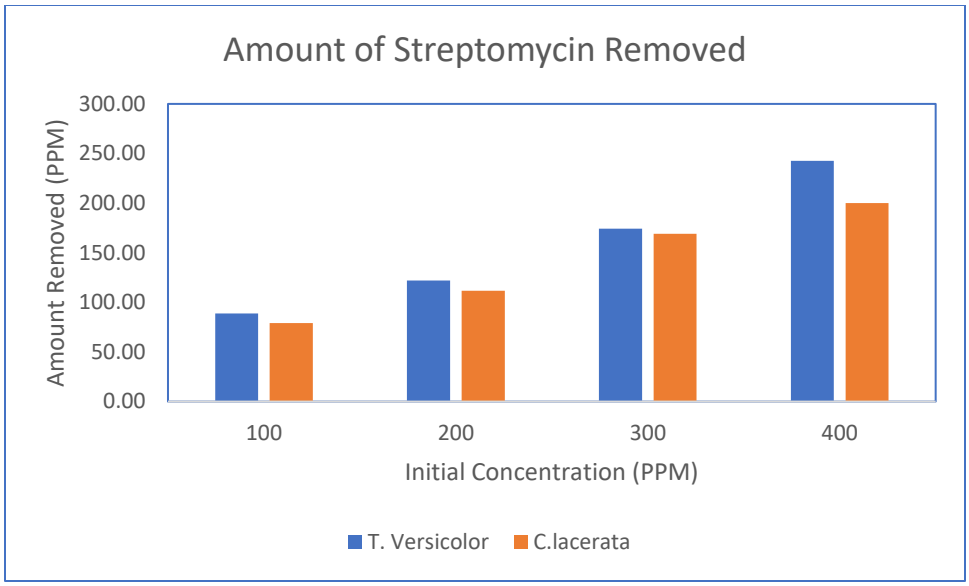

Figure 11: Bar Graph showing amount of streptomycin removed by T. versicolor and C. lacerata at different concentration of 100 to 400 ppm

CHAPTER FIVE

5.0 Conclusion

Antibiotics are considered a serious concern worldwide as it has led to various diseases and fatalities. The excessive use and improper disposal of antibiotics have been found to pose serious environmental and health concern to humans and animals alike. The inability of wastewater treatment plants to remove antibiotic is a major threat to the ecosystem because wastewater considered treated and released into the surface water is still contaminated with antibiotics. The continuous exposure of humans and animals to antibiotics in the environment leads to the presence of multi-antibiotic resistant bacteria in the environment (water, soil and sediments) which can lead to a global antibiotic resistance problem.

This study demonstrates that WRF could be a suitable model for the biological remediation of streptomycin found in wastewater. I found that the WRF studied can effectively remediate low concentrations of streptomycin found in WWTP. The WRF investigated demonstrated the capability to uptake streptomycin from water and use it as a nutrient and energy source at the various concentrations (100-400 ppm) examined. The results showed that T. versicolor showed a high bio-remediation potential for streptomycin compared to C. lacerata at all the concentration examined. The highest increase in biomass weight and radii growth of the WRF was also observed in T. versicolor. My results showed that WRF remove proportionally more streptomycin at lower concentration than at higher concentration. This indicate that high concentration of streptomycin can inhibit the growth of WRF. The inhibitory effect of streptomycin on fungi should be further studied.

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Appendices

Appendix 1: ANOVA of fungi radial growth

Oneway Anova

Summary of Fit

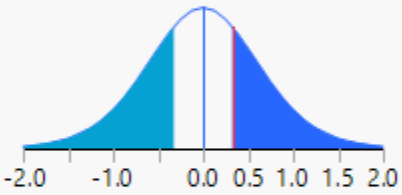
Rsquare	0.019312
Adj Rsquare	-0.05074
Root Mean Square Error	1.237942
Mean of Response	5.1125
Observations (or Sum Wgts)	16

Pooled t Test

T.v-C.I

Assuming equal variances

Difference	0.3250	t Ratio	0.525065
Std Err Dif	0.6190	DF	14
Upper CL Dif	1.6526	Prob > |t|	0.6078
Lower CL Dif	-1.0026	Prob > t	0.3039
Confidence	0.95	Prob < t	0.6961



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fungi	1	0.422500	0.42250	0.2757	0.6078
Error	14	21.455000	1.53250		
C. Total	15	21.877500			

Means for Oneway Anova

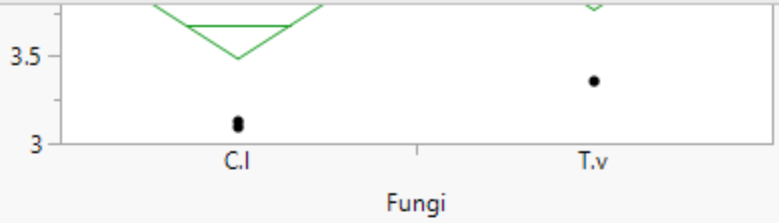
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C.I	8	4.95000	0.43768	4.0113	5.8887
T.v	8	5.27500	0.43768	4.3363	6.2137

Std Error uses a pooled estimate of error variance

Since (Prob > |t|) = p > 0.05, we cannot reject our null hypothesis. There is no statistically significant difference between the radial growth of C.lacerta and T. Versicolor

Appendix 2: ANOVA of fungi biomass weight

Oneway Analysis of Biomass weight By Fungi



Oneway Anova

Summary of Fit

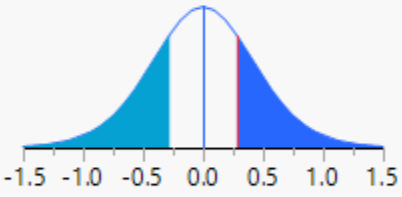
Rsquare	0.029498
Adj Rsquare	-0.03982
Root Mean Square Error	0.860383
Mean of Response	4.276188
Observations (or Sum Wgts)	16

Pooled t Test

T.v-C.I

Assuming equal variances

Difference	0.2806	t Ratio	0.652326
Std Err Dif	0.4302	DF	14
Upper CL Dif	1.2033	Prob > |t|	0.5248
Lower CL Dif	-0.6420	Prob > t	0.2624
Confidence	0.95	Prob < t	0.7376



Analysis of Variance

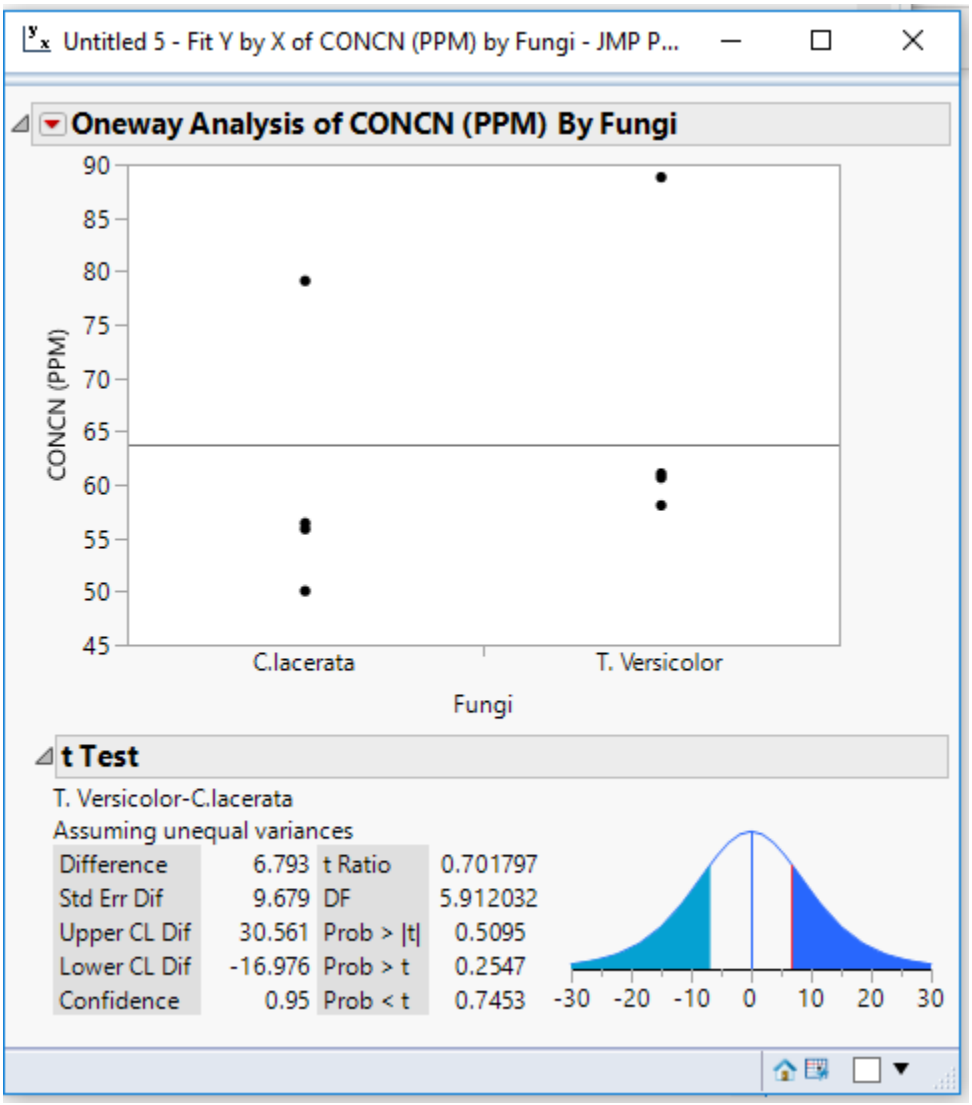
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fungi	1	0.315002	0.315002	0.4255	0.5248
Error	14	10.363615	0.740258		
C. Total	15	10.678616			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
C.I	8	4.13588	0.30419	3.4834	4.7883
T.v	8	4.41650	0.30419	3.7641	5.0689

Since (Prob > |t|) = p > 0.05, we cannot reject our null hypothesis. There is no statistically significant difference between the biomass weight of C.lacerta and T. Versicolor

Appendix 3: ANOVA of percentage removal of streptomycin by fungi



Since (Prob > |t|) = p > 0.05, we cannot reject our null hypothesis. There is no statistically significant difference between the removal of streptomycin by C.lacerta and the removal of streptomycin by T. Versicolor