

**SORPTIVE AND MICROBIAL PROPERTIES OF LOW-COST
ADSORBENTS USED IN THE EXTRACTION OF CIPROFLOXACIN AND
ISONIAZID FROM AQUEOUS SOLUTION**

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ABSTRACT

This work describes how coal fly ash (FA), kaolinite, perlite, talc and vermiculite were used to remove ciprofloxacin and isoniazid from aqueous solutions. The adsorptive features of the adsorbents were evaluated for ciprofloxacin and isoniazid with regards to the effects of contact time, pH, solid/liquid ratio and antibiotic concentration. All adsorbents were sterilised by dry heat before use to avoid the proliferation of antimicrobial resistance by the bacteria present on the adsorbents during experiments. The regression correlation coefficients indicate that the linearised form of the Langmuir isotherm gives the best fit for the sorption of both antibiotics onto FA and talc, ciprofloxacin onto kaolinite, and isoniazid onto perlite and vermiculite with R^2 values ranging from 0.908 - 0.999. The linearised form of the Freundlich isotherm best describes the sorption of ciprofloxacin onto vermiculite and isoniazid onto kaolinite with R^2 values of 0.999 for both. The linearised form of the Temkin isotherm best describes the sorption of ciprofloxacin onto perlite with an $R^2 = 0.997$. The values of the Freundlich exponent, $1/n$, range from 0.221 - 0.998, indicating a favourable adsorption of ciprofloxacin and isoniazid onto the adsorbents. The heat of sorption, B , calculated from the Temkin plots has values ranging from 0.018 - 10.460 J/mol, indicating a physical adsorption process (physisorption). Adsorption equilibrium on all adsorbents was achieved after 30 min for both antibiotics and the kinetic data obtained conforms best to the pseudo-second order equation with R^2 values ranging from 0.998 - 0.999. The removal of ciprofloxacin and isoniazid by all adsorbents except FA was strongly influenced by the pH suggesting that electrostatic interactions play a major role in the adsorption processes. All adsorbents except FA removed showed excellent adsorption of ciprofloxacin from aqueous solutions with all of them achieving removals ranging from 80 - 99%. The adsorbents were less efficient in removing isoniazid and kaolinite gave the highest removal of 55 %.

Furthermore, the microbial quality of the adsorbents was investigated and the results revealed that kaolinite, talc, perlite and vermiculite were heavily contaminated with microorganisms. FA was sterile. The fungi isolated from the mineral adsorbents were in concentrations ranging from 2.13×10^6 to 1.25×10^7 CFU/g and were mostly

moulds; *Penicillium* spp., *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Cladosporium* spp. and *Rhizopus oryzae*. One yeast was isolated and was identified as *Candida albicans*. The bacteria identified were in concentrations ranging from 4.96×10^6 - 1.19×10^9 CFU/g. *E. coli*, *Enterobacter cloacae*, *Exiguobacterium* spp., *Pseudomonas aeruginosa*, *Bacillus* spp. and *Serratia liquefaciens*. The leachability index (LI) values obtained for adsorbents indicated that it is highly unlikely that microorganisms could be leached out of the adsorbents by rain. Heat inactivation of the microorganisms at a 105 °C was totally unsuccessful. However, it was established that a dry heat dose of 160 °C for at least 15 min was sufficient to eradicate all microorganisms present in the adsorbents. The *D-values* for coliform bacteria from all samples were very similar ranging from 1.7-2.2 min indicating homogeneity in heat resistance by the microorganisms. The *Pseudomonas aureginosa* isolated had a *D-value* of 2.2 min. The fungi isolated from the samples had *D-values* ranging from 2.1-3.2 min.

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1 CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Water constitutes about two-thirds of the planet of which only 2.5 % is fresh water and, about 66% of all the fresh water is trapped in glaciers and ice caps (Postel et al., 1996). As the global population increases, there is an even greater demand for the already inadequate fresh water supply (Wyman, 2013). Between 1940 and 2000 the amount of fresh water used by human beings quadrupled as the population doubled (Fitzhugh and Richter, 2004). Human beings and their activities are by far the biggest consumer of freshwater with agriculture being the main culprit, followed by the industrial sector and then domestic uses (Hybel et al., 2015; Rosegrant et al., 2002). In addition to draining the fresh water supply, human activities also worsen the incidence by which water is polluted. Water pollution is directly proportional to the volumes of water consumed in agriculture, industries and households highest to lowest, respectively (Arenas-Sánchez et al., 2016; Ongley, 1996). In South Africa, poor sanitation especially sewage disposal from improper dwellings, self-care products, pharmaceutical and household chemical products are some of the leading domestic causes of groundwater pollution (Govender et al., 2011).

Over the years, the presence of pharmaceutical products in wastewater has become an area of great focus in research mainly because of the concerns raised by their detrimental effects on the environment, adverse effects on marine life and threats to human health (Grzybowski, 2011; Liu et al., 2011; Moore et al., 2011). Most pharmaceutical products will eventually find their way into surface waters because they are mainly excreted in urine and faeces and are therefore carried to water treatment plants in domestic sewage (Govender et al., 2011; Carlsson et al., 2006). Pharmaceutical products and their metabolites can be divided into 24 therapeutic classes. Literature reveals that very limited research has been done on the presence the antibiotic (antimicrobial agents) class in wastewater (Mompelat et al., 2009). The presence of antibiotics in wastewater is a great cause of concern mainly because of the ultimate development of resistance by bacterial organisms to the antibiotics and also direct toxicity to organisms (Peng et al., 2006).

1.2 Pharmaceuticals as water pollutants

Pharmaceuticals are a diverse group of chemical entities belonging to different classes and comprising of all human and veterinary drugs (Seifrtová et al., 2009). The emergence of bioactive pharmaceuticals and their metabolites in wastewater and various environmental compartments has triggered considerable interest, making it an area of great research focus over the years (Jones et al., 2005; Yu et al., 2016). The pollution is mainly attributed to the drastic increase in the use of active pharmaceutical ingredients as a result of the emergence of infectious diseases (HIV/AIDS) and opportunistic infections (*Pneumocystis pneumonia*) (Ngumba et al., 2016), drug misuse and abuse (Jurado et al., 2012; Schlüter et al., 2007), an aging population (Daughton and Ternes, 1999), off-label prescribing (Baandrup and Kruse, 2016), self-medication (Ortiz de García et al., 2013), lifestyle and cosmetic pharmacy (Khiari et al., 2014).

The most attention is focused on antibiotics and steroidal hormones because of their potential to give rise to mutant resistant strains of microorganisms and disruption of endocrine functions in non-target species, respectively (Pailler et al., 2009). Despite the fact that antibiotic resistance is most likely to develop within the patient under therapy, the post-therapeutic ecological consequences in the environment and in non-target organisms cannot be ignored (Lindberg et al., 2004; Yu et al., 2016). Most pharmaceuticals are said to be “pseudo-persistent” which means that they are always present in the environment even if their half-lives are short as a result of the relentless discharge (Yao et al., 2015). Their chemical and non-volatile nature results in them being retained in the aqueous environment (Hernando et al., 2006). However, their detrimental effects to the environment are not dependent on them being persistent in the environment, they emanate from the relentless introduction of these pharmaceuticals into the environment mainly via wastewater treatment plants and therefore legislative measures need to be put in place to control this type of pollution. At present, there are no regulations pertaining to pharmaceutical traces as pollutants therefore very little or no environmental monitoring of these chemical stressors has been implemented in most countries including South Africa (He et al., 2015). In places such as Australia, Canada, the USA and European countries where

preventative measures have been implemented, the regulations are more stringent and only apply to controlled substances and cytotoxic drugs and still preclude their release in sewage (World Health Organisation, 2011).

The fact that very little is known about long-term environmental effects of these compounds necessitates the need for more research on their fate, ecotoxicology and potential removal technologies (Ortiz de García et al., 2013; Tewari et al., 2013). Addressing the problem requires expertise from various stakeholders and communication across various disciplines of human and animal healthcare, environmental science and ecology, wastewater treatment, engineering and policy making sectors is essential. The aim of this work is to investigate the extraction mechanisms, kinetics and efficiencies of different adsorbents in eliminating antibiotics from wastewater hence establish if the processes are applicable at large scale.

1.3 Pharmaceutical contaminants in wastewater and the environment

With the increased global demand for freshwater (Kolpin et al., 2002), there is a high probability that most of the wastewater which reaches municipal WWTP is treated and then recycled for reuse by humans and animals through various sources (Wiener et al., 2016). This has given rise to concerns about the potentially harmful effects posed to human beings as a result of continuous exposure to traces of various pharmaceutical contaminants present in domestic water supplies (Inyang and Dickenson, 2015). Another facet to the matter is the lack of adequate scientific data to establish an accurate ecological and health risk assessment of the presence of pharmaceutical contaminants and their metabolites in domestic and environmental waters (Boix et al., 2016).

The safety and integrity of water sources have been under close scrutiny over the past few years (Baum et al., 2015). There are quite a few risks posed to humans by sub-therapeutic traces of pharmaceuticals, for example researchers have reported anaphylactic shock being triggered in some individuals following exposure to

pharmaceuticals in drinking water (Christensen, 1998; Webb et al., 2003). The resistance acquired by environmental bacteria from the chronic exposure to antibiotics can be transferred to clinical pathogens via gene transfer (Forsberg et al., 2012). A number of investigations on the effects oestrogens in aquatic environments revealed that the drugs can cause the feminization of male fish (Dong et al., 2014; Joss et al., 2005; Yan et al., 2012). Changes in the metabolome of *Gammarus pulex*, a low trophic level aquatic invertebrate, due to exposure to some pharmaceuticals has been reported (Gómez-Canela et al., 2016). In 2004, it was reported that the reduced vulture population in Pakistan had been caused by diclofenac-induced fatal renal failure resulting from the birds ingesting the drug from eating carcasses of treated animals (Oaks et al., 2004).

Pharmaceutical contaminants in wastewater are found in very minute concentrations ranging from ng/L to mg/L (Hernando et al., 2006; Xiong et al., 2017b) and therefore their detection and analysis requires the use of expensive and sophisticated instrumentation (Ortiz de García et al., 2013) that is not always available in WWTP especially those in developing countries. This poses a number of challenges because the most advanced wastewater treatment technologies are incapable of completely removing all drugs present in wastewater (Blair et al., 2013; Dong et al., 2016; Paxéus et al., 2016). The drugs at sub-therapeutic concentrations may still have effects on non-target organisms, long-term exposure to traces of drugs might have side effects which are not evident in short-term studies, the presence of some pharmaceuticals in drinking water may result in interactive effects i.e. antagonistic and/or synergistic action with those administered for therapeutic purposes (Pietrogrande and Basaglia, 2007), and there is potential emergence of antimicrobial resistance (Jones et al., 2005). The environmental toxicology of these chemicals has not been exhaustively established, however the possible threats to human and environmental health that have been seen over the years requires an extensive integrated approach into the matter so as to cover the knowledge gap and develop sensitive analytical methods to monitor their amounts, evaluate the risks they pose, determine their fate and ultimately implement feasible strategies to eliminate them from wastewater.

1.4 Routes of entry

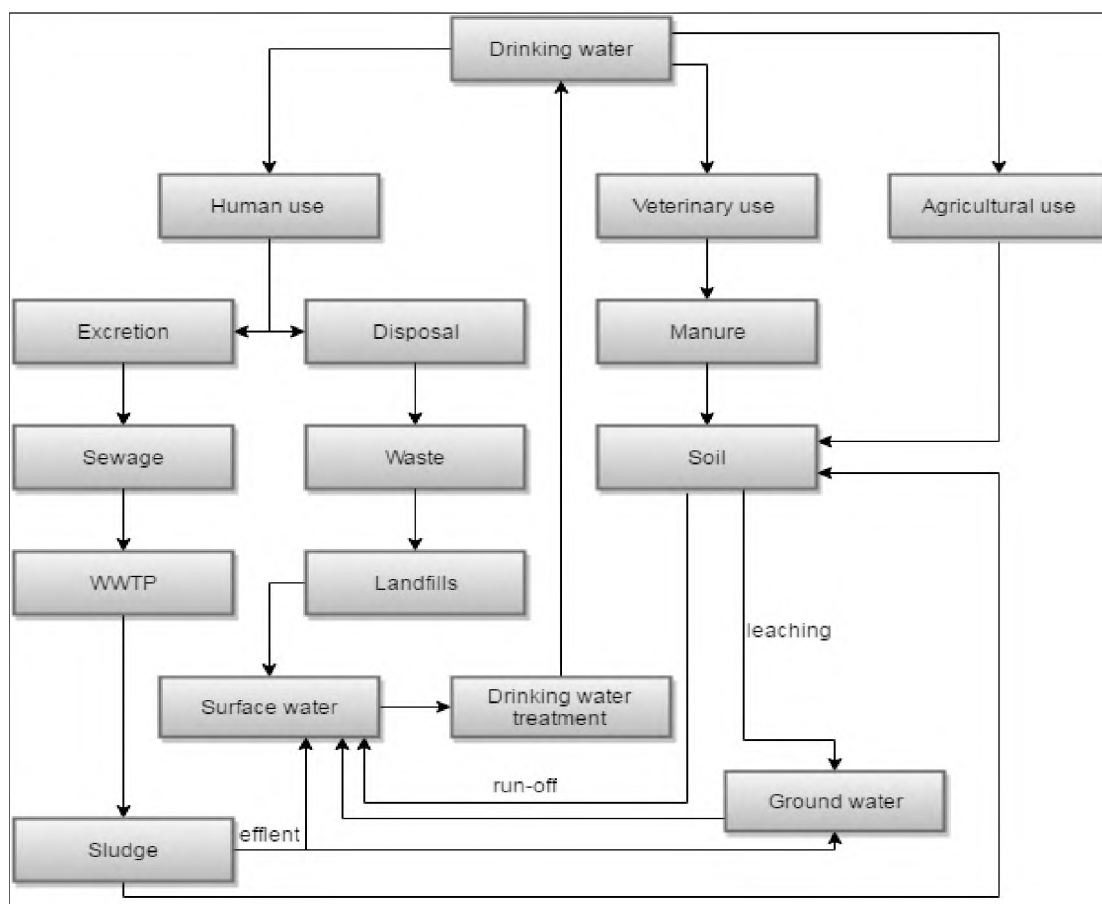


Figure 1: Main routes by which pharmaceuticals enter the environment. (Ebele et al., 2016) (Drawn using draw.io)

The main routes by which pharmaceuticals enter the environment are shown in Fig. 1 above. The major route is via faecal excrement and urine of humans and animals under antibiotic chemotherapy (Li et al., 2012; Tang et al., 2015). The compounds are released mostly unchanged, or as metabolites mostly conjugated to polar molecules which can be easily retransformed to the original active compound (Heberer, 2002). These compounds are diluted by the large volumes of water to sub-therapeutic concentration (Al-Ahmad et al., 1999; Jones et al., 2005; Segura et al., 2009). Once in the aqueous environment, pharmaceuticals are distributed to various facets of the environment (Batt et al., 2006). The most significant contributors of pharmaceutical pollutant are domestic and hospital wastewaters (Brown et al., 2006). Other sources include farming practices, industrial activities and disposal of

used or expired medicines (Baquero et al., 2008). Most pharmaceutical products have low volatility, therefore, spread throughout the environment mixed with water as aqueous solutions or suspensions, some may get adsorbed onto soil particles and enter a food chain (Comerton et al., 2009). In WWTPs pharmaceutical products may be adsorbed to sludge particles and find their way to farming land where sludge is used as crop fertiliser (López-Mosquera et al., 2000).

All environmental waters i.e. wastewater, surface water, ground water and drinking water are contaminated by pharmaceuticals (Houeto et al., 2012). Effluent from domestic, hospital and industrial wastewater, farming practices such as animal husbandry including dairy farming and aquaculture are amongst the leading contributors of antibiotics into the environment (Segura et al., 2009; Vulliet and Cren-Olivé, 2011) and mostly end up in WWTP which serve as reservoirs of resistant bacteria and potential reactors of resistant gene transfer, ultimately giving rise to multidrug-resistant organisms (Li et al., 2009; Zhang et al., 2009).

Hospital wastewater was reported to have more bacteria resistant to fluoroquinolone antibiotics than sewage treatment plant (STP) water (Nageswara Rao et al., 2008). In animal husbandry, antibiotics are used for the treatment and prophylaxis of various ailments and also in sub-therapeutic levels as growth enhancers (Kurie Mitani, 2006), a practice that was been banned across the European Union countries due to consequential risks that it poses to consumers and the potential to lead the proliferation antibiotic resistance (Farouk et al., 2015; Kemper, 2008).

Most pharmaceutical industries in developing countries also release highly contaminated effluents into domestic sewage and environmental waters without prior treatment mainly because of the lack of treatment technologies and poor enforcement of environmental regulations (Rehman et al., 2015).

In fish farms, antibiotics are used as feed supplements directly introduced into fish tanks mixed with food (Kim et al., 2016). As a result, antibiotics especially those with high partition coefficients, $\log K_a > 2$, such as erythromycin ($\log K_a = 3.6$) (Tran et al., 2016b) can accumulate on sediments by hydrophobic partitioning and eventually leach out into the environment (Chen et al., 2015; Kim et al., 2016). Some hydrophilic antibiotics such as ciprofloxacin that speciate as a function of pH ($pK_a 1 = 5.90$, $pK_2 = 8.89$), can also be adsorbed onto sediments by non-hydrophobic

interactions such as electrostatic forces (Xiong et al., 2017a). This practice might also result in human exposure as a result of consumption of the contaminated fish products (Kim et al., 2016). Antibiotics can also be directly introduced into the environment by application of animal manure or sewage sludge as a plant fertilizer (Fatta-Kassinos et al., 2011; Frey et al., 2015; Zhao et al., 2010), dissolved in runoff from animal farms and leachate from landfills containing disposed expired or unused antibiotics as events of rain (Feitosa-Felizzola and Chiron, 2009; Golet et al., 2001). WWTP effluent is rich in pharmaceutical contaminants and the major channel by which antibiotics are spread to the rest of the environment (Michael et al., 2013).

1.5 Environmental concerns

Once in the aqueous environment antibiotics go through various biological, chemical and physical processes which add a certain degree of complexity to their fate and distribution throughout the environment (Rehman et al., 2015). Bacterial processes can biodegrade some of these antibiotics, light sensitive compounds can be photodegraded and sorption can lead to the accumulation onto sediments and sludge. An understanding of the behaviour of the compounds during these processes is important as it helps to predict the concentration of the various in WWTP effluent and sludge and potential to be introduced into the environment. The nature of the aqueous environment together with the physicochemical properties of the antibiotic compounds also play an important role as they determine whether the antibiotics will succumb to the processes (including the employed treatment) or persist in the environment (Michael et al., 2013). For example, fluoroquinolones, sulfonamides, trimethoprim and cephalosporins are resistant to microbial biodegradation and tend to persist in WWTP and other environmental compartments (Nageswara Rao et al., 2008). In addition, fluoroquinolones have strong adsorptive properties and tend to accumulate on sediments and other organic matter thus elevating their persistence in environmental matrices (Kurie Mitani, 2006). Depending on the nature of the adsorbent, sorption can take place on multiple sites such as non-polar, polar, anions, and cations and surface bound Fe and Al. Hydrophobic partitioning and non-hydrophobic interactions such as electron donor-acceptor complexes, cation

exchange, cation bridging, and surface complexation are the main mechanisms by which adsorption occurs (Wang et al., 2016).

The persistence of antibiotics in the environment and chronic exposure of organisms to these chemical stressors has also proven to have ecotoxicological effects. Studies have reported adverse effects in aquatic organisms; *Daphnia magna*, shrimp and fish (Yahiat et al., 2011). The inhibition of photosynthesis by fluoroquinolone antibiotics (Aristilde et al., 2010) has been reported and concerns have also been raised about antibiotics causing reduced biodegradation of plant material (Richardson and Ternes, 2011). This clearly indicates an alteration in natural ecological processes which may result in the deterioration of the ecosystem. Sulfamethoxazole, ofloxacin and lincomycin have been reported to be mutagenic to freshwater algae. Ofloxacin also proved to be genotoxic (Isidori et al., 2005; Yao et al., 2017).

1.6 Antibiotics

Antibiotics are chemical entities constituting an integral part of modern medicine and an essential line of defence against pathogenic bacteria and fungi by eradicating or inhibiting their growth (Davies and Davies, 2010) and up to 200 000 tonnes are produced every year, worldwide (Rehman et al., 2015). A huge proportion of antibiotics that are ingested end up in wastewater and are amongst the most detected pharmaceuticals in WWTP around the world (He et al., 2015; Tewari et al., 2013). They are classified as recalcitrant bio-accumulative environmental pollutants (Chen and Zhou, 2014) and the rate of degradation of most of these compounds cannot offset their accumulation (Ncibi and Sillanpää, 2015).

Most antibiotics prescribed and dispensed cause side effects which differ in severity according to the agent used, however in most cases these side effects are outweighed by the therapeutic benefits and are therefore overlooked during chemotherapy (Slavkin, 1997). The continuous exposure to unspecified sub-therapeutic doses of antibiotics, on the other hand, merely presents risks and no benefits at all. Due to their extensive use and incomplete elimination antibiotics have been detected in various environmental waters (Golet et al., 2001).

Ciprofloxacin and norfloxacin have been detected in effluent samples from an urban WWTP in Switzerland, collected after primary and tertiary in concentrations ranging 249-405 ng/L and 45-120 ng/L, respectively, in surface water (Kolpin et al., 2002), in river water (Chen and Zhou, 2014), and in sludge (Radjenović et al., 2009). The major concern of the presence of antibiotics in the environment is the proliferation antimicrobial resistance genes and antimicrobial resistance bacteria, which reduce the therapeutic potential against human and animal bacteria pathogens (Ngumba et al., 2016).

1.6.1 Antibiotic resistance

The prevailing emergence of antimicrobial resistance amongst bacteria is an area of primary concern especially with regards to the release of antibiotics into the environment (Merola et al., 2014). Resistance is the acquired ability by bacterial population to render an antibiotic ineffective (Kemper, 2008). This is a result of a change in bacterial DNA which occurs when bacteria are subjected to an antibiotic concentration that will not kill them therefore possibly exerting selective pressure that will result in the development of antimicrobial resistance in bacteria (Yao et al., 2015). Antimicrobial resistance has become a great challenge in clinical therapy mainly because it compromises the effectiveness of antibiotics resulting in therapeutic failure, elevated health costs, increased morbidity and mortality rates (Munier et al., 2015; Zhang et al., 2009). For example, pathogens such as multi-drug resistant (MDR) *Klebsiella pneumoniae* cannot be treated with any antibiotic currently on the market (Giamarellos-Bourboulis et al., 2006). The resistant genes can be horizontally transferred from animal to human pathogens and also across different classes of antibiotics used for veterinary and human health, especially when the antibiotics have the same mechanism of action (Davies and Davies, 2010). Horizontal gene transfer is a mechanism by which bacteria can disseminate novel traits including acquired antimicrobial resistance (Jain et al., 2016). It is usually carried out by mobile DNA elements such as plasmids, transposons and integrons (Aleksun and Levy, 2007). When bacteria acquire resistance genes to protect them against various antibiotics, they are capable of employing several biochemical types of resistance

mechanisms such as antibiotic inactivation, target modification, alteration in permeability and the by-pass of metabolic pathways (Giedraitiene et al., 2011).

1.6.2 Quinolone antibiotics - Ciprofloxacin

The history of quinolones dates back to the discovery of nalidixic acid in 1963 but it experienced high therapeutic failure rates due to poor bioavailability and susceptibility to resistance by common pathogens belonging to *Salmonella spp.*, *Staphylococcus spp.*, *Pseudomonas spp.*, *Proteus spp.*, and *Escherichia spp.* (Peraman et al., 2015). Hence there was a need for further optimisation by structural modification to produce a compound with improved pharmacokinetics, a broad spectrum of activity and less adverse effects such as ciprofloxacin, a very potent fluoroquinolone (Plevová et al., 2015). Fluoroquinolones are amongst the most the most prescribed antibiotics worldwide (de Lastours and Fantin, 2014).

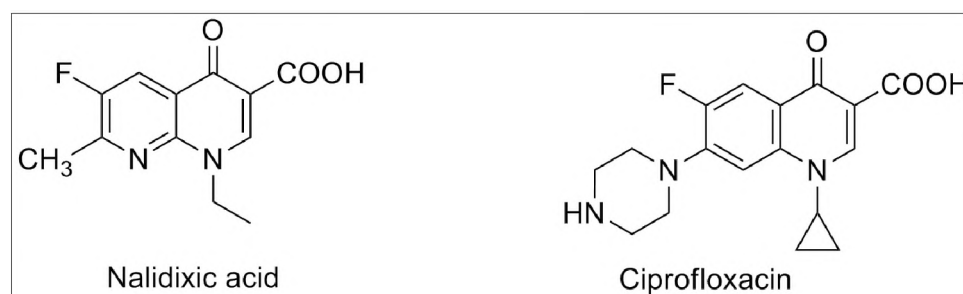


Figure 2: Structures of nalidixic acid and ciprofloxacin (Drawn using ChemDraw)

The structures of nalidixic acid and ciprofloxacin are shown in Fig. 2. Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(piperazinyl)-quinolone-3-carboxylic acid] is a synthetic broad-spectrum antibiotic with activity against a wide range of Gram-negative and some Gram-positive microorganisms (Attia et al., 2016). It is a second generation fluoroquinolone and is used to treat various bacterial infections such as urinary tract infections, respiratory tract infections, acute uncomplicated cystitis and chronic bacterial prostatitis (Masadeh et al., 2015). Ciprofloxacin is the most widely used fluoroquinolone antibiotic in both human and veterinary medicine

(Antonin et al., 2015; Jalali, 2016). Depending on its concentration, it can have bacteriostatic or bactericidal effects on microorganisms. Some physicochemical properties of ciprofloxacin are presented in Table 1. Its mechanism of action involves the blocking the microbial DNA from undergoing ATP-dependent supercoiling by competitively inhibiting the responsible enzyme, DNA gyrase, ultimately blocking DNA replication which leads to the death of the microorganism. It also inhibits the enzyme topoisomerase IV which is responsible for the partitioning of the microorganism's chromosomes (Cambau et al., 2009; Fisher et al., 1989; Pan et al., 1996)

Table 1: Physicochemical properties of Ciprofloxacin (Hassani et al., 2015; Ngumba et al., 2016)

Molecular formula	C ₁₇ H ₁₈ FN ₃ O ₃
Molecular weight (g/mol)	331.346
Lambda max (nm)	276
Water Solubility (g/L)	13.5 (sparingly soluble)
Log K _{ow}	0.28

The features which are most significantly responsible for the antibacterial activity of ciprofloxacin are the fluorine attached to C₆ which enhances penetration of the drug into the bacterial microorganism and activity against anaerobes, piperazine at C₇ which lengthens serum half-life against Gram-positive bacteria and, the cyclopropyl group ring at N₁ which generally enhances general potency (Denadai and Cass, 2015; Domagala, 1994).

An understanding of the pharmacokinetics of a particular drug is essential because it helps give a good estimate of the quantity of drug and metabolites discharged from the body during therapy (Ortiz de García et al., 2013). Following oral administration, ciprofloxacin is rapidly absorbed reaching peak serum concentrations in 1 to 2 hours and has a bioavailability of about 70% (Vance-Bryan et al., 2012). There is a direct correlation between the dose of ciprofloxacin via the oral or intravenous route and the serum concentrations (Bergan et al., 1987). Two-thirds of serum clearance is achieved via the kidneys by glomerular filtration and tubular secretion (39.70 L/h) (Szalek et al., 2012). The other third is by non-renal clearance which involves

metabolic degradation, biliary excretion and transintestinal elimination (Rohwedder et al., 1990). As a result of hepatic metabolism in the human body, ciprofloxacin forms four active metabolites, M1 (desethyleneciprofloxacin), M2 (sulfociprofloxacin), M3 (oxociprofloxacin) and, M4 (fomylciprofloxacin) (Roberge et al., 2000). Following the oral or intravenous administration ciprofloxacin to humans and animals, about 50-90% of the unchanged drug is excreted in the ratio 4:1 in urine and faeces, respectively, and about 11% of the drug is excreted in the form of metabolites in both urine and faeces (Chang et al., 2016; Dey et al., 2015; Shah et al., 1996). Oxo-ciprofloxacin is mostly found urine whilst sulfo-ciprofloxacin is the most abundant metabolite in faeces (Al-Omar, 2005).

Ciprofloxacin like most antibiotics is amphoteric and has pK_a values of 6.09 and 8.74 (Yu et al., 1994). The speciation of ciprofloxacin as a function of pH is shown in Fig. 3. It is non-volatile and exists as a zwitterion in neutral conditions, cationic form in acidic conditions and anionic form under basic conditions (Breda et al., 2009). This poses challenges in the treatment of wastewater because the strategy to be used depends on the pH of the wastewater for it to be successful (Semerjian and Ayoub, 2003). Ciprofloxacin is also not biodegradable and is known to be very persistent in the aqueous environment (Hassani et al., 2015). Fluoroquinolones have been found in various aquatic environments across the world including hospital effluent (approx. 60-12 000 ng/L), WWTP effluent (2-580 ng/L), surface water (5-1 300 ng/L) (Wammer et al., 2013). In general, the partition of the antibiotics between the aqueous and organic phase is the determining factor whether the compound will remain in the effluent or accumulate in sludge. Substances with a higher $\log K_{ow}$ (>4) are most likely to be adsorbed onto sludge by hydrophobic partitioning (Jones et al., 2005). However, this is not always the case with pharmaceuticals and only applies to hydrophobic drugs, hydrophilic pharmaceuticals with a low K_{ow} (<4) are expected to remain in the aqueous phase (Kim et al., 2016) for example, ciprofloxacin has a $\log K_{ow} = 0.28$ (Ahmed et al., 2015), but tends to accumulate on sludge by electrostatic interactions (Tran et al., 2016a). It has also been reported that despite the low $\log K_{ow}$ of ciprofloxacin, up to 94 ± 19 % of the drug is eliminated from wastewater by adsorption onto sludge and this is mainly attributed to the hydrogen bonds responsible for the sorption properties of the drug, although hydrophobic interactions have also been reported (Golet et al., 2003).

Ciprofloxacin is also capable of forming complexes with metal ions such as Ca^{2+} , Mg^{2+} , Fe^{2+} and Al^{3+} (Neuvonen et al., 1991) and can, therefore, persist in limestone, ferrous and clay environments. Twenty-four hours after administration, 50 % of the oral dose and 80 % of the intravenous dose is released in urine as the unchanged parent compound or as active metabolites. The co-administration of ciprofloxacin and medicines containing Ca^{2+} and Mg^{2+} may result in the precipitation of ciprofloxacin, reducing the bioavailability of the drug and an increase in the amount eliminated in faeces. Consequently, the desired effects of the antibiotic are compromised, leading to prolonged therapy and possible proliferation of antimicrobial resistance (Dey et al., 2015). Ciprofloxacin has been found in concentrations of 49 to 405 ng/L in Swiss wastewater and 40 to 2 148 ng/L in Canadian wastewater (He and Blaney, 2015).

The worldwide median concentration of ciprofloxacin detected in freshwater media is 0.164 mg/L and the highest concentration ever reported is 6.5 mg/L (Xiong et al., 2017b). Ciprofloxacin has been found in sewage effluents, hospital wastewaters and river water worldwide, at concentrations ranging from 3 to 489 ng/L (Lee et al., 2007). Ciprofloxacin was one of the dominant antibiotics detected in a WWTP in Brisbane, Australia and that conventional WWTPs only remove a fraction of the antibiotics in wastewater resulting in them always being present in the effluent (Watkinson et al., 2007). In another study investigating the causes of *umuC* genotoxic effects in wastewater from a University hospital identified ciprofloxacin to be the most genotoxic (Hartmann et al., 1998). Investigations done in a number of WWTP in Wisconsin, USA, detected six antibiotics including ciprofloxacin and the concentrations were similar to those detected in Europe and Canada (Karthikeyan and Meyer, 2006). It has also been reported that raw and treated wastewater originating from domestic and industrial sources in northern Portugal revealed the development of resistance by enterococci for the antibiotics ciprofloxacin, tetracycline and erythromycin and wastewater treatment did not remove these strains but it positively selected ciprofloxacin resistant phenotypes (Da Silva et al., 2006). Therefore, the presence of ciprofloxacin in aqueous environments can lead to the proliferation of antibiotic resistance in microorganisms which has detrimental effects on human health (Johnson et al., 2015). The literature on the occurrence of most pharmaceuticals in aqueous environments in Africa and South East Asia is

limited, however, the use of antimicrobials in these countries is very high (Matongo et al., 2015; Tran et al., 2016b).

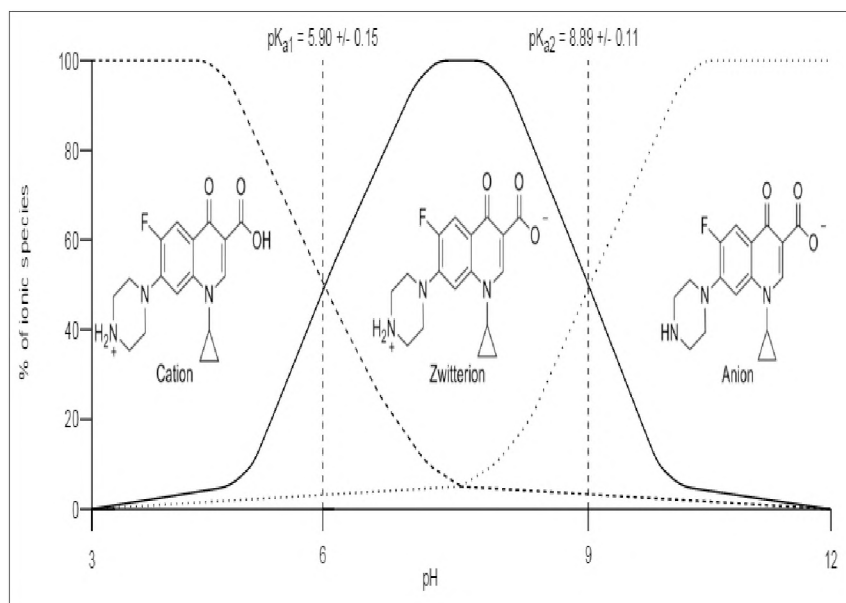


Figure 3: Speciation of ciprofloxacin as a function of pH. (Genç and Dogan, 2015). (Redrawn using Draw.io)

1.6.2.1 Bacterial resistance to Ciprofloxacin

Resistance of bacteria to fluoroquinolones is achieved by different types of mechanisms including, decreased penetration of drug into the microorganism resulting from altered expressions of porins or efflux pumps, enzymatic inactivation of the drug and, alteration of target as a result of mutations or a protection mechanism resulting in the reduced affinity for the drug (Mérens and Servonnet, 2010). The resistance to ciprofloxacin is mainly conferred by chromosomal mutations in the quinolone resistance-determining regions of the *gyrA* and *parC* genes which encode for the subunits of DNA gyrase and topoisomerase IV, respectively (de Lastours and Fantin, 2014). Efflux pumps also contribute significantly to resistance to fluoroquinolones. These can be drug specific or multidrug efflux transporters and are encoded by genes carried in plasmids which are widely distributed within the microorganism and can be passed on to other species by horizontal gene transfer (Nakaminami et al., 2010). Virtually all bacterial species have an energy-dependent

pump that can expell several classes of antimicrobial agents from the microorganism. The overexpression of these efflux pumps in bacterial species may lead multidrug resistance, such the NorA efflux pump in *Staphylococcus aureus* (Hooper, 2000). In addition to the four known chromosome-dependant efflux systems responsible for fluoroquinolone resistance, Yamane et al.; 2007 reported a new plasmid-mediated efflux pump, QepA, in an *E. coli* isolate from Japan to be also responsible for fluoroquinolone resistance (Yamane et al., 2007). Almost all species of bacteria are capable of developing resistance to fluoroquinolones with hospital-acquired methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa* showing the most resistance and an increase in resistance by *Escherichia coli*, *Salmonella spp.*, *Campylobacter spp.*, *Neisseria gonorrhoeae* (Acar and Goldstein, 1997; Rushdy et al., 2013).

1.6.3 Isoniazid

The structure of isoniazid is shown in Fig. 4. Isoniazid (pyridine-4-carbohydrazide) is an anti-tubercular drug belonging to the nucleoside reverse transcriptase inhibitor class of antibiotics (Bhandari and Kaur, 2013). Its structure comprises of hydrazine group and the pyridine ring which are essential for activity against *Mycobacterium tuberculosis* (Jena et al., 2014). Despite its susceptibility to resistance (Aguiar et al., 2015), isoniazid remains the cheapest, most effective and most prescribed drug for TB chemoprophylaxis and the first line combination chemotherapy of all forms of TB (Chen et al., 2009; Verma et al., 2015). This is because isoniazid has a high selectivity to *Mycobacterium tuberculosis*, remarkable bacteriostatic capacity (minimum inhibitory concentration (MIC) is approximately 0.36-0.73 μ M), inexpensive and good patient compliance (Huang et al., 2015).

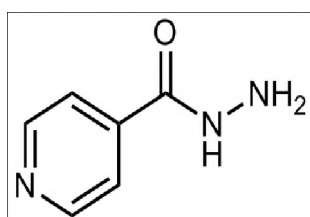


Figure 4: The structure of isoniazid. (Drawn using ChemDraw)

Some physical properties of isoniazid are presented in Table 2.

Table 2: *Some physical properties of Isoniazid (Bhandari and Kaur, 2013; Jena et al., 2014)*

Formula	C ₆ H ₇ O ₃
Molecular weight (g/mol)	137.14
Aqueous solubility at 25 °C (g/L)	230 (freely soluble)
Melting point (°C)	171-173
Log <i>P</i> at 25 °C	-0.402

The speciation of isoniazid as a function of pH is shown in Fig. 5. Chemically, isoniazid has 3 pK_a values: 1.8 for the basic pyridine nitrogen, 3.5 for the hydrazide nitrogen, and 10.8 for the hydrazide group. At the pH 7.4, the interactions between the drug and the lipid membrane model are expected to be mainly due to the hydrophobic and electrostatic interactions (Pinheiro et al., 2014). However, some authors only report 2 pK_a values. It speciates as a function of pH and has two pK_a values, pK_{a1} = 3.53 and is a result of the protonation of the heterocyclic nitrogen atom under acidic conditions. pK_{a2} = 11.40 and is ascribed to the proton loss from the amide nitrogen atom aggravated by basic conditions. At pH < 5, isoniazid exists as three species in equilibrium: one neutral and two positively charged. In the pH range 5-9, isoniazid has an uncharged molecular form. Deprotonation occurs gradually with increasing pH above 9 resulting in a negatively charged species (Blokhina et al., 2015).

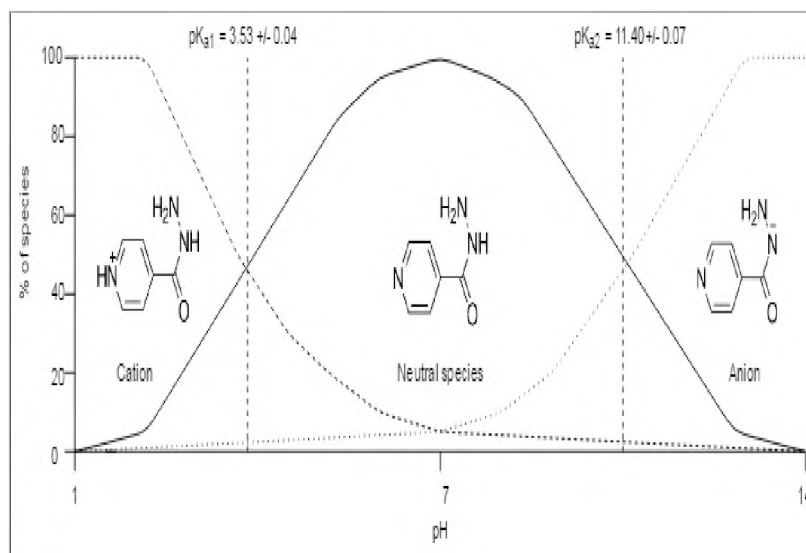


Figure 5: Speciation of isoniazid as a function of pH. (Drawn using Draw.io)

Despite numerous meticulous studies published over the years, the precise mechanism of action of isoniazid remains unknown (Campen et al., 2015). However, there is a general consensus by many authors that isoniazid impedes the biosynthesis of mycolic acids which are vital long fatty acid chains specific to the *Mycobacterium tuberculosis* cell wall and the following sequence of reactions are a widely accepted possible mechanism of action of isoniazid (Aguiar et al., 2015; Marques et al., 2013; Sousa et al., 2014). First, the isoniazid prodrug is activated, via oxidation by the heme catalase-peroxidase (KatG) enzyme found in *Mycobacterium tuberculosis*, to a highly reactive intermediate species, an isonicotinic acyl radical or anion. The intermediate then covalently binds to the cellular coenzyme, nicotinamide adenine dinucleotide (NAD⁺) generating an isonicotinoyl-NAD adduct which inhibits the NADH-dependent enzyme 2-trans-enoyl-acyl carrier protein reductase (InhA) of *Mycobacterium tuberculosis* (Aguiar et al., 2015; Jena et al., 2014). InhA is a renowned primary target of isoniazid that catalyses the final step of fatty acid elongation and therefore its inhibition halts mycolic acid synthesis ultimately resulting in the death of the microorganism (Kanetaka et al., 2015).

Following oral administration, the metabolism of isoniazid occurs in the liver and small intestines via three main pathways; acetylation, hydrolysis and deamination (Adole et al., 2013; Sasu et al., 2015). Acetyl isoniazid, the major metabolite, is a product of the extensive acetylation of isoniazid by the liver enzyme N-

acetyltransferase and, it can be metabolised to isonicotinic acid, acetyl hydrazine and hydrazine (Zhang et al., 2012). To maintain an effectual plasma concentration, frequent dosing or a high daily dose must be administered because isoniazid has a short half-life of 1-4 hours (Huang et al., 2015). This causes problems because not only is isoniazid and its metabolites hepatotoxic (Adole et al., 2013), they are excreted from the body mainly in urine, with about 50-70% of the oral dose being eliminated within 24 hours of administration, therefore, a large concentration of the drug accumulates in domestic and hospital sewage (Bhandari and Kaur, 2013). Isoniazid together with other anti-TB drugs have been detected in the effluent from a pharmaceutical industry in Pakistan (Aldaf and Ali, 2010). Very little has been reported in literature on the occurrence of isoniazid in the environment. However, isoniazid is most likely to occur in large concentrations in South African waters due to its extensive use. This is because the presence of pharmaceuticals in the environment is dependent on social, cultural, technological and agricultural factors (Matongo et al., 2015). For example, the HIV/AIDS pandemic and prevalence of TB in South Africa will result in an elevated release of the drugs used to treat these diseases (Sasu et al., 2015).

The release of isoniazid into wastewater poses an environmental threat mainly due to the fact that the drug is not readily biodegradable and the lack of sewage treatment in most developing countries will result in the isoniazid reaching wastewater treatment plants where it is exposed to bacteria and may find its way into municipal water for reuse (Sasu et al., 2015).

1.6.3.1 Resistance to Isoniazid

The development of resistance to isoniazid is an intricate process that is usually conferred by the resistance-inducing mutations which occur in the *inhA* and *katG* gene within the *Mycobacterium tuberculosis* (Huang et al., 2014; Manning et al., 2015). The *katG* encodes the KatG enzyme responsible for activating the isoniazid prodrug and therefore alterations in this gene have been reported to be the most common and chief cause isoniazid resistance (Kamachi et al., 2015).

1.6.3.2 Tuberculosis and microbial resistance

Tuberculosis (TB) which has been declared a global health emergency (World Health Organisation, 2013), is a highly infectious chronic disease caused by the bacillus *Mycobacterium tuberculosis* and is responsible for the more deaths than AIDS and malaria combined (Forte et al., 2012). At present, about a third of the global population has latent TB and it will progress to the active form in 5 - 10 % of these people (Roodgar et al., 2015). In 2012, the World Health Organisation (WHO) reported 8.6 million new cases of TB and 1.3 million deaths from the disease worldwide. In South Africa alone, there were 4.6 million to 5.9 million new cases of TB i.e. more than half of the total global cases and of these tragic figures, 1.8-2.3 % of the cases had the multidrug drug resistant (MDR) strains of *Mycobacterium tuberculosis* (World Health Organisation, 2013). The global burden of TB is escalated by the HIV pandemic, emergence of drug-resistant strains, poverty and the lack of access to affordable quality healthcare especially in medium to low-income countries (Hoza et al., 2015; Jena et al., 2014).

The proliferation of antimicrobial resistance strains of *Mycobacterium tuberculosis* poses great challenges to TB treatment. Multi-drug resistant (MDR) strains exhibit resistance to at least isoniazid or rifampicin, the two main drug regimens used in the first line treatment of TB (Hamze et al., 2015). Two other resistant strains of *Mycobacterium tuberculosis* have been reported, extremely drug resistant (XDR) strains and totally drug resistant (TDR) strains. In addition to being resistance to at least isoniazid or rifampicin, XDR strains are resistant to quinolones and some of the second line drugs used in TB chemotherapy. TDR are resistant to all clinically tested agents (Chollet et al., 2015). The emergence of resistant strains immensely worsens health and economic burdens that arise from TB as clinical interventions are forced to resort to second line drugs which require a longer duration of treatment and are more expensive (Campen et al., 2015). This increased the incidence of resistant strains of TB has become a major cause of concern especially with HIV co-infection whereby the devastating end result is death (Alemayehu et al., 2014; Wang et al., 2015).

1.7 Wastewater treatment

The first stage of wastewater treatment is the pre-treatment of effluent in an STP before it enters the WWTP (Garrido-Baserba et al., 2015). However, most developing countries are lagging behind in terms STP infrastructure and technologies and total absence in some places resulting from the lack of skilled personnel, stunted technological development and inadequate funds (Ngumba et al., 2016). This has led to the release of raw sewage heavily contaminated with antibiotics and other pharmaceuticals from households and hospitals being released directly into WWTP, surface and groundwater (Muserere et al., 2014).

Domestic sewage can be divided into two categories; greywater and blackwater. Greywater is wastewater from sinks, showers, baths and laundry whilst blackwater is wastewater from the toilet contaminated with faecal matter and urine, and therefore the chief route of pharmaceuticals into WWTP (Paulo et al., 2013). Traditionally, WWTPs are designed to eliminate nutrients, particulate and organic matter from wastewater and are thus incapable of eliminating antibiotics and other pharmaceuticals from wastewater (Clara et al., 2005; Giannakis et al., 2015).

The complex nature of effluents makes it difficult for a single process to provide adequate wastewater treatment. Current practices involve the use of a combination of many different processes in order to achieve the desired water quality in the most economical way. Wastewater treatment comprises of three main stages; primary, secondary and tertiary treatment which vary from plant to plant. The primary treatment involves the mechanical removal of solids by processes such as filtration and sedimentation. Secondary treatment employs the biological processes to eliminate biodegradable material and may use bacterial organisms in the bioreactors (Shannon et al., 2007). This is worrisome because it presents a platform for the continuous exposure to antibiotics which might lead the proliferation of antibiotic resistance amongst the bacteria (Michael et al., 2013). The elimination of pharmaceuticals in the primary and secondary stages of WWTP are attributed to sorption to sludge, biodegradation and to a very small extent volatilization (Khan and Ongerth, 2002). The tertiary treatment usually involves disinfection by means of chlorination or UV irradiation. WWTP links all environmental waters e.g. surface

water, groundwater with sewage effluent it provides a means by which antibiotics, resistant bacteria and genes can spread throughout the environment (Mao et al., 2015; Schlüter et al., 2007). Despite the potential environmental problems that they might cause, the health benefits from antibiotics are irreplaceable. Therefore, the only strategy to control the problem is to use only the when necessary and find means to counteract the pollution in water in order to minimise the contamination of other aquatic environments.

1.8 Adsorbents as a treatment strategy for removal of antibiotics

The removal of antibiotics from wastewater is dependent on their chemical properties, the treatment strategy employed and the concentration in the water (Verlicchi et al., 2012). Advanced treatment technologies such as ozonisation and advanced oxidation processes (AOPs) are very effective at eliminating antibiotics from wastewater (Giannakis et al., 2015). However, the major drawback of these processes is the high operational cost which makes them unsustainable at large scale. Adsorption processes utilising activated carbons and clay minerals provides environmentally friendly and versatile methods, the materials are locally abundant, relatively cheap, simple to design, easy to operate (Ahmaruzzaman, 2008) and very effective, achieving removals ranging between 90 - 100% (Ahmed et al., 2015). Adsorption can be a chemical or physical phenomenon that involves the accumulation of matter from a fluid onto the surface of a solid. It is influenced and controlled by the materials' properties which include surface charge, surface free energy, surface roughness, the balance between hydrophobic and hydrophilic groups, and specific chemical groups on the surface (Xu et al., 2014). Steps in the adsorption process involve: (1) solute transport in the bulk-adsorbate movement by the stagnant liquid film surrounding the adsorbent (bulk transport), (2) film diffusion-adsorbate transport along the film (film transport), (3) pores diffusion-adsorbate diffusion through the porous structure to the active sites (intra-particle transport), (4) adsorption-interaction between adsorbate and porous structure (adsorption onto the adsorbent) (Homem and Santos, 2011). The main mechanisms involved in the adsorption phenomenon are electrostatic interactions, hydrophobic interactions,

hydrogen bonds, partitioning, pore-filling, π - π interactions etc. (Tan et al., 2015). Some common adsorbents are biochar (Inyang and Dickenson, 2015), activated charcoal and pumice stone. The biggest challenge to adsorption is the antibiotics compete with dissolved organic molecules for active sites on the adsorbent and this compromises the efficiency of the adsorbent (Yan et al., 2013). The removal efficiency of the adsorption process depends on the physicochemical properties of the adsorbates, nature and porosity of the adsorbent, temperature and the constitution of the aqueous matrix (Shah and Imae, 2016). These can be manipulated in various ways such as ammonisation, acid treatment and microwave treatment, in order to enhance adsorptive interactions (Peng et al., 2015).

1.8.1 Removal of antibiotics using activated carbon

Amongst all the adsorbent materials proposed, activated carbon is the most popular for the removal of pollutants from wastewater. Its surface properties make it an excellent widely used adsorbent (El-Sharkawy et al., 2016). Activated carbons, in the granular or powdered form, have been widely used as adsorbents for organic micropollutants and their application to pharmaceutical pollutants has become of great interest over the years (Rivera-Utrilla et al., 2013). However, despite its high porosity, surface area and adsorption capacity, the production costs of AC are very high, making it the most expensive adsorbent. Its regeneration is also difficult and has a higher carbon footprint relative to other adsorbents.

1.8.2 Low-cost adsorbents and removal of antibiotics

Due to the problems mentioned above, alternative adsorbents to replace the costly activated carbon have become a major area of focus over the past years. Research has focused on various natural solid materials, which are capable of removing pollutants from the contaminated wastewater at low cost. An adsorbent can be considered as low-cost if it requires little processing and is abundant in nature, and it is a by-product or waste material from an industrial process. Certain waste

products from industries and agricultural operations, natural materials and bio-adsorbents represent potentially economical alternative adsorbents. Most potential low-cost adsorbents have been tested and proposed for phenolic compounds removal (Ahmaruzzaman, 2008; Ali et al., 2012; Gupta and Suhas, 2009; Lin and Juang, 2009; Rafatullah et al., 2010).

1.8.2.1 Clays

Clay minerals such as kaolinite, talc, rectorite, perlite, vermiculite and montmorillonite are cheap, layered structures with large surface area and exchangeable cations. Hence, there has been an increasing interest, over the past few years, in their possible use in wastewater treatment (Hassani et al., 2015). The proposed mechanisms of interactions between clays and pharmaceuticals such as fluoroquinolones which are capable of existing as cationic species depending on the pH of the solution include hydrogen bonding, cationic bridging, cationic exchange and ligand exchange with cation exchange dominating between the protonated piperazinyl amine groups and cations on the clay surface (Yan et al., 2013). Sorption of isoniazid onto sepiolite, loughlinite and palygorskite has also been reported (Akyuz et al., 2010). In this work, we investigate the removal of ciprofloxacin and isoniazid from aqueous solution using four randomly selected clays; kaolinite, talc, vermiculite and perlite.

1.8.2.2 Perlite

Perlite is a naturally occurring glassy volcanic alumino-silicate rock mineral with a very low density when expanded ($0.08 - 0.24 \text{ gcm}^{-3}$) that is commonly used in the various fields such as ceiling tile, sound proofing, pipe insulation and cryogenic insulation (Celik et al., 2016; Maaloufa et al., 2015). It is mostly made up of amorphous silica ($\geq 70 \text{ \% wt. SiO}_2$), low amounts of other metal oxides (Al_2O_3 , K_2O , Na_2O , and Fe_2O_3), free and chemically bonded water ranging $2 - 5 \text{ \% wt.}$ (Pilatos et al., 2016). Perlite is capable of expanding up to $10 - 30$ times its original volume

when heated to temperatures above 760 °C (Celik et al., 2016), resulting from the synchronised steam release and structure softening (Pilatos et al., 2016). The chemical composition of the mineral is not altered during expansion, only the chemically bonded water is released (Shmuradko et al., 2012). Characteristics of perlite such as strong adsorbability, porous nature, high chemical resistance, light weight, low toxicity and low cost make it a potential wastewater treatment strategy (Kolvari et al., 2015). A number of sorption studies have been reported in literature (Akkaya, 2013; Silber et al., 2012; Talip et al., 2009; Tekin et al., 2010).

1.8.2.3 Vermiculite

Vermiculite is a naturally occurring mineral belonging to the phyllosilicate group comprising of flat shiny flake sheets (Rashad, 2016). It is mainly made up of SiO₂ which constitutes 37 - 42 % wt., metal oxides such as MgO, Al₂O₃, Fe₂O₃ and FeO in smaller proportions and water ranging from 8 - 18 % wt (Rashad, 2016). The composition may vary depending on the ore deposit and has a pH of about 7 (Rashad, 2016). Vermiculite has a series of features such as high cation-exchange capacity (90 cmol/kg), chemical and mechanical stability, large surface area and low cost, showing prospects in adsorption of cations (Tian et al., 2016). These make it attractive for use in the remediation of wastewater. Vermiculite is a greatly abundant mineral with very low production costs and easy to transport and store due to its light weight. (Gülbiçim et al., 2017; Saleh et al., 2016). About 408 thousand metric tonnes of vermiculite were produced around the world in 2015 and South Africa is one of the six leading producers of the mineral (Kimball, 2016). The largest operating vermiculite mines in South Africa are located in the Phalaborwa region in the Limpopo province (Muiambo et al., 2010). Similar to perlite, vermiculite is capable of expanding 8 - 20 times its original size when exposed to temperatures up 600 - 1000°C (Shoukry et al., 2016). Vermiculite has been successfully used in the adsorption of heavy metals and dyes from aqueous environments (Dzene et al., 2016; Saleh et al., 2016; Stawiński et al., 2016).

1.8.2.4 Talc

Talc is a soft powdered, tri-layered, hydrated magnesium silicate, which may contain a small portion of aluminium, iron 2, iron 3 and/or manganese (Sprynskyy et al., 2011). It is used in the cosmetic industry as a topical dermatological protector and as an excipient for oral pharmaceutical preparations (Ohenoja and Illikainen, 2015). In medicine, sterile talc is used as a sclerosing agent that is administered inter-pleurally in the treatment of pneumothorax and pleural effusion (Haini et al., 2016). Talc is mined from rock ore and in its purest has the formula $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$ (Sprynskyy et al., 2011) with a theoretical mineral composition of 31.9% MgO, 57.8% SiO_2 and 4.75% H_2O of the total weight (López-Galindo et al., 2007). Three sheets make up the talc structural unit, a brucite layer stratified between two silica layers which are tetrahedrally linked (Yao et al., 2016). The structural units stack up and are held together by Van der Waals forces (Ohenoja and Illikainen, 2015). It is a very light material with a density of 2.7 g/cm^3 (Fiume et al., 2015). Talc is seldom pure when it is mined, many associated minerals from the rock ore such as calcite (CaCO_3), dolomite ($\text{CaMg}(\text{CO}_3)_2$), and chlorites ($\text{Mg}_5(\text{Al,Fe})(\text{Al,Si})_4\text{O}_{10}(\text{OH})_8$) in talc deposits (Castillo et al., 2015). In general, talc particles layered on their basal planes consists of siloxane groups ($-\text{Si-O-Si}-$) and are hydrophobic but the edge faces are hydrophilic due to the presence of hydroxyl groups ($-\text{SiOH}$, and $-\text{MgOH}$) (Ahmed et al., 2013). Talc has been reported to remove ions and heavy metals from aqueous solutions (Ahmed et al., 2013; Awual et al., 2014).

1.8.2.5 Kaolinite

Kaolinite is a layered material with a tetrahedrally coordinated silica sheet linked through oxygen atoms to a second octahedrally coordinated aluminate sheet with apical oxygen atoms and hydroxyl groups. (Liu et al., 2016). The flat surface is negatively charged over the entire pH range, whilst the edges are positive at low pH, but negative at high pH, due to the protonation/deprotonation of the hydroxyl groups making them potential reactive sites (Duarte-Silva et al., 2014). The edge effects arise due to the fracture of the lattice network and contain silica and alumina like

sites (Lapčík et al., 2012). The oxygen atoms found in the interlayers of kaolinite are hydrophobic and therefore provide binding sites for hydrophobic compounds such as aromatic hydrocarbons (Tandlich and Balaz, 2011). Besides widespread use of in the paper industry, refractory pigments and ceramics (Magriotis et al., 2014), kaolinite is also administered orally to act actively as a gastrointestinal protector or anti-diarrhoeic, applied topically as a skin protectant, and as excipient in tablet and capsule formulation (Viseras et al., 2007). The chemical formula for kaolinite is $\text{Al}_2\text{O}_3 \cdot 2\text{SiO}_2 \cdot \text{H}_2\text{O}$, it is extracted from kaolinized granite by washing it out using high-pressure water hoses resulting in a clay stream which is then pumped into a separation pump where impurities are removed (Greenwood et al., 2007). In contrast with talc, the mineral composition of pure kaolinite is; 46.55 % SiO_2 , 39.49 % Al_2O_3 and 13.96% H_2O of total weight and most common minerals associated with crude kaolinite are feldspars, quartz and mica (López-Galindo et al., 2007). The largest known deposits of kaolinite in South Africa are found in the Makana area in the Eastern Cape province which are underexploited (Malatji, 2010), and yet the area is dominated by poverty (Davenport et al., 2011). Therefore, the use of kaolinite in wastewater treatment has the potential to create employment and improve the economy in this area. A number of sorption studies using kaolinite have been reported (Fleury et al., 2017; Han et al., 2016; Kong and Wang, 2016; S. Wang et al., 2016).

1.8.2.6 Waste derived materials/by-products from industry

The waste materials/by-products from industries could potentially find use as low-cost adsorbents since they are abundant in nature, inexpensive, and require little processing.

1.8.3 Coal fly ash (FA)

FA is a by-product of pulverised coal combustion found mainly in thermal power electricity plants and steel industries (Ahmaruzzaman, 2010). Majority of the power

generation in South Africa is derived from coal and therefore, thermal power stations in the country generate millions of tonnes of FA every year (Doucet et al., 2016). Approximately 95 % of this by-product is not beneficiated resulting in a very high percentage of it being dumped into ash dams and landfills (Ojumu et al., 2016). FA poses a pollution threat and in South Africa, it is considered a hazard to the environment. This is because of significantly large amounts of elements, ions and compounds some of which are toxic and radioactive, accumulating in FA during the combustion of coal (Osmanlioglu, 2014). Toxic trace elements such as, B, Cr, Mo, Pb and Se are of greatest concern because when the ash dumps are infiltrated by rain or wastewater these elements are leached out and pollute the surrounding environment, therefore, the use of FA in different applications is economically viable and has environmental relevance (Behin et al., 2016). Repeated human exposure to FA can also result in health problems such as skin, nose, throat and respiratory tract infections as well as arsenic poisoning (Yao et al., 2014).

FA consists mostly of an unburned mineral portion of coal and a small proportion of unburned carbon particles (Norris et al., 2015). The composition, mineralogy, surface chemistry and reactivity of FA are of great importance in the investigation and the development of various applications of FA (Yao et al., 2014). It exists as solid or hollow fine powder particles which are predominantly spherical and mostly amorphous in nature (Blissett and Rowson, 2012). Properties of the coal being burnt and the way FA is handled and stored greatly influences the chemical composition of the FA (Pandey and Singh, 2010). There are four different types of coal: anthracite, bituminous, sub-bituminous, and lignite. The coals differ in the ash component, heat value, geological origin and chemical composition. Anthracite is rarely burnt in power stations and therefore very little anthracite FA exists (Ahmaruzzaman, 2010). The normal ranges of chemical composition for FA produced from bituminous, sub-bituminous and lignite coal types are presented in Table 3.

Table 3: The normal ranges of chemical composition for FA produced from bituminous, sub-bituminous and lignite coal types (Ahmaruzzaman, 2010)

Component (% wt.)	Bituminous	Sub-bituminous	Lignite
SiO ₂	20-60	40-60	15-45
Al ₂ O ₃	5-35	20-30	10-25
Fe ₂ O ₃	10-40	4-10	4-15
CaO	1-12	5-30	15-40
MgO	0-5	1-6	3-10
SO ₃	0-4	0-2	0-10
Na ₂ O	0-4	0-2	0-6
K ₂ O	0-3	0-4	0-4
LOI	0-15	0-3	0-5

About 75 % of the coal deposits in Africa are found in South Africa and over 95 % of these coal reserves are bituminous and mostly found in the Karoo basin (Van Niekerk et al., 2008). Raw fly ash is used as a cement additive, brick manufacturing, backfilling material and the huge proportion which remains unused can be potentially used to remove heavy metals, dyes and anions from wastewater (Koshy and Singh, 2016). The use of FA in the different applications is advantageous and addresses various problems caused by its disposal. The costs of disposing this by-product are minimised; secondly, less land is used up by ash dumps; thirdly, financial gains from selling the FA; fourthly, replacing some scarce or expensive natural raw materials; and lastly suppress environmental hazards posed by dumping FA as well as improve ecological cycles (Teixeira et al., 2016; Zhou et al., 2016).

In most cases, the leachate obtained from FA is highly alkaline due to the presence of liming agents such as CaO and treating acid mine drainage (AMD) with FA removes heavy metals by precipitation and reduces the SO₄²⁻ concentration by over eighty percent (Madzivire et al., 2011). Heavy metals are persistent pollutants that are resistant to biodegradation and are easily accumulated in living organisms even at low concentrations, causing poisoning. They are mostly discharged into surface waters as hydrated ions which are readily absorbed and very toxic as they disrupt enzymatic processes (Visa, 2016). The major chemical components of FA and its physical components such as porosity, particle size distribution and surface area gives FA the potential to be used in metal remediation in wastewater treatment (Pizarro et al., 2015). However, the use of FA poses the risks associated with the

leaching out of heavy metals (As, Ag, Cr, Cd, Cu, Pb, Se, Zn) from the FA and therefore pre-treatment (mostly acid treatment) of the adsorbent, might be necessary to improve its environmental characteristics and augment its potential beneficial use (Zhang et al., 2016).

Inorganic contaminants such as phosphorus, fluoride, and boron in addition to heavy metals which exist in wastewater can be removed by FA (An et al., 2016). Pharmaceuticals such as female hormones have been successfully removed from wastewater using FA (Norris et al., 2015). Various metals such as Cu, Pb, Zn, Mn, Cd, Cr and Ni have been successfully removed from wastewater using FA as an adsorbent (Blissett and Rowson, 2012).

1.9 Adsorption kinetics

Adsorption on the solid-liquid interface is characterised by external mass transfer (boundary layer diffusion) and/or intra-particle diffusion. The following three consecutive steps are involved:

- (1) Film diffusion: transport of the solute from bulk medium to the adsorbent exterior surface.
- (2) Particle diffusion: solute diffusion into the pore of adsorbent except for a small quantity of adsorption on the exterior surface and parallel to this is the intraparticle transport mechanism of the surface diffusion.
- (3) Adsorption of solute onto the inner surfaces of the pores and capillary spaces of the adsorbent.

The last step is considered to be an equilibrium reaction. Of the three steps, the third step is assumed not to be the rate-limiting step of the adsorption process. The overall rate of adsorption will be controlled by the slowest step, which would be either film diffusion or pore diffusion controlled. Film transport is usually the rate-limiting step observed when low-cost adsorbents are used and kinetic modelling is

used to determine the rates of limiting steps in order to optimise the adsorption processes (Ahmed et al., 2015; Ganesapillai and Simha, 2015).

The pseudo first-order rate model expressed as:

$$\log(q_e - q_t) = \log q_e - \left(\frac{k_1}{2.303}\right) t \quad (1)$$

The second-order rate model expressed as:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left(\frac{1}{q_e}\right) t \quad (2)$$

The intra-particle diffusion model expressed as:

$$q_t = k_{id} t^{0.5} + C \quad (3)$$

Where q_e and q_t (mg/g) are the amounts of antibiotic adsorbed per unit mass of adsorbent at equilibrium and time t (min), respectively, k_1 (min^{-1}) is the rate constant of the pseudo-first-order model, k_2 (mg/g.min) is the rate constant of the pseudo-second-order model, k_{id} (mg/g.min^{0.5}) is the rate constant of the intra-particle diffusion model. Values of k_1 and q_e can be calculated from the slope and intercept of the linear plot of $\log(q_e - q_t)$ versus t , respectively. Values of k_2 and q_e can be calculated from the intercept and slope of the linear plot of t/q_t versus t , respectively. The value of k_{id} can be calculated from the slope the linear plot q_t versus $t^{0.5}$ and C (mg/g) is the intercept (Nithya et al., 2016). The adsorption kinetic models are employed to establish the rate limiting step of the sorption process (Ganesapillai and Simha, 2015).

1.10 Adsorption isotherms

Adsorption isotherms provide the equilibrium relationship between the adsorbates and adsorbents and are thus necessary to design and optimise the adsorption process (Tan et al., 2015). Furthermore, adsorption parameters provide constant values

which enable us to compare the adsorption capacities for different adsorbents (Can et al., 2016). The Langmuir, Freundlich and Temkin equations are commonly used mathematical models which fit adsorption data and characterise equilibrium adsorption (Can et al., 2016; Chung et al., 2015; Shafqat and Pierzynski, 2014). The linearised forms of the Langmuir, Freundlich and Temkin adsorption isotherms are represented by the following equations;

$$\frac{1}{q_e} = \frac{1}{q_m} + \frac{1}{K_l C_e q_m} \quad (4)$$

$$\ln q_e = \ln K_f + \frac{1}{n} \ln C_e \quad (5)$$

$$q_e = B \ln K_t + B \ln C_e \quad (6)$$

Where q_e is the equilibrium adsorption capacity of the adsorbent, C_e is the concentration of antibiotic in the aqueous phase at equilibrium, q_m (mg/g) and K_l (L/mg) are Langmuir parameters representing the monolayer capacity and adsorption constant, respectively. K_f (mg/g) and n are Freundlich parameters representing the adsorption constant and the sorption coefficient, respectively. K_t (L/g) is the Temkin isotherm equilibrium binding constant and B (J/mol) is the Temkin's heat of adsorption (Can et al., 2016).

The conceptual basis of the Langmuir model is that maximum adsorption corresponds to monolayer pattern of adsorbate layer on the adsorbent surface and extends beyond its original approach to describe gas-solid phase sorption (Ganesapillai and Simha, 2015). It assumes that there is no change in adsorption energy and the adsorbates do not move or interact with each other on the adsorbent's surface (Song et al., 2013). The Langmuir model constant (K_l) which represents the affinity between the adsorbate and the adsorbent and is used to establish the dimensionless separation factor (R_l) given by;

$$R_l = \frac{1}{1 + K_l C_o} \quad (7)$$

Where C_o (mg/L) is the initial concentration of adsorbate. R_l value indicates the adsorption nature of unfavourable if $R_l > 1$, linear if $R_l = 1$, favourable if $0 < R_l < 1$ and irreversible if $R_l = 0$ (Ganesapillai and Simha, 2015; Rangabhashiyam et al., 2014).

The Freundlich model represents the isothermal variation as a function of surface coverage which is not restricted to one layer and occurs on heterogeneous sites (Chung et al., 2015; Passé-Coutrin et al., 2009; Song et al., 2015). This model assumes that adsorption energy exponentially decreases when the adsorption sites on the adsorbent are exhausted (Rangabhashiyam et al., 2014). The Freundlich exponent, $1/n$ is a function of the strength of adsorption in the adsorption process and ideally, it should have a value less than one during a normal adsorption process which is an indication that the adsorption sites are homogenous in energy and there is no interaction between the adsorbed species. When $1/n$ assumes a value greater than 1, then the interactions become weak and adsorption becomes unfavourable (Can et al., 2016).

In contrast to the Freundlich isotherm, the Temkin model works on the assumption that the heat of sorption as a function of temperature is linear rather than exponential (Rangabhashiyam et al., 2014). In liquid phase adsorption, the Temkin model works on the assumption that the free energy of adsorption is simply a function of surface coverage and the value of B indicates whether the adsorption is a physical or chemical process (Abdelnaeim et al., 2016).

1.11 Microflora on adsorbents of natural origin

Mineral adsorbents either synthesised in the laboratory or mined from naturally occurring deposits, therefore their compositions and microbiology vastly differ. Minerals used in the pharmaceutical industry are mostly synthetic analogues as it is cheaper from an economic point of view to synthesise them than to retrieve them from natural deposits (Carretero and Pozo, 2010). However, clay minerals are an

exception because it is extremely difficult and costly to synthesise them. Natural clays are layered aluminosilicates (Mueller, 2015) which exhibit a high degree of expandability, high water retention capacity, strong adsorption and good biocompatibility (Bellou et al., 2015). The microorganisms most frequently found on minerals are *Bacillus*, *Streptococcus*, Gram-negative bacteria and yeasts and moulds (de la Rosa et al., 1995). Aerobic and facultatively anaerobic bacteria belonging to the *Bacillus* spp., *Pseudomonas* spp., and *Arthrobacter* spp., as well as anaerobic bacteria belonging to clostridia sulfate-reducing and iron-reducing, have been isolated in kaolinite (Osipov and Turova, 1997).

The minerals found in clays affect microbiological activities and survival in different ways. The enhanced survival of bacteria by clays as a result of the process of desiccation was proposed as the major mechanism by which microorganisms are protected from dying by clays (Dandurand et al., 1994). Clays are also capable of adsorbing bacteria onto their surfaces. About 97% of all prokaryotes are affiliated with minerals in suspension (Hong et al., 2013) and this is mainly influenced and attributed to the surface charge distribution and the electrostatic double layer surrounding the clay particles and the microorganisms. Therefore, bacteria will have different adhesion behaviours on different clays due to the different surface electrical properties on the clay surfaces and different surface chemistry resulting in different bond formation (Mueller, 2015).

The sorption of bacteria onto clay surfaces is an abiotic physiochemical process and depends on the chemistry of the bacteria and the absorbent (Cao et al., 2011). These interactions have the potential to alter the activity and distribution as well as influence the survival of the microorganisms. The adsorption of bacteria onto clays results in conformational changes of the proteins on the bacterial surfaces. Bacteria cell walls consist of polysaccharides, lipids, nucleic acids and proteins which create a highly heterogeneous outer surface capable of forming various interactions with adsorbents such as hydrogen bonds (Hong et al., 2013). For example, a study by Bellou et al., 2015 revealed that human adenoviruses, coliphages and possibly other enteric viruses are capable of being adsorbed onto clay surfaces particularly kaolinite and bentonite (Bellou et al., 2015). Therefore, in light of the potential proliferation of antimicrobial resistance, adsorbents should be sterile prior to mixing with aqueous solutions with traces of antibiotics.

1.12 Sterilisation

Sterile is defined as being free of viable microorganisms (bacteria, fungi and viruses) (Vetten et al., 2014), however in practice this cannot be ascertained because the absolute sterility of a product can only be proven by testing every product produced which is not feasible, therefore the sterilization technique employed must ensure that the Sterility Assurance Level (SAL) is achieved (Rogers, 2012). SAL is the maximum probability of one in a million of a product being contaminated with microorganisms commonly known as the 10^{-6} probability of non-sterility (Shalaby et al., 2013; Vetten et al., 2014) In thermal sterilisation, SAL it can be calculated by the following equation:

$$\text{Log } SAL = \text{Log } N_o - \frac{T}{T_{10}} \quad (8)$$

Where N_o is the average microbial or spore population, calculated from a statistically representative sample. T is the exposure time to a selected constant temperature and T_{10} value is relative to the longest time resistant sample microorganism, whose inactivation is intended (Botelho et al., 1988).

Probability is used in the definition of SAL (Sandle, 2013a). It is difficult to prove that all living forms have been eradicated from a product mainly because microorganisms can be present but undetectable because they have been incubated in an unfavourable environment or their existence has never been established (Sandle, 2013a). Possible contaminants include viable cells, non-viable particles, chemicals and pyrogens (Vetten et al., 2014). Sterilisation can be defined as a process which effectively eradicates transmissible agents from items, materials or products to render them free of viable microorganisms including bacterial endospores (Li and Farid, 2016). The most common sterilisation procedures include heat (moist or dry), gas, radiation (gamma or electron), UV light, filtration and chemical methods (Tohfafarosh et al., 2016). Regulatory, economic and scientific aspects play a crucial role in the choice of the sterilisation method (Yavuz et al.,

2016). The main factors affecting sterilisation are the initial number/concentrations of microorganisms, the resistance of microorganisms, physicochemical factors, exposure time and storage.

1.12.1 Dry heat sterilisation

Dry heat sterilisation is the process by which sterilisation is achieved by thermal conduction whereby heat is absorbed by the outer surface of a microorganism/item and then passed inwards to the next layer until the entire microorganism/item reaches the peak temperature required to achieve sterilisation. The suggested mechanism of destruction is dehydration leading to a thermal oxidation, and at higher temperatures incineration (Sandle, 2013b).

The dry heat process is one of the most used methods to sterilise and/or dehydrogenate thermostable pharmaceutical materials products such as oils, ointments, powders, glass, metal equipment, etc. The processes are less complex than moist heat processes although they require higher base temperatures and longer time exposures (Moesby et al., 2005). Typical dry heat sterilisation cycles are run for 2 h at 160 °C, 1 h at 170 °C, 30 min at 180 °C. However, temperatures as low as 105 - 135 °C can kill microbes with longer exposure times (Rogers, 2012). An effective dry heat sterilisation process must achieve a microbial survival probability of 10^{-12} (United States Pharmacopeia, 2006).

In addition to sterilising, dry heat is also capable of removing or deactivating bacterial endotoxin, a process known as depyrogenation which has proven to be a challenge with other sterilisation methods. Pyrogens are substances capable of inducing a vigorous inflammatory response, most notably spiking a fever in mammals and the most common type are bacterial endotoxin (Opal, 2007). Bacterial endotoxins are cell wall components (lipopolysaccharides) of Gram-negative bacteria such as *Escherichia coli*, *Salmonella spp.*, and *Pseudomonas spp.* which can be shed off from living bacterial cells (Brawner et al., 2016; Moesby et al., 2008). However, most of it is released when the cell undergoes lysis so it is therefore mostly associated with dead bacterial cells.

1.12.2 Steam sterilisation

The principle of steam sterilisation is based on the manipulation of the boiling point of water by adjusting pressure to achieve higher temperatures than those which can ordinarily be achieved. Steam is highly penetrative and provides at least 7 times more heat than dry heat at the same temperature. Steam sterilisation is usually applied to products which require terminal sterilisation, stainless steel and liquids. The mechanism involves the transfer of latent heat of condensation to the load resulting in rapid heating. The typical steam sterilisation method is at a temperature of 121 °C for 15 min (Rogers, 2012; Sandle, 2013c).

1.12.3 The death value (*D-value*)

The death value (*D-value*) expresses microorganism resistance to a sterilising agent. The *D-value* is the amount of time required to reduce the number of viable cells by 90% or one log at a particular constant temperature (base temperature) as shown in Fig. 6. It is the rate at which a microorganism dies off during a sterilisation process (Fleischman, 2015). For example, the *D-value* of spores of *B. subtilis var. niger* at 170 °C (D_{170}) could typically be 1.5 min (Sandle, 2013b).

The *D-value* can be expressed by the following equation:

$$D - value = \frac{Time}{\log N_0 - \log N_T} \quad (9)$$

Where N_0 is the initial microbial or spore population prior to heat exposure and N_T is the surviving microbial or spore population after the time of heat exposure (Deák, 2014).

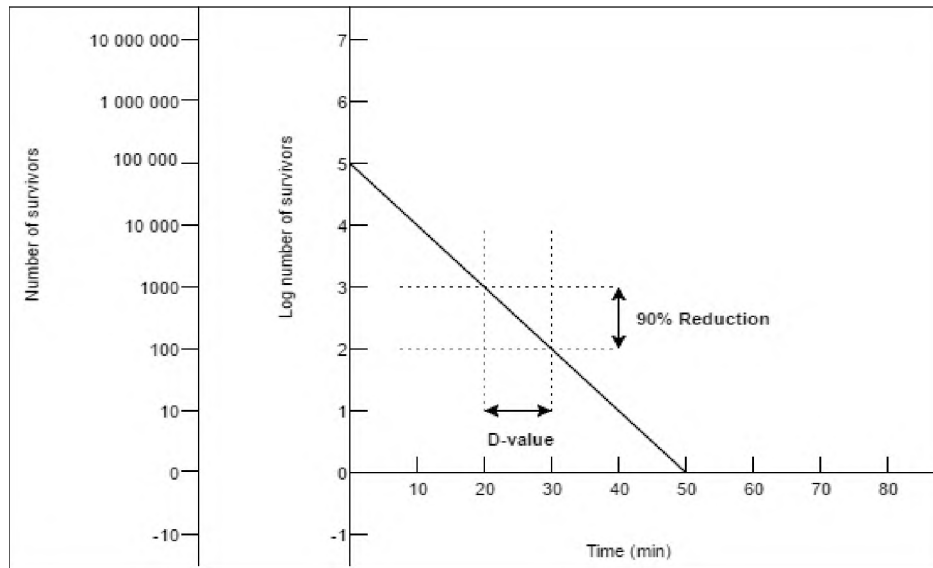


Figure 6: Death rate curve of microorganisms - the decimal reduction value (*D*-value). *D*-value = number of min taken to reduce a bacterial or spore population by 90 % or one logarithm. Reproduced from (Rogers, 2012) and drawn using Draw.io

Significance of *D*-value in the validation of the sterilisation process

1. Each microorganism in an environment has a distinctive *D*-value when subjected to a particular sterilisation method.
2. It is used to calculate the *Z*-value and biological factor *F*.
3. It is used to mathematically deduce the number of log reductions of the microbial population.

1.12.4 The *Z*-value

The *Z*-value is described by Fig. 7 and represents the elevation of temperature required to reduce the *D*-value of an organism by 90% and is solely used in the validation of a heat sterilisation method (Chen et al., 2011; Fleischman, 2015). It can be represented by the following equation:

$$Z = \frac{T_X - T_O}{\log D_O - \log D_X} \quad (10)$$

Where D_o is the D -value at the initial temperature T_o , D_x is the D -value at a later temperature T_x (Rogers, 2012).

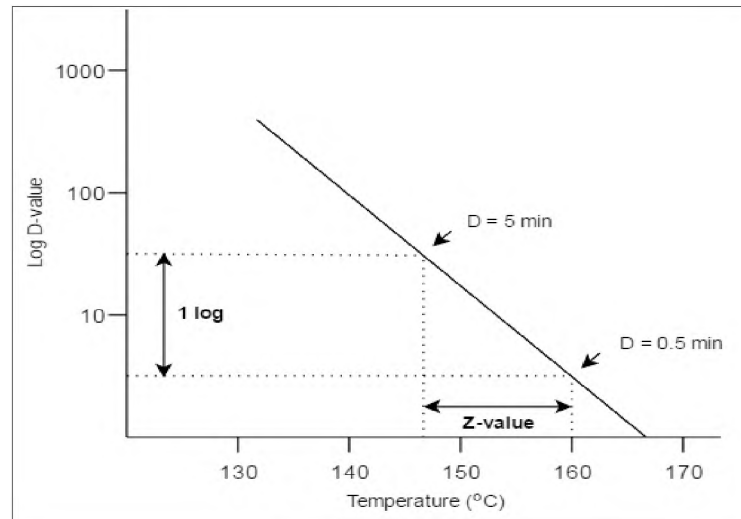


Figure 7: The Z -value. Z -value = the number of degrees required to reduce the D -value of an organism by 90 % or one logarithm. Reproduced from (Rogers, 2012) and drawn using Draw.io

The D -value and Z -value are kinetic parameters which describe the inactivation of the microorganisms and are used to show the efficiency and effectiveness of the process. They completely define the heat resistance characteristics of microorganisms (Deák, 2014).

The lethality rate (F_H) is the equivalent sterilisation time spent at base temperature and can be calculated using the Patashnik method whereby lethality (L) is represented by the equation:

$$L = 10^{\frac{T-T_b}{Z}} \quad (11)$$

and

$$F_H = \Delta t \times L \quad (12)$$

Where Δt is the cycle time, T is the actual cycle temperature, T_b is the base temperature and Z is the microbial death rate constant (Van Doornmalen and Kopinga, 2009).

1.12.5 Resistance of bacteria to dry heat

Some bacteria, such as *Clostridium spp.*, *Bacillus spp.*, *Coxiella spp.*, *Actinomycetes spp.*, and some *Mycobacteria spp.* (Berthelot et al., 2013) are capable of sporulating which enables the bacterial cell to survive under extreme conditions such as limited nutrients and elevated temperatures for extended periods of time (Zhou et al., 2015). Sporulation is the ability of bacteria to form dormant spores that make them resistant to acute treatment when not enough stress is applied (Smelt and Brul, 2014). The dormancy ends when conditions become favourable and any damaged to the DNA is repaired when the cell returns to life via spore germination which rekindles metabolic activities within the cell (Berthelot et al., 2013). However, if the stress levels were too high and the cell was irreversibly damaged during the inactive spore state the cell succumbs to the damage and dies. Stress can make bacteria super dormant and incapable of germinating under normal conditions (Zhou et al., 2016). The sterilisation technique used must, therefore, ensure that the cell is completely destroyed and will not be revived under any condition.

The spore's unique physical structure and chemical composition are responsible for the resistance to stress (Zhou et al., 2016). It is comprised of different layers with the outermost layer being the exosporium, coats, outer membrane, cortex, germ cell, inner membrane and the inner part being the central core (Reineke et al., 2013). These may differ across various species of bacteria but still play the same role. The coat and cortex act as protective barriers (Díaz-González et al., 2015) and the core produces stability proteins for DNA stabilisation (e.g. α/β -type small acid soluble proteins (SASPs) (Zhou et al., 2016). Spores are significantly much more resistant to dry heat than moist heat treatment but most factors which are responsible for dry heat resistance have not been thoroughly investigated and the mechanisms of resistance are not known.

2 CHAPTER 2: ADSORPTIVE REMOVAL OF CIPROFLOXACIN AND ISONIAZID FROM AQUEOUS SOLUTION

2.1 Introduction

The prevailing emergence of antimicrobial resistance amongst bacterial microorganisms remains a serious health and economic problem. It is estimated that 700 000 deaths occur worldwide every year as a result of antibiotic resistance (Bengtsson-Palme and Larsson, 2016). There are ongoing and major concerns that antimicrobial resistance is caused and/or aggravated by, amongst others, the presence of antibiotics and their transformation products in the environment (Ashbolt et al., 2013; Tran et al., 2016). Resistance can spread among bacteria via vertical and horizontal gene transfer and can spread from human and animal sources to the receiving environment (He et al., 2014). However, in most countries, the rules and regulations governing pharmaceutical pollution apply to controlled substances and cytotoxic drugs and do not address the antibiotics and the risk of resistance (World Health Organisation, 2011).

Antibiotics are extensively used worldwide in human and veterinary medicine for therapeutic, prophylactic and growth-enhancing purposes (Chen et al., 2016). Consequently, they are relentlessly discharged into the environment and are therefore considered to be “pseudo-persistent” environmental pollutants (Yao et al., 2015). Antibiotics in wastewater from domestic, hospital, farm and industrial effluent (Wu et al., 2016) are released into wastewater treatment plants (WWTP) which act as the major port of entry of antibiotics into different facets of the environment (Luo et al., 2014). The conventional treatment methods employed in WWTPs are incapable of completely removing antibiotics from wastewater (Jia et al., 2016). Furthermore, WWTPs link all environmental waters and considered “hotspots” for the spread of antibiotics, antibiotic-resistant bacteria and genes across the entire environment (Rizzo et al., 2013). Antibiotics have been detected in wastewater in concentrations ranging from ng/L to mg/L (Hijosa-Valsero et al., 2011; Xu et al., 2015; Xiong et al., 2017b). Therefore, development of a simple and efficient treatment antibiotic removal from contaminated wastewater is

fundamental to reduce the potential proliferation of antimicrobial resistance among bacteria.

Isoniazid (pyridine-4-carbohydrazide) is an anti-tubercular drug believed to belong to the nucleoside reverse transcriptase inhibitor class of antibiotics (Bhandari and Kaur, 2013). Its structure comprises of hydrazide group and the pyridine ring which are essential for activity against *Mycobacterium tuberculosis* (Jena et al., 2014). Despite its susceptibility to resistance (Aguar et al., 2015), isoniazid remains the cheapest, most effective and most prescribed drug for TB chemoprophylaxis and the first line combination chemotherapy of all forms of TB (Chen et al., 2009; Verma et al., 2015).

Ciprofloxacin [1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(piperazinyl)-quinolone-3-carboxylic acid] is a synthetic broad-spectrum antibiotic with activity against a wide range of Gram-negative and some Gram-positive microorganisms (Attia et al., 2016). It is a second generation fluoroquinolone and is used to treat various bacterial infections such as urinary tract infections, respiratory tract infections, acute uncomplicated cystitis and chronic bacterial prostatitis (Masadeh et al., 2015). Ciprofloxacin is the most widely used drug in its class in both human and veterinary medicine (Antonin et al., 2015; Jalali, 2016). Ciprofloxacin, even at very low concentrations found in wastewater, can lead to the proliferation of antibiotic-resistant bacteria (Yu et al., 2016a).

The application of adsorption technology to remove wastewater pollutants has proven to be one of the most versatile water treatment methods and is highly efficient, environmentally friendly and easy to operate (Yu et al., 2016b). Activated charcoal is the widely preferred adsorbent due to its excellent adsorption properties. However, the high costs incurred during its production reduces the feasibility for its use at large scale (Sun et al., 2016). Recently, there has been an increased interest in investigating the sorption of different classes of organic pollutants onto low-cost adsorbents (Hassani et al., 2015; Norris et al., 2015). Naturally occurring rock minerals such as kaolinite, perlite, talc and vermiculite and waste-derived materials such as coal fly ash (FA), a mineral residue, are potential alternatives owing to their porous nature, chemical inertness, relatively low costs and availability in

commercially bulk quantities (Ahmaruzzaman, 2010; Kolvari et al., 2015; Tian et al., 2016). Therefore, these adsorbents can hopefully find application as low-cost, effective materials for the removal of antibiotics from various effluents, such as domestic, industrial and hospital wastewaters. This will also create employment in areas where the minerals are found and also boost the economy. For example, the the largest kaolinite deposits in South Africa are found in the Makana area in the Eastern Cape and are mostly unexploited (Malatji, 2010), yet the area is dominated by poverty (Davenport et al., 2011).

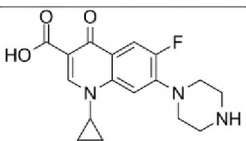
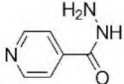
This chapter describes in detail the removal of ciprofloxacin and isoniazid from aqueous media by adsorption onto kaolinite, perlite, talc, vermiculite and FA batch systems. The adsorption of antibiotics at the solid-liquid interface is strongly influenced by the nature of the structural groups present on the solid surface, the molecular structure of the antibiotic being adsorbed (the adsorbate), and the environment of the aqueous phase. Therefore, the effect of varying parameters such as solid/liquid ratio, pH, contact time, the initial antibiotic concentration was also investigated systematically. The linearised forms of the Langmuir, Freundlich and Temkin isotherms were used to analyse the data.

2.2 Materials and methods

2.2.1 Materials and reagents

The physicochemical properties of ciprofloxacin and isoniazid used are presented in Table 4.

Table 4: *Physicochemical properties of ciprofloxacin and isoniazid* (Genç and Dogan, 2015; Hamjinda et al., 2016; Ràfols et al., 2012).

Antibiotic	Chemical Structure	Molecular mass (g/mol)	pK _a	Log o/w
Ciprofloxacin (C ₁₇ H ₁₈ FN ₃ O ₃)		331.34	pK _a 1 = 5.90 ± 0.15 pK _a 2 = 8.89 ± 0.11	0.28
Isoniazid (C ₆ H ₇ N ₃ O)		137.14	pK _a 1 = 3.53 ± 0.04 pK _a 2 = 11.40 ± 0.07	-0.65 ± 0.03

FA was obtained in its raw state from Ash Resources (Pty.) Ltd. (Randburg, South Africa). Ground talc was purchased from Micronized Products (Pty.) Ltd. (Johannesburg, South Africa). Raw kaolinite was obtained from the local St. Rowan Mine (Grahamstown, South Africa). Perlite and vermiculite, both in the expanded form, were obtained from Sunshine Nursery (Grahamstown, South Africa). All glassware, ciprofloxacin (purity $\geq 98\%$, CAS # 85721-33-1), isoniazid (purity $\geq 99\%$, CAS # 54-85-3), ammonium oxalate (99.99 %), Büchner funnels (Corning size number No. 40), porcelain high form crucibles (15 mL), mortar and pestle, Suprasil® 3500 μL quartz cuvettes (200-2500 nm spectral range) and Dow Corning® high-vacuum silicone grease were purchased from Sigma Aldrich (Pty.) Ltd. (Johannesburg, South Africa). Hydrochloric acid (HCl) (32 %), glacial acetic acid (97 %), potassium chloride (KCl) (99.5 %), sodium chloride (NaCl) (99.5 %), calcium chloride (CaCl_2) (98 %), ethylene glycol monoethyl ether (EGME) (99.5 %), commercial buffers (pH 4 ± 0.02 , 7 ± 0.02 and 10 ± 0.02 at 20°C), and ammonium chloride (NH_4Cl) (99 %), ammonium hydroxide (NH_4OH) (25 %), ammonium (NH_4^+) test kits, isopropyl alcohol (99 %), silver nitrate (99.8 %), urine jars (40 mL) were purchased from Merck (Pty.) Ltd. (Johannesburg, South Africa). Milli-Q water was obtained from a Milli-Q® RiOs™ and Academic A10 Water Purification system (Merck Millipore, Port Elizabeth, South Africa.).

Masses were weighed using a Pioneer™ PA1214 analytical balance with 0.0001 g accuracy and Pioneer™ PA2102 analytical balance with 0.01 g accuracy purchased from Ohaus Corporation (Pine Brook, NJ, USA). Mechanical shaking was done using a model number TS-520D orbital shaker purchased from Already Enterprise Inc. (Taipei, Taiwan). The electric muffle furnace was purchased from Weiss-Gallenkamp (Loughborough, United Kingdom). The UFE 700 oven was purchased from Memmert, (Schwabach, Western Germany). pH was measured using a Crison pH meter basic 20 coupled with an ultrathin glass Crison electrode purchased from Crison Instruments (Alella, Spain). Parafilm™, Whatman number 1 filter paper and heavy duty aluminium foil were purchased from Spellbound Labs (Port Elizabeth, South Africa). The model STR-MH magnetic stirrer was purchased from FMH Laboratory Products (Midrand, South Africa). The Cintra 10 GBC 916 UV/vis spectrophotometer was purchased from GBC Scientific Equipment (Pty.) Ltd. (Melbourne, Australia). The 315 μm mesh sieve

and mechanical shaker were purchased from Retsch GmbH (Haan, Germany). The Rocker 300 vacuum pump was purchased from Rocker Scientific Co., Ltd. (New Taipei City, Taiwan).

2.2.2 Preparation of adsorbents

The adsorbents were ground using a mortar and pestle and mechanically passed through a 315 μm mesh sieve to ensure uniform particle size. Afterwards, the adsorbents were washed with Milli-Q water in the ratio 1:10 (g/mL), by stirring on a model STR-MH magnetic stirrer at speed 5 for 24 hours (h). The suspensions were then separated by light suction vacuum filtration using a Buchner funnel lined with Whatman number 1 filter paper and dried for 12 h in a Memmert ULM600 oven at 105 °C. The adsorbents were air-dried at room temperature (25 ± 1 °C) for 24 h.

2.2.2.1 Characterisation of adsorbents

Characterization of the adsorbents was done by determining total organic carbon content using the standard method (Schinner et al., 1996) and loss on ignition (LOI) method (Heiri et al., 2001), pH (Rowell, 1994), the cation exchange capacity (CEC) using the ammonium acetate saturation method (Sumner and Miller, 1996), specific surface area (SSA) using the modified ethylene glycol monoethyl ether / calcium chloride (EGME/ CaCl_2) method (Tandlich and Balaz, 2011). All procedures were performed in triplicates for each adsorbent and reported as means $\pm$ standard deviations (S.D.). Surface morphology was observed on a Tescan VEGA LMU scanning electron microscope (SEM) (Tescan, Czechoslovakia), mineralogical composition using (XRD) patterns recorded using a Bruker D8 Discover machine (Bruker, Germany) equipped with the PSD Lynx Eye detector, using Cu-K radiation ($\lambda = 1.5405 \text{ \AA}$, nickel filter). The Fourier transform infrared (FTIR) spectra were obtained at room temperature from an average of 10 individual scans using Perkin Elmer version 10.4 spectrophotometer (Perkin Elmer, Port Elizabeth, South Africa) in the spectral range of 4000 to 650 cm^{-1} having a resolution of 4 cm^{-1} by the KBr disc method.

Furthermore, XRD patterns and FTIR spectra were used to investigate the adsorption mechanisms.

2.2.3 Loss on ignition (LOI)

Porcelain crucibles were acid washed (phosphate detergent, HCl 10 % and distilled water) and dried in a Memmert UFE 700 oven at 105 °C for 24 h. The crucibles were then placed in a desiccator containing silica gel for 24 h. The dried crucibles were weighed using a Pioneer™ PA214 analytical balance and the mass was recorded (M_0). Two grams (2 g) of the adsorbent sample was added to the dried crucibles and the total mass was recorded (M_1). The crucibles were put in the oven at 105 °C for 24 h and placed in a desiccator for 2 h before recording the final total mass (M_2). The dry weights (W_s) of the adsorbents were calculated using the equation:

$$W_s = \frac{M_2 - M_0}{M_1 - M_0} \quad (13)$$

The crucibles containing the dry adsorbent samples were ignited at 400 °C in a Gallenkamp App9B 4152 SP muffle furnace for 4 h. Two empty crucibles were used as controls. The percentage LOI (%) was calculated using the following equation:

$$LOI = \frac{M_s - M_c}{M_s} \times 10 \quad (14)$$

Where M_s is the dry weight of adsorbent after drying at 105 °C and M_c is the dry weight of adsorbent after ignition at 400 °C.

2.2.4 pH studies

2.2.4.1 Sample preparation

In an attempt to standardise conditions, pH of the adsorbent samples was measured in 0.01 M CaCl_2 , 1M KCl and distilled water (dH_2O) (Rowell, 1994). Each adsorbent

was weighed using a Pioneer™ PA214 analytical balance, added into a urine jar and subsequently mixed with each solution according to the following ratios (g/mL) 1:3 [adsorbent: dH₂O], 1:6 [adsorbent : dH₂O], 1:3 [adsorbent : 0.01 M CaCl₂] and 1:3 [adsorbent : 1 M KCl]. All mixtures contained 10 ± 0.1 g adsorbent. The mixtures were shaken occasionally by hand over a period of 15 min at room temperature (25 ± 1 °C) and left to stand for 5 min.

2.2.4.2 Measurement of pH

The pH-measurements were carried out using an ultrathin glass a Crison pH meter basic 20 coupled with an ultrathin glass Crison electrode. Three-point electrode calibrations through commercial buffers at pH 4.0, 7.0 and 10.0 were done before and after pH-measurement of each adsorbent sample titration.

2.2.5 Cationic Exchange Capacity

2.2.5.1 Preparation of 1 M ammonium acetate (NH₄OAc) saturation solution

In a fume hood, 117 mL of glacial acetic acid (97 %) was diluted to approximately 1 000 mL with dH₂O in a 2 000 mL conical flask and the solution was continuously mixed by a magnetic stirrer. Using a measuring cylinder, 138 mL of concentrated NH₄OH was added to the conical flask followed by approximately 800 mL dH₂O. pH was adjusted to 7.0 by adding NH₄OH in a dropwise fashion. The contents of the conical flask were transferred to a 2 000 mL volumetric flask and the volume was made up to the mark using dH₂O.

2.2.5.2 Preparation of 1 M potassium chloride (KCl) solution

Using a Pioneer™ PA2102 analytical balance, 74.5 g of KCl was weighed and then transferred into a 250 mL beaker. About 200 mL dH₂O was added to the beaker and stirred with using a glass rod until all the KCl dissolved. The solution was transferred to a 1 000 mL volumetric flask and the beaker rinsed several times with dH₂O to

ensure all the solution was transferred. The contents of the volumetric flask were made up to the mark using dH₂O.

2.2.5.3 Measurement of cationic exchange capacity

Using a Pioneer™ PA2102 analytical balance, 10 g of adsorbent was weighed and transferred into a 500 mL Erlenmeyer flask. Using a graduated 250 mL glass measuring cylinder, 250 mL of 1M NH₄OAc (pH 7) was added to the flask and the flasks were sealed with Parafilm™ and aluminium foil. The mixtures were mechanically shaken on an orbital shaker at 100 rpm for 24 h, after which the mixtures were left equilibrate for 12 h at room temperature (25±1 °C). Thereafter, the suspensions were separated by light suction using a Buchner funnel lined with Whatman number 1 filter paper (caution was taken to ensure that the adsorbents do not dry and crack). The adsorbents were the leached with 1M NH₄OAc until a negative calcium (Ca²⁺) test is obtained in the leachate.

Calcium test: Three drops each of 1M NH₄Cl (pH 7), 10 % ammonium oxalate, and dilute NH₄OH were added to 10 mL of leachate in a Pyrex test tube and the solution was heated to 90 °C over a Bunsen burner flame. The presence of calcium is indicated by a white precipitate or turbidity of the solution.

The leachate was discarded. The adsorbents were then leached with 1M NH₄Cl four times and once with 0.25M NH₄Cl. About 150 - 200 mL isopropyl alcohol (99 %) was used to wash out the electrolytes from the adsorbents, and then the leachate was tested for chloride (Cl⁻).

Chloride test: Three drops of 0.10 M AgNO₃ were added to 10 mL of leachate. The presence of chloride ions is indicated by a white precipitate. After the test for Cl⁻ became negative based on visual observation, the adsorbents were allowed to drain thoroughly. The adsorbed ammonium (NH₄⁺) was extracted by leaching the adsorbent with 80 mL of 1 M KCl by adding just enough to cover the surface of the adsorbent in the Buchner funnel at a time and letting it leach through before the next addition.

The leachate was transferred into a 250 mL volumetric flask and made up to the mark using dH₂O.

Ammonium test: The leachate was mixed with the reagents in the NH₄⁺ test kit according to manufacturer's instructions and the reaction mixture was transferred into a Suprasil® 3500 µL quartz cuvette. The concentration of the NH₄⁺ was determined spectrophotometrically at 660 nm using a Cintra 10 GBC 916 UV/vis spectrophotometer in accordance with the US EPA method 350.1 available at <https://www.epa.gov/sites/production/files/2015-06/documents/epa-350.1.pdf>.

The absorbance obtained was paralleled with a five-point ammonium chloride calibration curve prepared by plotting the linear fit of absorbance as a function of concentrations in the range 1 - 10 mg/L. The background influence of the aqueous phase was eliminated by performing an automatic baseline correction with a blank solution. The CEC (mEq/100 g) was calculated using the following equation (Ross and Ketterings, 2011):

$$CEC = conc(NH_4) + (0.25 L \times 10 g \text{ adsorbent}) \times \left(\frac{mEq NH_4}{18 mg NH_4} \right) \times 100 \quad (15)$$

2.2.6 Specific surface area measurements

2.2.6.1 Drying of the adsorbent samples

For each adsorbent, three 100 mL glass jars, with open lids, and 150 g of CaCl₂ were placed in a UFE 700 oven at 105 °C for 24 h, and then cooled in separate desiccators with the lids sealed with high vacuum grease for 2 h, respectively. The mass of each dry glass jar, with its lid closed, was recorded using a Pioneer™ PA214 analytical balance. The glass jars were transferred into the desiccator with oven dried CaCl₂ for 48 h with their lids open, and afterwards, the mass of each glass jar was recorded with its lid closed. Approximately 1.1 g of each adsorbent was weighed and transferred into each glass jar. The jars were returned into the desiccators containing CaCl₂ for 24 h, with their lids open, over CaCl₂ to dry the adsorbent samples. After 24 h, the individual glass jars were covered with lids and weighed using a Pioneer™ PA214 analytical balance to determine the dry weight of each

sample. The mass of the glass jar and the adsorbent sample was recorded for each sample. Two empty jars were used as negative controls.

2.2.6.2 Preparation of CaCl₂/EGME solvate

Using a mortar and pestle 150 g CaCl₂ was ground and mechanically passed through a 315 µm mesh sieve. The CaCl₂ was baked at 160 °C for 24 h in an oven. The CaCl₂ was removed from the oven, left to cool for 5 min and then mixed with 150 mL aliquot of EGME to obtain the CaCl₂/EGME solvate which was used to maintain a constant pressure in the desiccator.

2.2.6.3 Measurement of Specific Surface Area

The CaCl₂/EGME solvate was immediately placed into an empty desiccator (to create a stable EGME atmosphere over the adsorbent samples) and the adsorbent samples were left to equilibrate, with lids open, for 30 min. The desiccator was then evacuated at approximately 6.7 Pa using a vacuum pump for 45 min. The desiccator was then sealed and the samples were left to equilibrate with the EGME atmosphere for 24 h. The desiccator was re-pressurized and the masses of the glass jars, with lids closed, were using a Pioneer™ PA214 analytical balance. The glass jars were returned into the desiccator which was then sealed and evacuated as described above. The operations were repeated every 24 h until a constant weight of the samples was obtained (0.0001g precision). The specific surface area (m²/g) was calculated using the following equation (Segré, 2013):

$$SSA = \frac{W_a}{2.86 \times 10^{-4} \times W_s} \quad (16)$$

Where W_a (g) is the mass of ethylene glycol monoethyl ether retained on the adsorbent sample, W_s (g) is the mass of the dry adsorbent sample and 2.86×10^{-4} g is the mass of EGME required to form a monolayer on a one square meter surface.

2.2.7 Adsorption Studies

2.2.7.1 Kinetics Studies

All adsorption experiments were carried out by batch equilibrium technique at room temperature (25 ± 1 °C) in duplicates. Aqueous stock solutions of concentration 1.0 g/L were prepared by dissolving the antibiotic powders in MilliQ water. In the adsorption kinetics studies, 0.1 ± 0.001 g and 1.0 ± 0.01 g were added to sterile 30 mL centrifuge tubes containing 10 mL ciprofloxacin and isoniazid solution, respectively. The concentration of the antibiotic, which was obtained from the dilution of the stock solutions, was 25 mg/L. The mixtures were shaken on a Junior Orbit Shaker (LAB-LINE Instruments Inc., Melrose Park, ILL) at a constant speed of 220 rpm for a fixed time interval (5, 10, 15, 30, 60, 120, 240 and 480 min) in the dark. After shaking, the mixtures were separated by spinning on a Mixtasel centrifuge (Selecta®, Barcelona, Spain) at 700 RCF for 10 min and passing the supernatants through a 0.45 µm pore size PVDF membrane syringe filter (Sigma-Aldrich (Pty.) Ltd., Johannesburg, South Africa). Antibiotic solutions which were not mixed with adsorbents were used as controls to assess the influence of phase separation on the initial antibiotic concentrations and the effect of physical losses such as evaporation and sorption onto glass on the experimental results. These were found to be negligible. The filtrates were then diluted accordingly with MilliQ water. The concentrations of the antibiotic solutions were determined spectrophotometrically using a Cintra 10 GBC 916 UV/vis spectrophotometer (GBC Scientific Equipment (Pty) Ltd., Melbourne, Australia) wavelengths of maximum absorbance, 277 nm and 262.5 nm for ciprofloxacin and isoniazid, respectively. The wavelengths of maximum absorbance of the antibiotics were predetermined spectrophotometrically after eliminating the background influence of the aqueous phase by performing an automatic baseline correction with a blank solution. Calibration curves were established by using ten standards in the range 0 to 7.5 mg/L and 0 to 27 mg/L for ciprofloxacin and isoniazid, respectively.

2.2.8 Equilibrium Studies

To investigate the effects of the solid/liquid ratio, dosages of 0.100, 0.175, 0.250 g were used for ciprofloxacin solutions and 0.5, 1.0 and 1.5 g were used for isoniazid solutions. Influence of pH was evaluated by adjusting the initial pH of the antibiotic solutions ranging from 3 to 12 using 0.1 M aqueous solutions of HCl or NaOH.

To obtain adsorption isotherms, solutions of different antibiotic concentrations (5 - 250 mg/L) were made by diluting the stock solutions with MilliQ water. The ciprofloxacin and isoniazid solutions were mixed with 0.1 ± 0.001 g and 1.0 ± 0.01 g adsorbent, respectively. The mixtures were shaken at 220 rpm for 40 min to allow adsorbents to reach equilibrium adsorption. This time was sufficient to reach apparent sorption equilibrium. The initial pH of antibiotic solutions was at the optimum for maximum adsorption for each adsorbent. The amounts of antibiotic adsorbed and % removal were calculated by mass balance using the formula:

$$q_e = \frac{C_0 - C_e}{w} V \quad (17)$$

$$\% \text{ removal} = \frac{C_0 - C_e}{C_0} \times 100 \quad (18)$$

Where q_e (mg/g) is the amount of antibiotic adsorbed at equilibrium, w (g) is the weight of the adsorbent, V (L) is the volume of antibiotic solution, C_0 (mg/L) is the initial antibiotic concentration in solution and C_e (mg/L) is the concentration of antibiotic remaining in solution at equilibrium.

2.3 Results and discussion

The characterization of the adsorbents was done by determining the pH, LOI, SSA and CEC as describe above. The results obtained are discussed in detail in this section. Table 5 below shows the summary of results obtained for pH, LOI, SSA and CEC.

Table 5: Physicochemical properties of adsorbents presented as the mean $\pm$ standard deviation ($n = 3$)

Parameter	FA	Talc	Perlite	Kaolinite	Vermiculite
Dry weight (%)	99.53 $\pm$ 0.01	100.0 $\pm$ 0.00	99.01 $\pm$ 0.02	98.08 $\pm$ 0.04	97.59 $\pm$ 0.03
LOI (%)	0.60 $\pm$ 0.19	0.16 $\pm$ 0.10	0.86 $\pm$ 0.03	0.47 $\pm$ 0.20	0.20 $\pm$ 0.08
CEC (cmol/kg)	9.46 $\pm$ 0.95	12.02 $\pm$ 1.75	41.21 $\pm$ 3.42	15.15 $\pm$ 1.65	86.52 $\pm$ 4.98
SAA (m ² /g)	5.52 $\pm$ 2.05	6.34 $\pm$ 1.32	3.10 $\pm$ 0.98	20.21 $\pm$ 7.52	5.92 $\pm$ 1.99
pH [adsorbent:dH ₂ O] 1:3	11.99 $\pm$ 0.35	9.04 $\pm$ 0.40	8.11 $\pm$ 0.50	5.58 $\pm$ 0.47	8.61 $\pm$ 0.31
[adsorbent:dH ₂ O] 1:6	12.14 $\pm$ 0.64	9.01 $\pm$ 0.19	8.08 $\pm$ 0.22	5.73 $\pm$ 0.13	8.78 $\pm$ 0.64
[adsorbent:KCl] 1:3	12.24 $\pm$ 0.43	8.63 $\pm$ 0.33	6.83 $\pm$ 0.38	4.81 $\pm$ 0.41	5.92 $\pm$ 0.47
[adsorbent:CaCl ₂] 1:3	12.39 $\pm$ 0.20	8.42 $\pm$ 0.60	7.19 $\pm$ 0.21	5.25 $\pm$ 0.23	6.08 $\pm$ 0.26

2.3.1 LOI

LOI is a parameter which refers to the mass loss of solid materials as a result of, oxidation of carbon to carbon dioxide and ash when the material is heated at a specific temperature and is widely used to estimate the organic and/or carbonate content of different kinds of samples (Mu et al., 2016). The carbon content was determined by incinerating the adsorbents at 400°C for 4 h. This temperature is used so that carbonates do not oxidise and so only organic content is measured. The percentage loss in mass after incineration correlates to the carbon content of the adsorbent and gives an accurate rough estimate of the organic matter present in the adsorbent (Heiri et al., 2001). The dry weights obtained ranged from 97.59. - 100.00 %. Talc had the least moisture content as expected due to its highly hydrophobic nature (Lv et al., 2014). The LOI of the adsorbents ranged from 0.16 to 0.86 % indicating that all the adsorbents had a low carbon content. It is important to note that the total organic carbon content also affects the measured CEC and SAA in three complex ways: (1) Chemically reacting with the investigative fluid employed for CEC and SAA, (2) obstructing the investigative fluid from reaching active sites by blocking pores or coating the surface of the adsorbent, (3) developing nano-scale pores as a

result of thermal maturation and diagenesis of the organic matter. Obstruction of active sites might lead to a decrease in the measured SAA, whereas, chemical interaction and development of nanopores might increase the measured CEC and SAA (Saidian et al., 2016).

2.3.2 pH

pH is an important parameter which indicates the acidity or alkalinity of the adsorbent. The pH of the adsorbents was measured in dH₂O and in salt solutions (0.01 M CaCl₂ and 0.1 M KCl). Measuring the pH of the adsorbents in salt solution gives an interpretation for the degree of saturation of the adsorbents by cations other than hydrogen and provides standardised conditions and gives more accurate values (Rowell, 1994; Segré, 2013). The difference in the adsorbent pH (ΔpH) measured in dH₂O and in salt solution is known as the salt effect and is given by the following equation:

$$\Delta pH = pH_s - pH_{dH_2O} \quad (19)$$

Where pH_s is the pH of the adsorbent measured in salt solution and pH_{dH_2O} is the pH of the adsorbent measured in dH₂O. For acidic adsorbents with a net negative charge and net positive charge the increase in salt concentration causes cation exchange with hydrogen ions (H⁺) held on permanent charge sites of the adsorbents being displaced into the solution lowering the measured pH and adsorption of H⁺ onto reactive sites on the adsorbent resulting increase in the measured pH, respectively (Rowell, 1994). Furthermore, ΔpH values are positive in adsorbents with net positive charges and negative in adsorbents with a net negative charge, and its magnitude is directly proportional to the net charge (Rowell, 1994). The pH_{dH_2O} of the adsorbents ranged from 5.58 -12.14 and the pH_s measured in KCl and CaCl₂ ranged from 4.81 - 12.24 and 5.25 - 12.39, respectively. It is evident that despite the difference in the solid/liquid ratio, the pH_{dH_2O} of each adsorbent were very similar (± 0.2 units) and are very close to what has been reported in literature perlite (6.5 - 8.0) (Rashad, 2016), FA (12.4) (Komonweeraket et al., 2015), vermiculite (7.0) (Rashad, 2016), kaolinite (5.0) and talc (8.7) (Lin et al., 2016). Hence, the average value of the two ratios was used as the overall pH_{dH_2O} for each adsorbent. The different salt solutions

also produced similar pH_s (± 0.5 units) and the average was used as the overall pH_s for each adsorbent. All samples, with the exception of kaolinite had $pH_{dH_2O} > 7$ indicating that most of the adsorbents are alkaline in nature. Except for FA, all the pHs measured in salt solutions are lower than those measured in dH_2O . This was expected because most of the adsorbents have a net negative charge (Date et al., 2012). The ΔpH values are presented in Table 6 below.

Table 6: Salting effect of the different adsorbents

Adsorbent	ΔpH
FA	0.26
Talc	-0.50
Perlite	-1.09
Kaolinite	-0.24
Vermiculite	-2.70

In general, the salting effect is more pronounced in alkaline adsorbents (Date et al., 2012) and this is in correlation with the results obtained. Perlite and vermiculite had the most negative ΔpH values, - 1.09 and -2.70, respectively indicating that they had the most negative net charge. All alkaline adsorbents have alkaline pH in dH_2O and the ΔpH values are large whereas the acidic kaolinite exhibits a small ΔpH value (- 0.24) indicating a close to zero salt effect as expected (Date et al., 2012). It is interesting to note that perlite and vermiculite had the greatest deviation between pH_{dH_2O} and pH_s . It can be deduced from the high CEC values obtained for these two adsorbents are dominated by exchangeable cations such as Ca^{2+} , and therefore suggests that the significant decrease in pH is due to the common ion effect which reduces the amount of carbonate that dissolves (Rowell, 1994). The pH of FA exhibited a strong alkali pH of 12.19. This is because the alkali oxides which dissolve in water increasing the pH (Zhai et al., 2017).

2.3.3 CEC

The CEC essentially represents the total capacity of adsorbents to bind exchangeable cations such as Ca^{2+} , Mg^{2+} , K^+ , Cu^{2+} , Zn^{2+} and Fe^{2+} (Ross and Ketterings, 2011). The measurement of CEC measurements is based on the principle of using an index cation that is capable of replacing the other cations adsorbed on the adsorbents surfaces (Hadi et al., 2016). In clay minerals, the CEC corresponds to negatively charged sites on the surface of the adsorbents which adsorb and hold positively charged ions by electrostatic force (Hadi et al., 2016; Sharma et al., 2015). The CEC was determined by the ammonium acetate saturation method. Leaching the adsorbents with 1M NH_4Cl provides a more accurate estimation of the true CEC. This is because calcium containing soluble minerals is removed leaving most exchange sites available for occupation by the saturating cation (NH_4^+) (Sumner and Miller, 1996). Furthermore, CEC is also governed by the pH, organic matter content, crystal chemistry and morphology of lamellar particles in the adsorbent (Brigatti et al., 2013). Mineral clays can be divided into two groups, based on the presence (or absence) of interlayer adsorption sites. Those which lack interlayer adsorption sites such as kaolinite exhibit relatively low CEC values whereas those which have interlayer adsorption sites vermiculite exhibit high CEC values (Dzene et al., 2016).

Of all the adsorbents, vermiculite had the highest CEC of 86.52 cmol/kg and is in agreement with the values reported in literature (Li et al., 2015). This is because vermiculite exhibits a high negative permanent surface charge partially compensated by hydrated cations (Na^+ , Ca^{2+} and Mg^{2+}) positioned in the interlayer spacing of the solid which reversibly exchange with cations in solution (Brigatti et al., 2013; do Nascimento et al., 2016). Therefore, the NH_4^+ is intercalated within the interlayer of vermiculite and occupies all available exchangeable sites resulting in a high CEC values.

Talc is electrically neutral due to its low isomorphous substitution in tetrahedral and octahedral sites, and hence the interlayer space is empty. Contiguous layers are held together by van der Waals interactions (Brigatti et al., 2013). The CEC obtained for talc was 12.02 cmol/kg. The minimal CEC of talc is attributed to the pH-dependency

of the surface charges of talc (Li et al., 2015) and limited isomorphic substitution (Lv et al., 2014).

The CEC of perlite was 41.21 cmol/kg and is within the range reported in literature (35-50 cmol/kg,) (Silber et al., 2010). Perlite possesses permanent and variable charge components, mainly consisting of mixed oxides of SiO_2 and Al_2O_3 . The negative charge derived from dissociation of hydroxyl groups is typical of oxides surfaces which act as reactive sites for exchangeable cations (Silber et al., 2010).

The CEC obtained for kaolinite was 15.15 cmol/kg and is slightly larger than the values previously reported in literature (3-15 cmol/kg) (Sharma et al., 2015; Dzene et al., 2016). The major exchangeable cations found on the surface of kaolinite are Na^+ , Ca^{2+} , and Mg^{2+} (Li et al., 2015). However, the relatively low CEC is due to the absence of interlayer adsorption sites resulting in a small number of reactive sites only on the outer surfaces of kaolinite. The relative amounts of external adsorption sites, i.e., either located on the edges or on the (upper and bottom) flat basal surfaces of the particles, are mainly controlled by the morphology or shape of the particle, as its equivalent diameter and thickness (Dzene et al., 2016). For kaolinite, these charges are positive under acidic conditions and negative under alkaline conditions (Lin et al., 2016).

FA had the lowest CEC value (9.46 cmol/kg) of all the adsorbents. Cation adsorption onto FA mainly involves electrostatic forces. Therefore, the chemical composition and physical-chemical properties of FA has many similarities to volcanic material, which is the precursor of natural zeolites. However, its structure is dominated by amorphous spherical particles (Pandey and Bhattacharya, 2016). The CEC obtained for FA is very low, suggesting that FA had little capability to hold cations.

2.3.4 SSA

The SSA refers to the total area per unit mass of a solid material which is an essential parameter to quantify interaction processes at the liquid-solid interface (Saidian et al., 2016). The SSA of the adsorbent is a fundamental property that is often used as

an indicator of adsorbent behaviour to explain the physical and chemical phenomena that occur during the adsorption process. It is strongly related to other physical and chemical properties of adsorbents such as mineral content, organic matter content, porosity, CEC, hydrodynamic and geotechnical characteristics (Heister, 2014). In addition, there is a strong correlation between SAA, adsorbent texture, CaCO_3 content, and the salinity of the soil, which refers to the absence or presence of internal pores (Bayat et al., 2015). The SAA was determined using the modified EGME/ CaCl_2 method (Tandlich and Balaz, 2011). Unlike other methods such as gas adsorption with inert gases (such as nitrogen) which measure the external specific surface area, the EGME method employs a polar liquid to measure the total surface area of the adsorbents (Saidian et al., 2016). The number of days taken by each to get saturated with EGME is presented in Fig. 8. Kaolinite exhibited the highest SAA $20.21 \text{ m}^2/\text{g}$ which is within the range $9\text{-}62 \text{ m}^2/\text{g}$ previously reported (Saidian et al., 2016). Despite having the largest SAA, the CEC of kaolinite was relatively low due to the fact that it only possesses reactive sites on its outer surface as already discussed. The results revealed no significant difference in SAA of FA, talc and vermiculite, 5.52 , 6.34 , $5.92 \text{ m}^2/\text{g}$, respectively. Perlite exhibited the least SAA of $3.10 \text{ m}^2/\text{g}$. This is probably due to the uniformity in the dimensions of the particle sizes as a result of sieving, the similarity in organic matter content as proven by the relatively similar LOI, and similar mineralogy (silicates). The SAAs of the adsorbents are generally in agreement with the what has been reported in literature (Ouchiar et al., 2015; Tekin et al., 2010).

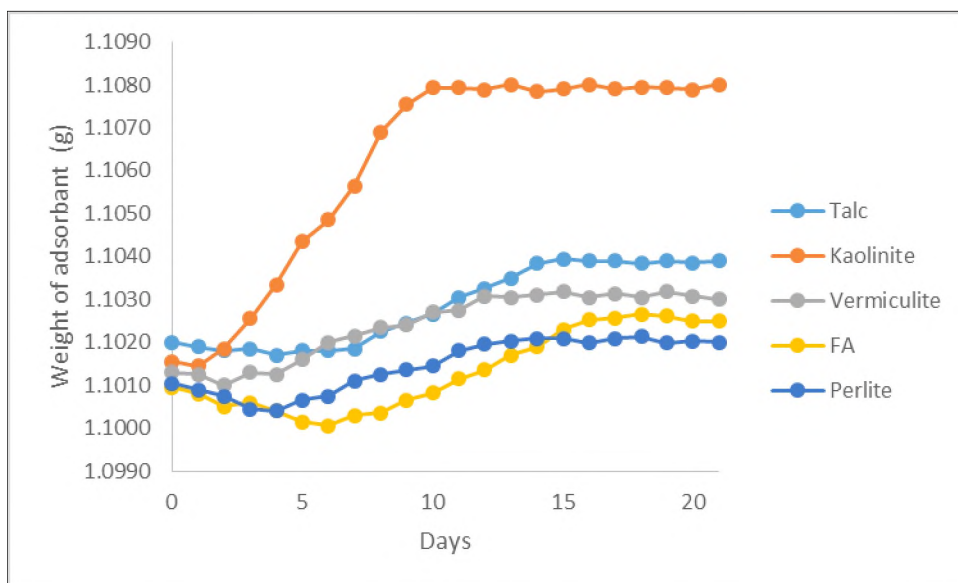


Figure 8: Number of days taken for the adsorbents to reach constant mass

2.3.5 SEM Analysis

The morphology of the adsorbents was examined by SEM analysis. The SEM images of the adsorbents are presented in Fig. 9. It can be seen that FA has spherical cenospheres of various sizes (Fig. 9a). Talc (Fig. 9b), perlite (Fig. 9c), kaolinite (Fig. 9d) and vermiculite (Fig. 9e) exhibit a well-defined flaky sheet-like structure characteristic of these minerals (Hongo et al., 2012). Perlite has high mesoporous rough structure. Talc has tabular, platy crystals occurring in aggregates. Kaolinite and vermiculite have hexagonal crystals. The results are similar to what is reported in literature (Doucet et al., 2016; Hongo et al., 2012; Sprynskyy et al., 2011; Zhang et al., 2017).

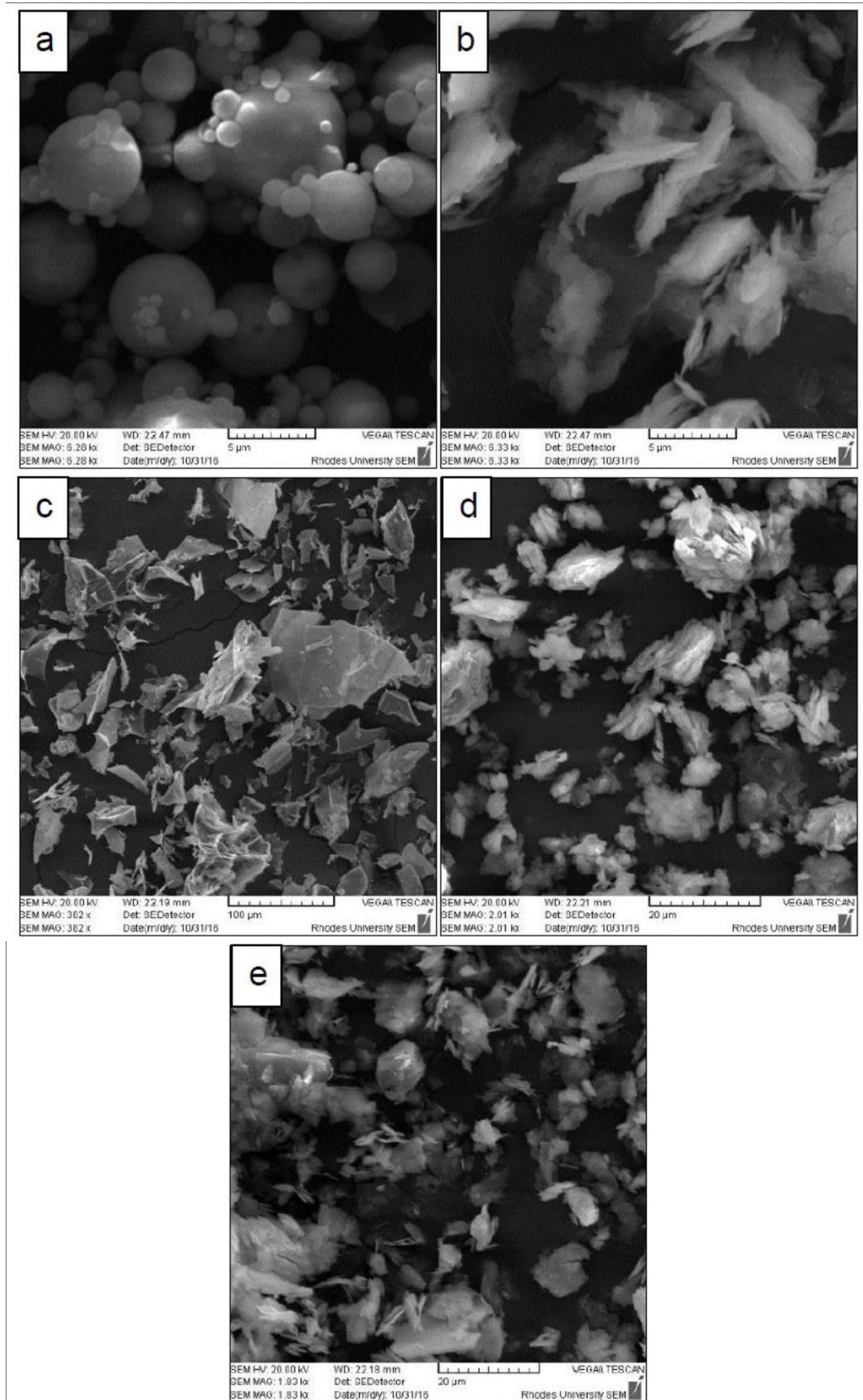


Figure 9: SEM images of (a) FA, (b) talc, (c) perlite, (d) kaolinite and (e) vermiculite

2.3.6 XRD Analysis

The adsorbents used originate from natural sources and their properties are influenced by the regional conditions of the source location. X-ray diffraction patterns of crystalline structures are characterised by many sharp peaks while those of the non-crystalline solids display diffuse humps. A hump indicates short-range order due to the irregular and non-repetitive arrangement of the atoms. Therefore, a hump in the XRD pattern indicates the amorphous nature of the material (Rohilla et al., 2011). The XRD patterns of the adsorbents are presented in Fig. 10.

The FA XRD patterns (Fig. 10a) show that the amorphous phase of aluminosilicate appear as a broad peak centred approximately at 22° (2θ scale) (An et al., 2016). It can be seen (Fig. 10a) that this hump is not present in the FA sample that was mixed with the ciprofloxacin solution and reduced markedly in intensity for the FA sample mixed with isoniazid solution. It is also clear that the main peak at 26.90° gained more than 10 times the intensity for the FA sample that was mixed with ciprofloxacin solution. These results suggest that the crystalline surface of fly ash changed after adsorption of ciprofloxacin and isoniazid.

The XRD patterns of kaolinite (Fig. 10b) show that a well-defined characteristic reflection of the crystalline nature of kaolinite is dominant in all samples (Zhang et al., 2017). The intense reflections at 26.67° (2θ scale) correspond to quartz indicating the presence of quartz in the kaolinite (Mohiuddin et al., 2017). There were no significant differences in mineral assemblages of raw kaolinite samples and those that were mixed with antibiotic solutions. We, therefore, concluded that the adsorption of ciprofloxacin and isoniazid onto kaolinite was merely a physical process on the external surface of the adsorbent. The sorption of antibiotics did not affect the crystal lattice of kaolinite.

The perlite XRD patterns (Fig. 10c) show that it is mostly crystalline and exhibits a characteristic broad band between 20° and 30° (2θ scale) (Thanh et al., 2011). The XRD spectrum did not indicate a significant difference in terms of crystalline

structures after mixing with the antibiotic solutions. This led to the conclusion that the crystalline texture of perlite was not affected by the adsorption of ciprofloxacin or isoniazid.

The XRD patterns of talc (Fig. 10d) also reveal the presence of amorphous material in talc and the presence of intensive peaks of quartz, characterising the presence of SiO₂ (Sprynskyy et al., 2011). Despite the increase and decrease in reflection without shifting of the reflection at 9.70° (2θ scale) after mixing with ciprofloxacin and isoniazid solutions, respectively. No differences can be observed in the diffraction patterns suggesting no change in the crystalline structure.

The vermiculite XRD patterns (Fig. 10e) show a hump with a centre around 20° (2θ scale) characteristic of the existence of amorphous material in the crystalline structure of vermiculite (Hongo et al., 2012). This hump narrowed and disappeared after shaking with ciprofloxacin and isoniazid solutions, respectively. The highly intense reflection at 46.36° also disappeared after shaking with the antibiotic solutions. This reveals a change in the crystalline structure of the vermiculite possibly as a result of intercalation of ciprofloxacin and isoniazid between the inner layers of the adsorbent (Spence and Kelleher, 2012).

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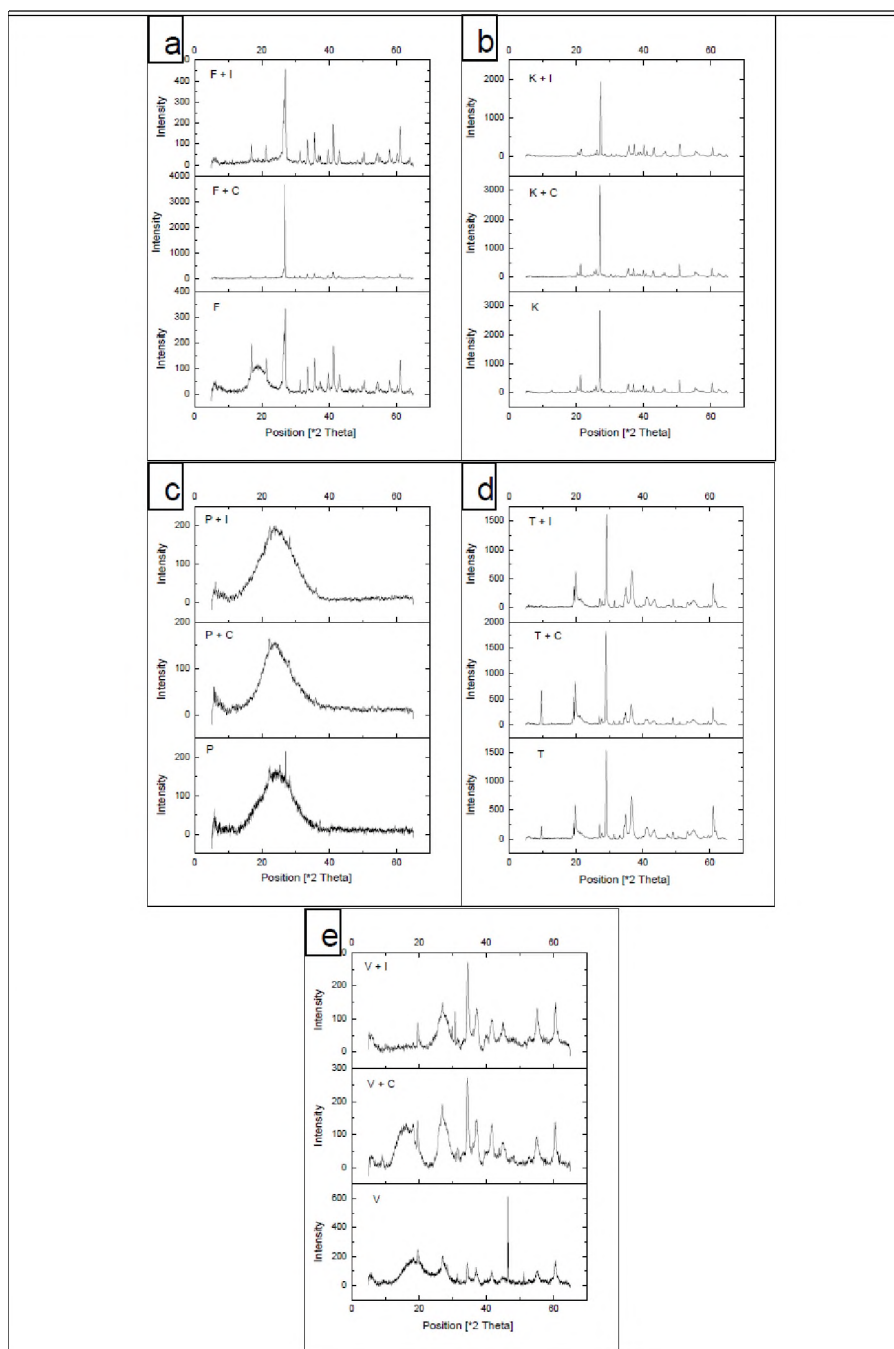


Figure 10: XRD pattens of adsorbents. (a) F is the XRD pattern of FA, F + C is XRD pattern of FA after mixing with ciprofloxacin solution, F + I is the XRD pattern of FA after mixing with isoniazid solution. (b) K is the XRD pattern of kaolinite, K + C is the XRD pattern of kaolinite after mixing with ciprofloxacin solution, K + I is the XRD pattern of kaolinite after mixing with isoniazid solution. (c) P is the XRD pattern of perlite, P + C is the XRD pattern of perlite after mixing with ciprofloxacin solution, P + I is the XRD pattern of perlite after mixing with isoniazid solution (d) T is the XRD pattern of talc, T + C is the XRD pattern of talc after mixing with ciprofloxacin solution, T + I is the XRD pattern of talc after mixing with isoniazid solution (e) V is the XRD pattern of vermiculite, V + C is the XRD pattern of vermiculite after mixing with ciprofloxacin solution, V + I is the XRD pattern of vermiculite after mixing with isoniazid solution

2.3.7 FTIR Analysis

FTIR spectral analysis can be used to identify specific functional groups present within the Si-containing or crystalline phases of the adsorbents. This assists in understanding the adsorption mechanisms and interactions between the antibiotics and the adsorbents. The IR spectra of the adsorbents are presented in Fig. 11. FA has a heterogeneous structure consisting of a glassy surface layer (SiO_2) and crystalline components such as quartz, mullite and magnetite (Böke et al., 2015). Hence, its FTIR (Fig. 11a) exhibits a broad band in the range $1200 - 900 \text{ cm}^{-1}$ with a centre around 1090 cm^{-1} which is attributed to the asymmetric stretching vibration peaks of Si-O-Si or Si-O-Al bonds of SiO_2 or AlO_4 tetrahedra which overlap in this region (Böke et al., 2015; Onutai et al., 2016). The shoulder visible between 1074 and 1069 cm^{-1} corresponds to the Si-O-Si stretching vibration. After shaking with the ciprofloxacin and isoniazid solutions, the peak of the centre of the broad band corresponding crystalline structure of FA shifted to lower wavenumber, around 1080 and 1060 cm^{-1} , respectively. This indicates a possible interaction of the oxygen with the antibiotics resulting in a greater number of non-bridging oxygen atoms (Shearer et al., 2016). The bands at 3340 and 1635 cm^{-1} are respectively assigned to the O-H stretching and bending of absorbed water indicating the presence of the antibiotic molecules on the surface of the adsorbent (Timakul et al., 2016; ul Haq et al., 2014).

The IR spectra of kaolinite (Fig. 11b) reveal that raw kaolinite exhibits bands in the range $3694 - 3620 \text{ cm}^{-1}$ corresponding to stretching vibrations of OH groups coordinated to Al^{3+} ions (Zhu et al., 2016). The peaks at 3694 , 3672 and 3620 cm^{-1} exhibited on FTIR spectra of kaolin are assigned to the in-phase stretching vibrations of inner surface OH groups. The peak at 3654 cm^{-1} is attributed to the out of phase stretching of inner O-H groups (Zhu et al., 2016). All three peaks are also present in the spectra of kaolin after shaking with both antibiotic solutions indicating that no intercalation took place, instead, hydrogen bonding or ligand exchange might have occurred on the external surface of kaolinite (Spence and Kelleher, 2012). Intercalation would have resulted in the reduction of electrostatic attractive forces between the antibiotics and the inner surface OH groups of the layers thus increasing the distance between the layers (Castrillo et al., 2015). The bands around 3400 and

1650 cm^{-1} correspond to stretching and bending vibrations of water molecules associated with the mineral surface, respectively. The peaks at 933 and 908 cm^{-1} , which are attributed to Al-OH deforming vibration and Al-OH bending vibration, move to higher wavenumber and an increase in intensity (Zhu et al., 2016). The presence of quartz in the kaolinite used is revealed by the low-intensity doublet with bands at 798 and 780 cm^{-1} (Król et al., 2016).

For perlite (Fig. 11c), the band at 1014 cm^{-1} of the perlite FTIR spectra represents Si-O-Si asymmetric stretching vibration (Sun and Wang, 2015) which shifted to a lower wavenumber after shaking with the antibiotic solutions. This suggests that coordination occurred, and based on the abundance of hydroxyl groups that exist on the inner surface of perlite, possibly hydrogen bonding and electrostatic attraction occurred with the functional groups on the antibiotics. It is also noteworthy that the bands at 778 and 3342 cm^{-1} are attributed to stretching of O-H and Si-OH, respectively (Sun and Wang, 2015), shifted to higher wavenumber suggesting a reduction in bond strength possibly resulting from weaker electrostatic attractions between the perlite and both antibiotics. The peak at 1045 cm^{-1} is the characteristic absorption peak of the telescopic vibration of Si-O-Si, and the shift to lower wavenumber after shaking with the antibiotic solutions is attributed to hydrogen bonding (Wei et al., 2014).

For talc (Fig. 11d), the bands ranging from 3700 - 3400 cm^{-1} are attributed to the stretching vibrations of O-H associated with Si (Si-OH) and Mg (Mg-OH) (Sprynskyy et al., 2011). Characteristically, the band at 3678 cm^{-1} is associated with O-H in the 3 Mg region ($\text{Mg}_3\text{-OH}$) of talc (Ngally Sabouang et al., 2015). This band showed a significant decrease in intensity after shaking with isoniazid solutions and increase in intensity after shaking ciprofloxacin solutions, respectively. This strongly suggests a possible interaction of the Mg-OH with the antibiotics. The same explanation can be given for shifts of wavenumbers of the band at 664 which is assigned to the O-H stretching vibrations associated with Mg-OH (Ngally Sabouang et al., 2015). A significant shift to lower wavenumber and a marked increase in the intensity of the band at 3400 cm^{-1} corresponding to Si-OH groups was observed after shaking with isoniazid solution. This is an expected binding site for isoniazid as the antibiotic is

most likely to bind to surface hydroxyls by hydrogen interaction through ring nitrogen lone pairs directly or indirectly through water bridges (Akyuz et al., 2010).

The IR spectra of vermiculite (Fig. 11e) show bands at 3414 and 999 cm^{-1} which represent the stretching vibrations of Si-OH groups and Si-O-Si groups of the silicate framework of vermiculite, respectively (Tran et al., 2015). The peak 1637 cm^{-1} is attributed to O-H bending (Oliveira et al., 2015). The band at 999 cm^{-1} increases in wavenumber and intensity after shaking with the antibiotic solutions which indicates possible electrostatic interaction. The peaks at 667 and 729 cm^{-1} are attributed to the stretching vibrations of X-O-Si, where X = Mg, Al or Fe and Si-O & Al-O, respectively (Hongo et al., 2012). They also shifted to higher wavenumber after shaking with the antibiotics solutions due to the interaction with the antibiotics. The adsorption at 1645 cm^{-1} is primarily due to water directly coordinated to the exchangeable cations of the clay (Tran et al., 2015). The significant shift of this band to lower wavenumber after shaking with ciprofloxacin suggests that cation exchange was one of the mechanisms of adsorption in the removal of ciprofloxacin by vermiculite.

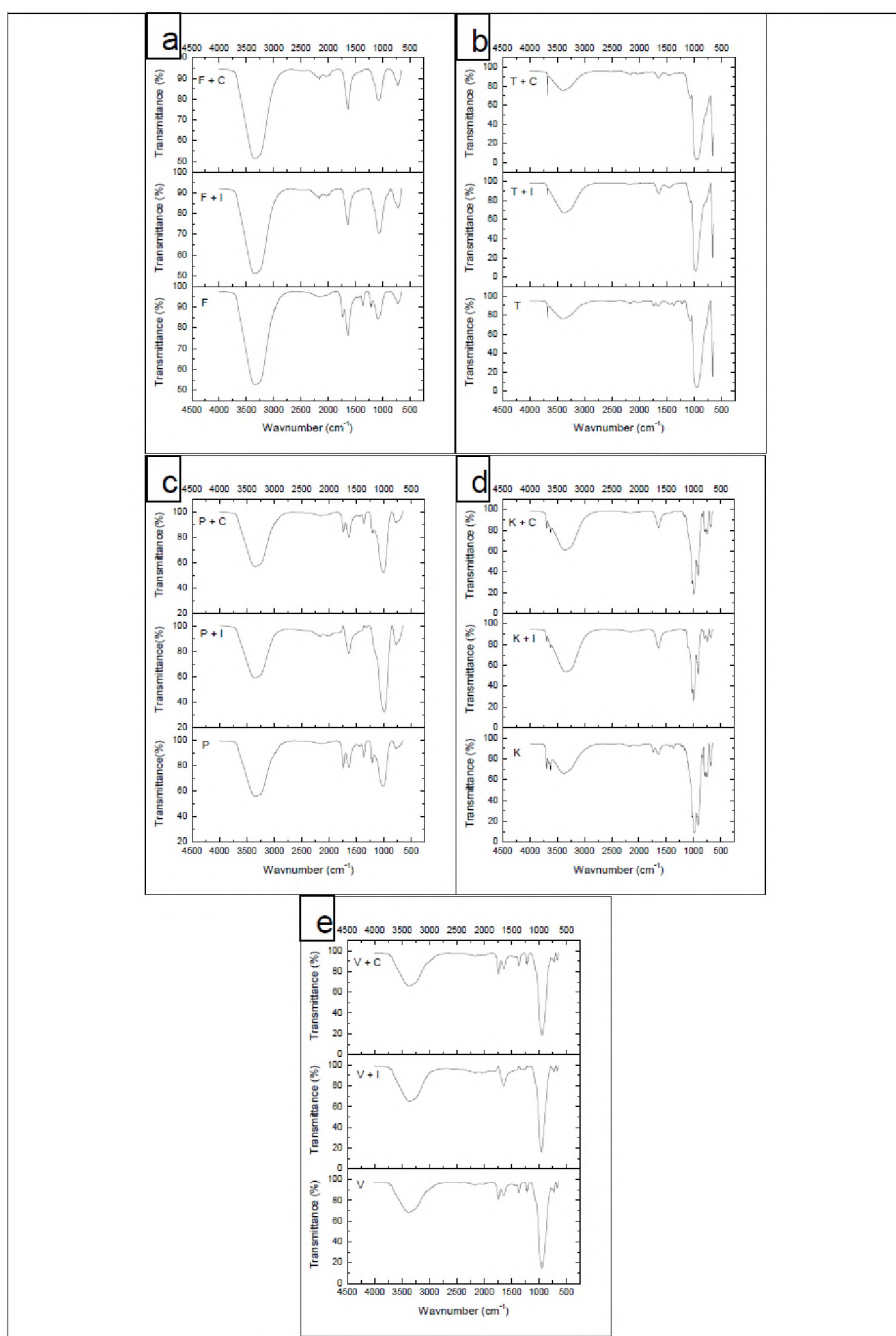


Figure 11: FTIR spectra of adsorbents. (a) *F* is the spectrum of FA, *F + C* is a spectrum of FA after mixing with ciprofloxacin solution, *F + I* is the spectrum of FA after mixing with isoniazid solution. (b) *T* is the spectrum of talc, *T + C* is the spectrum of talc after mixing with ciprofloxacin solution, *T + I* is the spectrum of talc after mixing with isoniazid solution. (c) *P* is the spectrum of perlite, *P + C* is the spectrum of perlite after mixing with ciprofloxacin solution, *P + I* is the spectrum of perlite after mixing with isoniazid solution (d) *K* is the spectrum of kaolinite, *K + C* is the spectrum of kaolinite after mixing with ciprofloxacin solution, *K + I* is the spectrum of kaolinite after mixing with isoniazid solution (e) *V* is the spectrum of vermiculite, *V + C* is the spectrum of vermiculite after mixing with ciprofloxacin solution, *V + I* is the spectrum of vermiculite after mixing with isoniazid solution solution

2.3.8 Adsorption kinetics

Contact time is a very important parameter that influences the effectiveness of the adsorption process. The concentration versus time plots for adsorption of ciprofloxacin and isoniazid onto the adsorbents are shown in Fig. 12. The adsorption is almost instantaneous and increased sharply initially, after which it slows as sorption equilibrium is reached after 30 min. This is because initially, the sorbent sites are vacant resulting in a higher frequency of interactions between the sites and antibiotic molecules. As time lapses the sorption sites continue getting occupied and gradually most sites become saturated. The few if any remaining vacant sites become more difficult to occupy due to repulsive forces between the antibiotic molecules on the surface of the adsorbent and those in the bulk solution (Singh and Choden, 2014). The highest rate and extent of adsorption of both ciprofloxacin and isoniazid are exhibited by kaolinite. Whereas, vermiculite and FA show both the lowest rate and extent of adsorption of isoniazid and ciprofloxacin, respectively. The rate of adsorption gives valuable information that is required to design batch adsorption systems. The data obtained from adsorption kinetics studies of solute uptake are used to optimise operating conditions for the full-scale batch process. Adsorption equilibrium for isoniazid and ciprofloxacin was achieved in 30 min by all adsorbents and was kept at 40 min for the rest of the experiments.

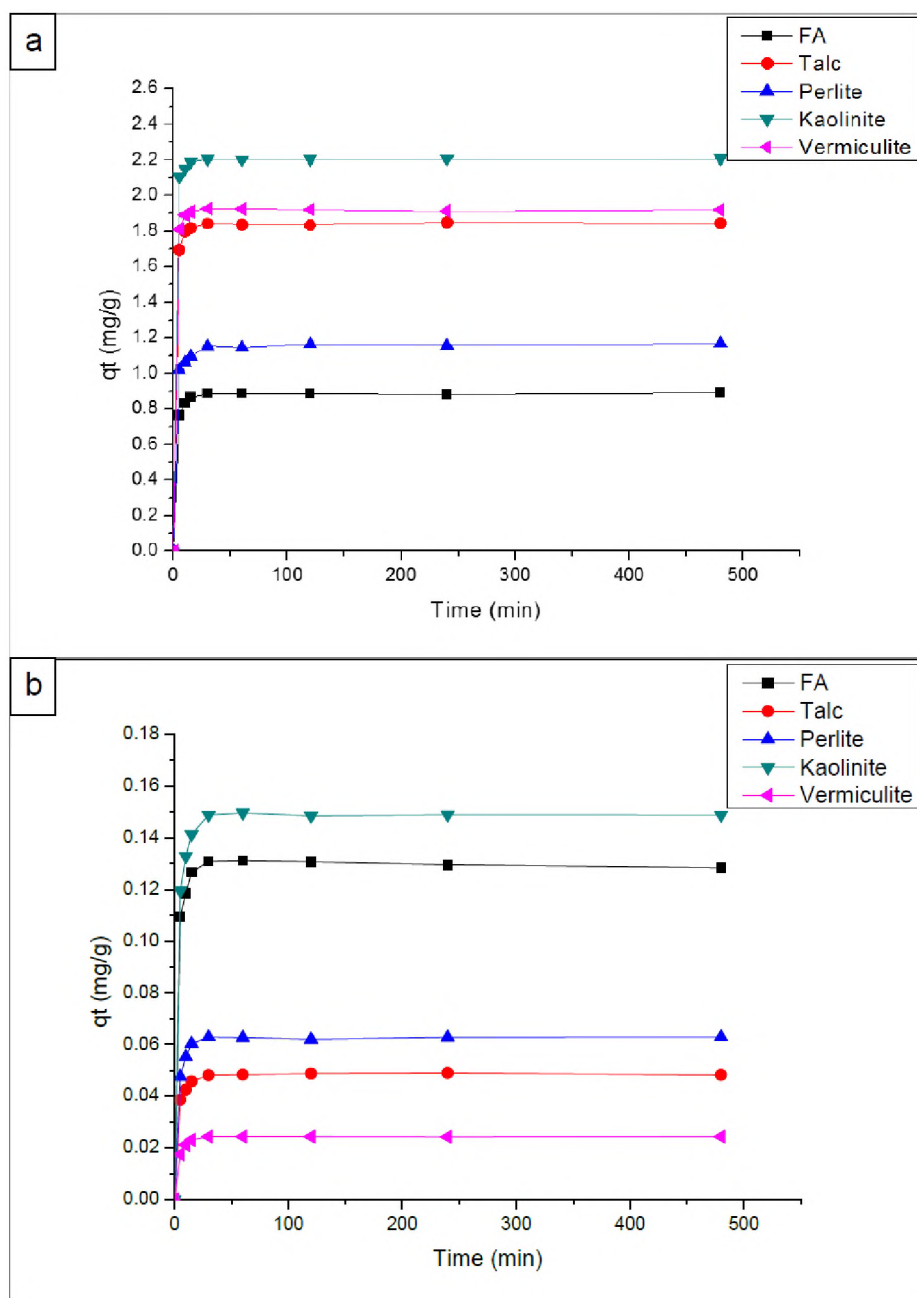


Figure 12: Sorption kinetics plots for adsorption of (a) ciprofloxacin and (b) isoniazid onto FA, kaolinite, perlite, talc and vermiculite. Amount adsorbed as a function of time presented as the mean ($n = 3$, $T = 25 \pm 1$ °C)

The non-equilibrium stages of adsorption were used to make a kinetic evaluation of the adsorption behaviours of ciprofloxacin and isoniazid by the adsorbents. The adsorption rate constants were estimated using the linearised forms of the following kinetic models based on the R^2 values and amount of antibiotic adsorbed per unit weight of adsorbent;

The pseudo first-order rate model expressed as:

$$\log(q_e - q_t) = \log q_e - \left(\frac{k_1}{2.303}\right) t \quad (20)$$

The second-order rate model expressed as:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left(\frac{1}{q_e}\right) t \quad (21)$$

The intra-particle diffusion model expressed as:

$$q_t = k_{id} t^{0.5} + C \quad (22)$$

Where q_e and q_t (mg/g) are the amounts of antibiotic adsorbed per unit mass of adsorbent at equilibrium and time t (min), respectively, k_1 (min^{-1}) is the rate constant of the pseudo-first-order model, k_2 (mg/g.min) is the rate constant of the pseudo-second-order model, k_{id} (mg/g.min^{0.5}) is the rate constant of the intra-particle diffusion model. Values of k_1 and q_e can be calculated from the slope and intercept of the linear plot of $\log(q_e - q_t)$ versus t , respectively. Values of k_2 and q_e can be calculated from the intercept and slope of the linear plot of t/q_t versus t , respectively (Nithya et al., 2016). The value of k_{id} can be calculated from the slope the linear plot q_t versus $t^{0.5}$ and C (mg/g) is the intercept (Nithya et al., 2016).

Fig. 13 shows the pseudo-first order, the pseudo-second order and intra-particle diffusion model plots for ciprofloxacin and isoniazid adsorption onto the forming adsorbents. Parameters and regression coefficients for the treatment of ciprofloxacin and isoniazid data according to each kinetic equation are presented in Table 7 and Table 8, respectively. Based on the R^2 values, the pseudo-second-order model gives the better fit for the adsorption of both ciprofloxacin onto all the adsorbents, with R^2 values ranging from 0.998 - 0.999, compared to the pseudo-first and intra-particle diffusion model. The data of equilibrium adsorbed amount calculated from the pseudo-second-order kinetics ($q_{e,c}$) are also close to the amounts adsorbed at equilibrium in experiments. This suggests that the sorption of

ciprofloxacin and isoniazid obey pseudo-second order kinetics. The pseudo-second-order gives a more accurate fit of the experimental data with giving almost perfect R^2 values because in the plots when the majority of the data is at equilibrium the data points are well aligned because the value of $t/q_t \approx t/q_e$ when $q_t \approx q_e$ (Simonin, 2016). It is also important to note that pseudo-first order and pseudo-second-order rate models do not give the adsorption mechanism. They only show the rate of adsorption with respect to shaking time with the adsorbents (Gupta et al., 2012). Therefore, the rationale behind fitting the kinetic data to the intra-particle diffusion model was to investigate the mechanisms of action responsible for adsorption.

The intra-particle diffusion model describes the process which follows after the solute molecules are transferred from the bulk aqueous phase to the surface of the adsorbent and involves diffusion into the interior pores. If the regression line of the plot q_t versus $t^{0.5}$ passes through the origin, then the rate of adsorption was controlled solely by intra-particle diffusion. Conversely, if the regression line does not pass through the origin as observed for the plots of q_t versus $t^{0.5}$ in Fig. 13, then intra-particle diffusion is not the rate limiting step governing the adsorption processes and other mechanisms are also controlling the adsorption process (Xie et al., 2016). This is further confirmed by the small values of C , in Table 7 and Table 8 indicating a small boundary layer effect (Ahmad and Haseeb, 2012). We, therefore, can conclude that the adsorption process is controlled by external mass transfer and intraparticle diffusion occurring simultaneously.

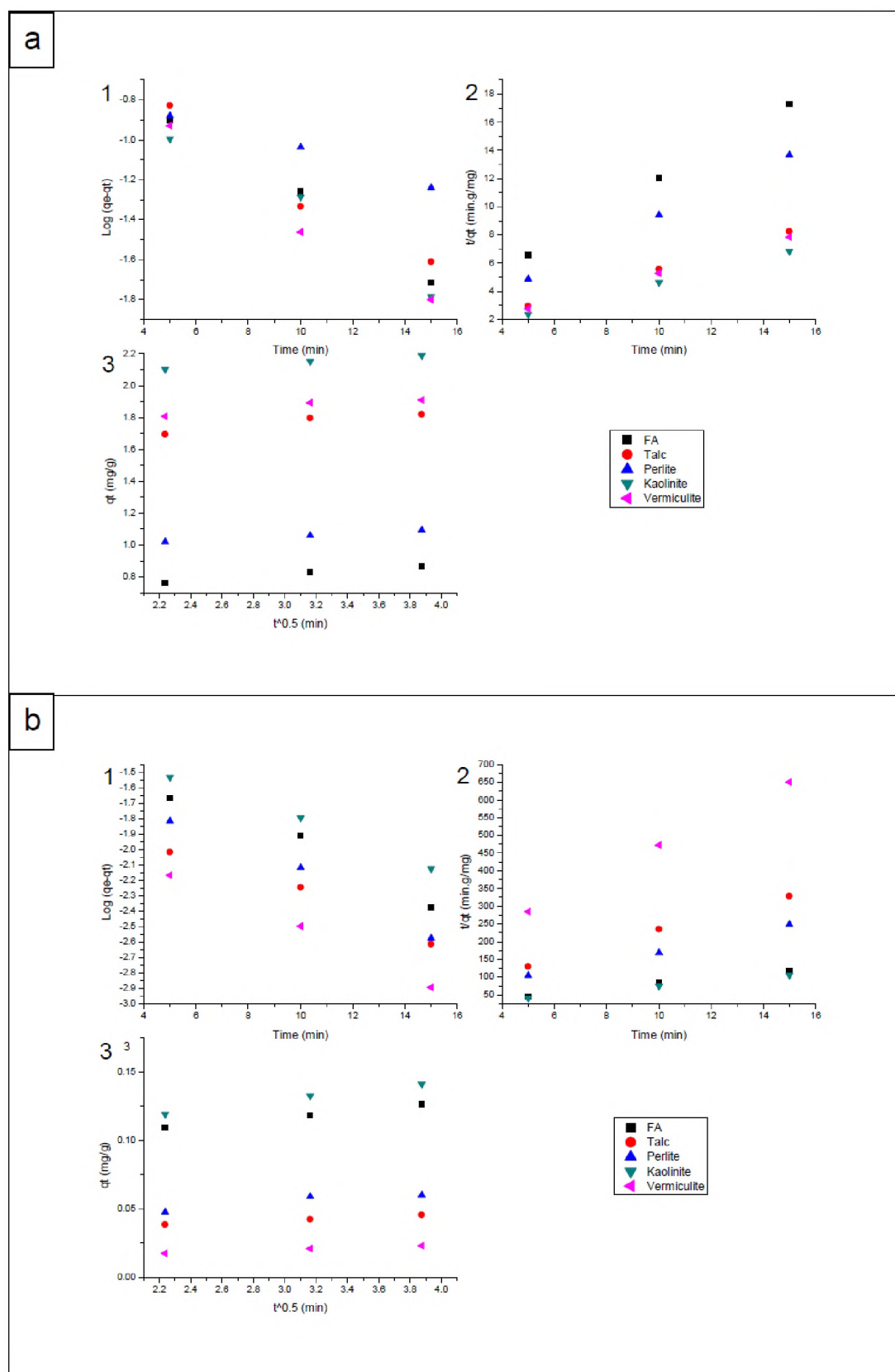


Figure 13: Linear regression kinetic plots (1) pseudo-first-order model, (2) pseudo-second-order model and (3) intraparticle diffusion model of (a) ciprofloxacin and (b) isoniazid on FA, kaolinite, perlite, talc and vermiculite ($n = 3$, $T = 25 \pm 1$ °C)

Table 7: Kinetic parameters for adsorption of ciprofloxacin onto FA, kaolinite, perlite, talc and vermiculite

Adsorbent	Pseudo-first order			Pseudo-second order			Intraparticle diffusion		
	K_1 (min ⁻¹)	$q_{e,c}$ (mg/g)	R^2	K_2 (mg/g. min)	$q_{e,c}$ (mg/g)	R^2	K_{id} (mg/g. min ^{0.5})	C (mg/g)	R^2
FA	0.1870	0.3309	0.9945	0.9343	0.9319	0.9999	0.0650	0.6190	0.9889
Talc	0.1803	0.3351	0.9719	0.9649	1.8875	0.9999	0.0776	1.5300	0.9222
Perlite	0.0829	0.2037	0.9943	1.4248	1.1375	0.9997	0.0454	0.9194	0.9989
Kaolinite	0.1819	0.2735	0.9776	1.3500	2.2341	0.9999	0.0521	1.9875	0.9996
Vermiculite	0.2006	0.2979	0.9842	1.2110	1.9662	0.9999	0.0635	1.6744	0.9270

Table 8: Kinetic parameters for adsorption of isoniazid onto FA, kaolinite, perlite, talc and vermiculite

Adsorbent	Pseudo-first order			Pseudo-second order			Intraparticle diffusion		
	K_1 (min ⁻¹)	$q_{e,c}$ (mg/g)	R^2	K_2 (mg/g. min)	$q_{e,c}$ (mg/g)	R^2	K_{id} (mg/g. min ^{0.5})	C (mg/g)	R^2
FA	0.1630	0.0527	0.9677	5.2455	0.1374	0.9987	0.0105	0.0857	0.9984
Talc	0.1370	0.0201	0.9830	12.052	0.0503	0.9986	0.0044	0.0287	0.9997
Perlite	0.1745	0.0387	0.9859	6.0785	0.0693	0.999	0.0077	0.0287	0.998
Kaolinite	0.1361	0.0594	0.9960	4.0844	0.1555	0.9995	0.0134	0.0899	0.9973
Vermiculite	0.1671	0.0161	0.9974	12.782	0.0273	0.9998	0.0034	0.0100	0.9894

2.3.9 Influence of pH

The adsorption mechanism is generally dictated by the physical and chemical interactions which occur between the adsorbate and adsorbent (Zhao et al., 2016). A number of mechanisms can be responsible for the adsorption of molecules from aqueous environments generally take place through (1) ion-exchange which involves the substitution of counter ions adsorbed on the surface of the adsorbent by ion with the same charge in solution, (2) ion-pairing which involves the adsorption of ions from solution onto oppositely charged sites unoccupied by counter ions (counter ion

exchange) and hydrophobic interactions which occur when the combination of mutual attraction between groups of the molecules and their tendency to escape from an aqueous environment becomes large enough to allow them to adsorb onto the solid adsorbent by aggregating their chains (Alkan et al., 2005).

The presence of highly charged groups on the surfaces of all the adsorbents makes them very sensitive to pH changes and so this results in a significant influence on the degree of interaction between the antibiotics and adsorbents. The influence of pH on the removal of ciprofloxacin and isoniazid from aqueous solutions by FA, kaolinite, perlite, talc and vermiculite is depicted in Fig. 14. There is a strong dependence on pH exhibited by the adsorption of ciprofloxacin and isoniazid by all adsorbents except FA. In general, the net surface charge of mineral oxides such as silica and alumina, tend to become more positive under acidic conditions i.e. when pH is lowered resulting in the adsorption of anionic species and a decrease in the adsorption of cationic species (Alkan et al., 2005). However, looking at Fig. 14., most ciprofloxacin was adsorbed within the pH range 3-5 by all adsorbents except FA. In this pH range, $\text{pH} < 5.90$, ciprofloxacin predominantly exists as a cationic species as a result of the basic nitrogen in the piperazine ring being protonated to form a positively charged ciprofloxacin ion ($\text{pK}_a 1$). Under basic conditions, $\text{pH} > 8.89$, the carboxylic acid functional group is deprotonated to form an anionic species ($\text{pK}_a 2$). At neutral pH, $5.90 \geq \text{pH} \geq 8.89$, ciprofloxacin occurs as a zwitterion (Chang et al., 2016; Nageswara Rao et al., 2008). This, therefore, suggests that cation exchange is the dominant mechanism of adsorption of ciprofloxacin by kaolinite, talc, perlite and vermiculite at lower pH values. There is a significant drop in the adsorption of ciprofloxacin as pH approaches $\text{pK}_a 1$ indicating that the cation is no longer the dominant species. The general minimum variation in adsorption of ciprofloxacin that is observed at $\text{pH} \geq \text{pK}_a 1$ suggests that the positive charge on the ciprofloxacin cation still contributes to adsorption via cation exchange mechanism and the rapid decline when pH was approaching $\text{pK}_a 2$ indicates that cation exchange is no longer the dominant mechanism of adsorption (Wu et al., 2010). Despite the decrease, the continued adsorption of ciprofloxacin at $\text{pH} \geq \text{pK}_a 2$ indicates that another mechanism is responsible for adsorption, possibly hydrogen bonding and/or hydrophobic interactions due to $\pi - \pi$ stacking. It is also highly likely that ciprofloxacin and

isoniazid can interact with the Si-O⁻ and Al-O⁻ functional groups on the surfaces of the adsorbents via complex formation and/or ion exchange (Wu et al., 2010). It is, therefore, important to note that although one mechanism dominates at certain pH, other mechanisms may also be involved adsorption process.

The nitrogen and oxygen groups present in the structure of isoniazid enable it to form complexes with adsorbents as a ligand. The amino group hydrogens and carbonyl group oxygen of isoniazid are capable of forming hydrogen bonds with the adsorbents (Akyuz and Akyuz, 2008). Isoniazid is also amphoteric and has multiple ionizable groups. It speciates as a function of pH and has two pK_a values, pK_a 1 = 3.53 and is a result of the protonation of the heterocyclic nitrogen atom under acidic conditions. pK_a 2 = 11.40 and is ascribed to the proton loss from the amide nitrogen atom aggravated by basic conditions. At pH < 5, isoniazid exists as three species in equilibrium: one neutral and two positively charged. In the pH range 5-9, isoniazid has an uncharged molecular form. Deprotonation occurs gradually with increasing pH above 9 resulting in a negatively charged species (Blokhina et al., 2015). As seen in Fig. 14. the high adsorption of isoniazid by kaolinite and perlite at pH 3 suggests that cation exchange is the main mechanism of adsorption and this is further confirmed by the drastic decline in adsorption when pH ≥ pK_a 1 of isoniazid since the cationic species is no longer dominating. The invariance in adsorption of isoniazid exhibited by all the adsorbents in the pH range 5-10 suggests that mechanism of adsorption is consistent throughout the range and since isoniazid exists predominantly as a neutral species in this range adsorption is most likely attributed to hydrogen bonding and hydrophobic interactions between the isoniazid and adsorbents. The decrease in adsorption observed at pH ≥ 10 is most likely due to electrostatic repulsion between the negatively charged isoniazid species and the highly negative surface charge of the adsorbent. Interestingly, the adsorption capacities were relatively constant throughout the entire pH range when FA was used as an adsorbent for both ciprofloxacin and isoniazid, indicating that pH had no significant influence on adsorption. This shows that FA has excellent environmental adaptability. Therefore, in contrast to the other adsorbents, hydrophobic interactions are probably more important than electrostatic forces in the adsorption of ciprofloxacin and isoniazid by FA. The pyridine ring of isoniazid has a high electron density which makes it

capable of forming hydrophobic bonds especially when the cyclic nitrogen forms a hydrogen bond the rest of the ring becomes more electronegative. The aromatic ring found in ciprofloxacin is also rich in delocalised electrons.

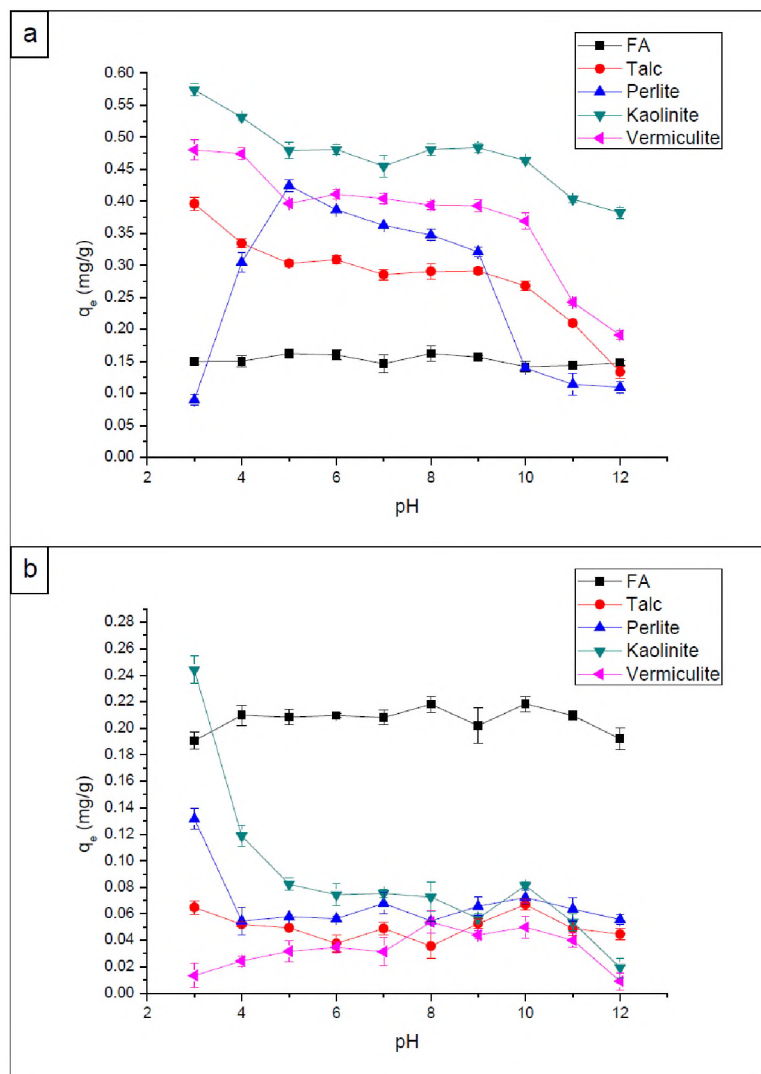


Figure 14: Effect of pH on the adsorption of (a) ciprofloxacin and (b) isoniazid onto FA, kaolinite, perlite, talc and vermiculite. Amount adsorbed at equilibrium (q_e) presented as the mean $\pm$ standard deviation ($n = 3$, $T = 25 \pm 1$ °C)

2.3.10 Effect of initial antibiotic concentration

The effect of the initial antibiotic concentration on the removal of ciprofloxacin and isoniazid by the adsorbents is presented in Fig. 15. The amount of both ciprofloxacin and isoniazid adsorbed per unit mass of adsorbent increases with the increase in

initial antibiotic concentration, which is most probably due to increased likelihood of contact between the solute and adsorbent particles resulting in a greater driving force to transfer the antibiotics from the bulk to the solid phase. The higher solute concentration also results in a steeper concentration gradient and hence greater removal (Singh et al., 2016). However, it is evident that the percentage of antibiotic removed decreases with the increase in initial antibiotic concentration. This is because at lower antibiotic concentrations, the ratio of the initial number of antibiotic molecules/ions to the available surface area of adsorbent is larger, and vice versa (Nithya et al., 2016). Kaolinite removed ciprofloxacin to concentrations below the detection limit for ciprofloxacin solutions which had an initial concentration less than 50 mg/L. The effect of initial antibiotic concentration on the removal of ciprofloxacin by kaolinite is presented in Fig. 16. The removal percentages of the ciprofloxacin after shaking for 30 min increased in the order FA < perlite < talc < vermiculite < kaolinite while those of isoniazid increased in the order vermiculite < talc < perlite < FA < kaolinite. The order remained the same despite the change in initial antibiotic concentrations.

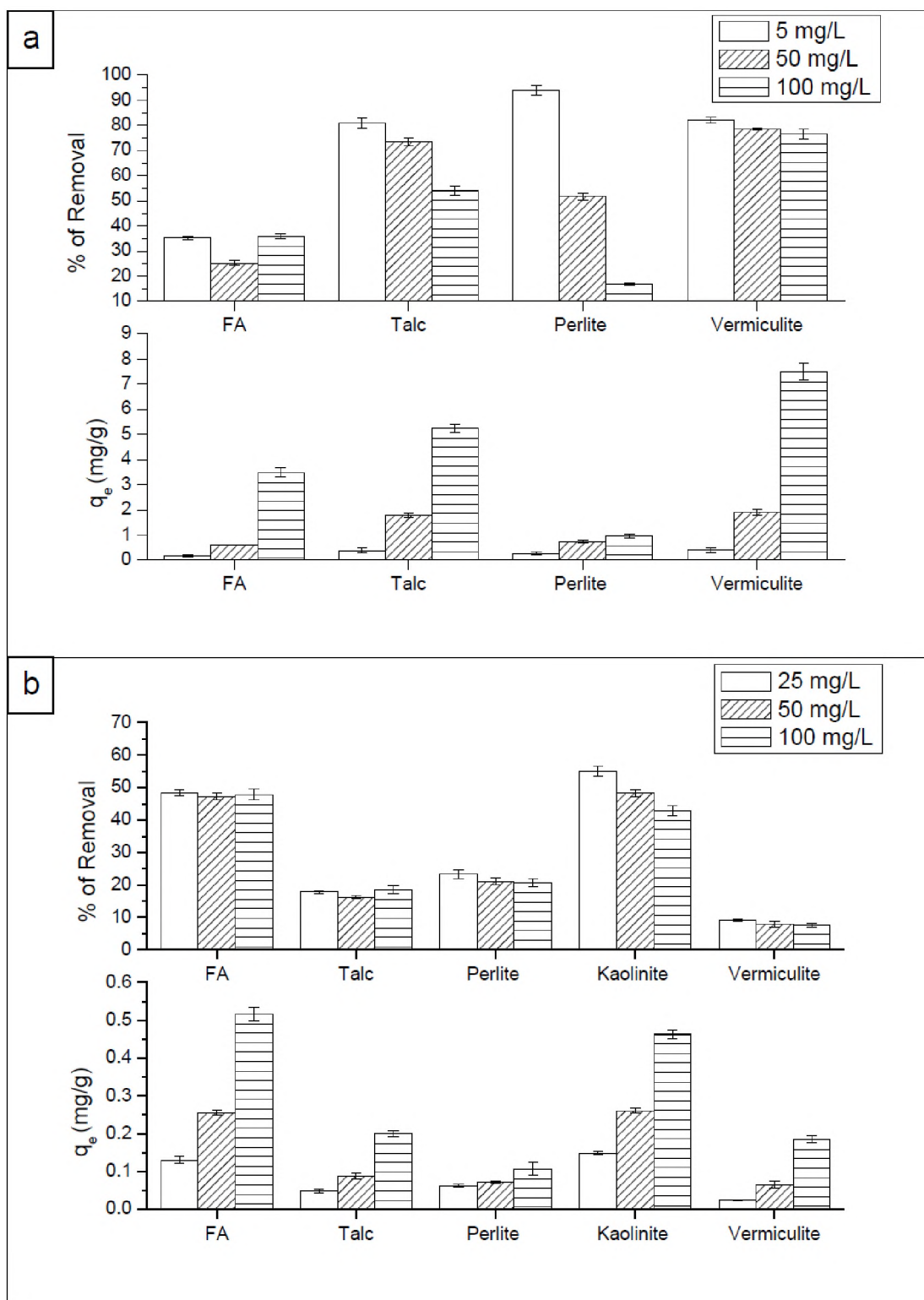


Figure 15: Effect of initial antibiotic concentration on the adsorption of (a) ciprofloxacin onto FA, perlite, talc, vermiculite and (b) isoniazid onto FA, kaolinite, perlite, talc and vermiculite. % removal and amount adsorbed at equilibrium (q_e) presented as the mean $\pm$ standard deviation ($n = 3$, $T = 25 \pm 1$ °C)

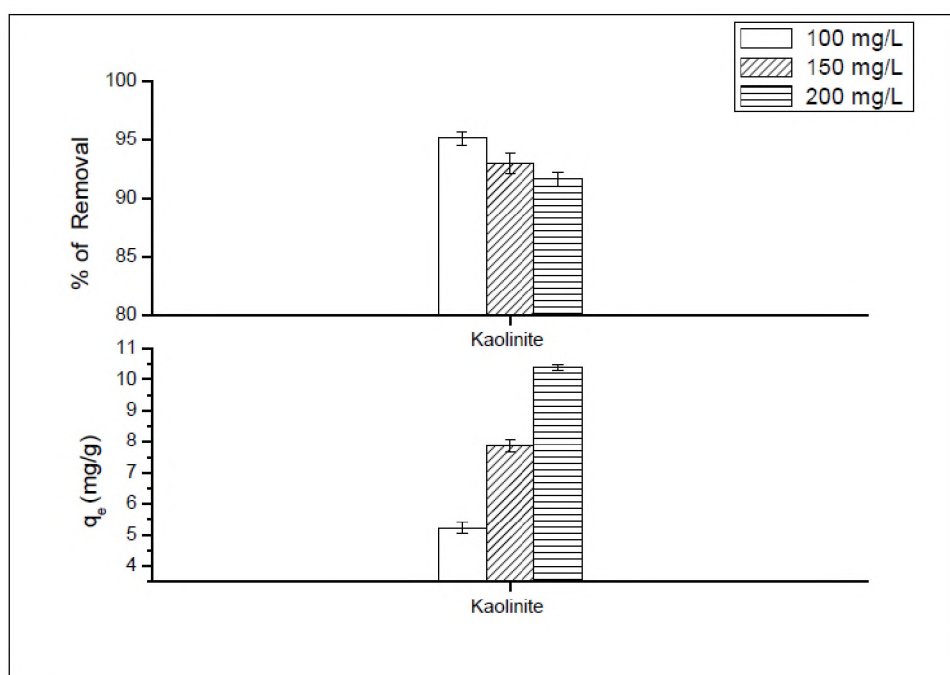


Figure 16: Effect of initial antibiotic concentration on the adsorption of ciprofloxacin onto kaolinite. % removal and amount adsorbed at equilibrium (q_e) presented as the mean $\pm$ standard deviation ($n = 3$, $T = 25 \pm 1$ °C)

2.3.11 Effect solid/liquid ratio

The adsorption of ciprofloxacin and isoniazid was studied by varying the solid/liquid ratio by adjusting the adsorbent dosage from 0.10 to 0.25 g and 0.5 to 1.5 g in 10 mL of antibiotic solution, respectively, while keeping other parameters constant. The effect of solid/liquid ratio on the removal of ciprofloxacin and isoniazid is presented in Fig. 17. It is clear that an increase in adsorbent dosage results in a greater percentage removal due to the increase in the surface areas available for adsorption. However, it is more interesting to note that the amount of antibiotic loaded per unit adsorbent at equilibrium tends to decrease with the increase in adsorbent dosage. This is because at higher doses aggregation of the adsorbent particles may occur resulting in a reduction of freely accessible sorption sites for the solute (Nithya et al., 2016). Hence, the adsorption capacity of adsorbent is not fully utilised.

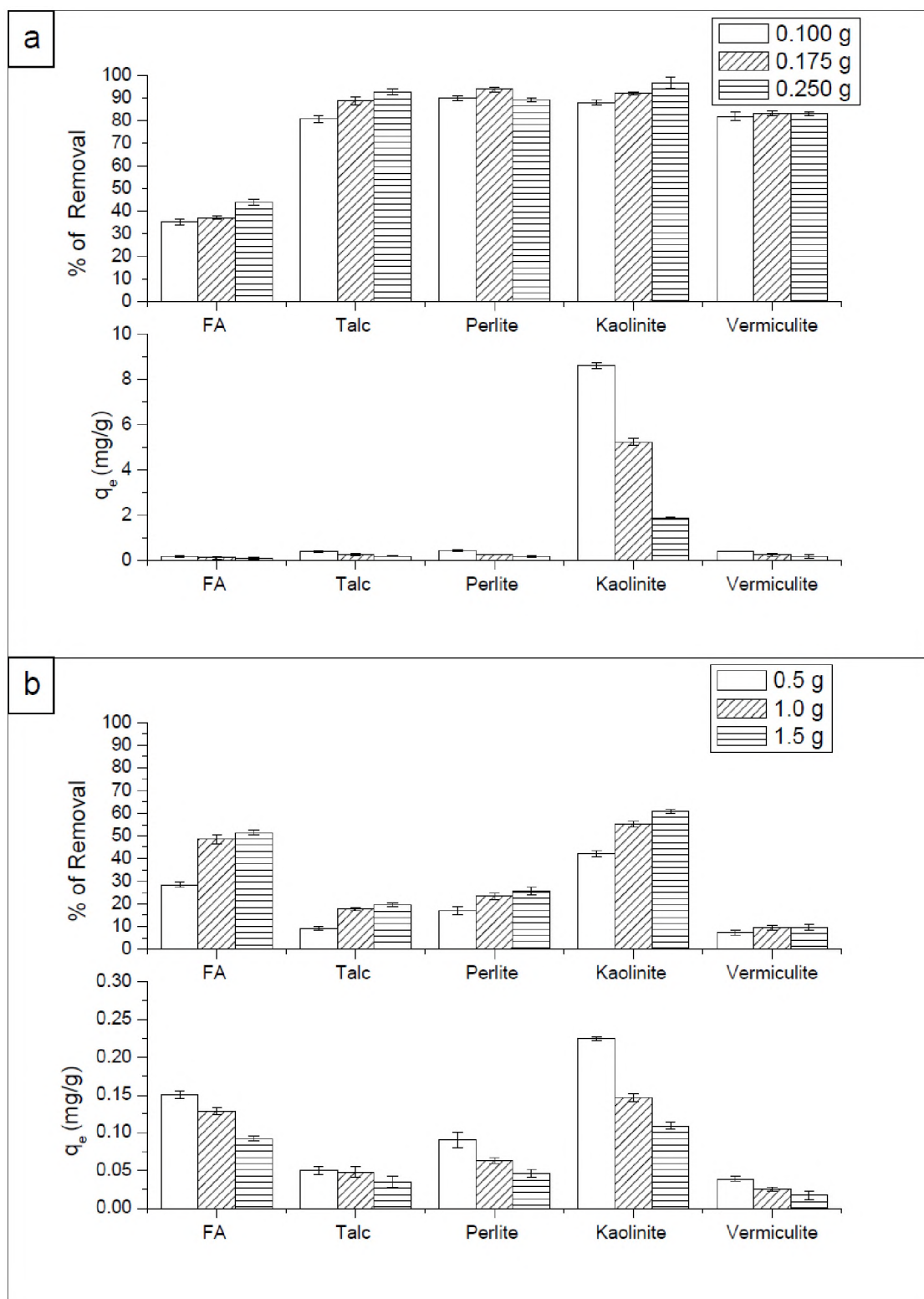


Figure 17: Effect of adsorbent dose on the adsorption of (a) ciprofloxacin and (b) isoniazid onto FA, kaolinite, perlite, talc and vermiculite. % removal and amount adsorbed at equilibrium (q_e) presented as the mean $\pm$ standard deviation ($n = 3$, $T = 25 \pm 1$ °C)

2.3.12 Adsorption isotherms

The reason for employing adsorption isotherms was to analyse the relationship between the equilibrium concentration of antibiotic in solution and the amount adsorbed onto the surface of the adsorbent. Furthermore, adsorption isotherms provide constant values which enable us to compare the adsorption capacities for different adsorbents. The linearised plots of the Langmuir, Freundlich and Temkin adsorption isotherms were used to analyse the experimental data and the parameters are presented in Table 9 and Table 10. The regression line correlation coefficients (R^2) of the plots were used to establish the model which best described the data.

$$\frac{1}{q_e} = \frac{1}{q_m} + \frac{1}{K_L C_e q_m} \quad (23)$$

$$\ln q_e = \ln K_f + \frac{1}{n} \ln C_e \quad (24)$$

$$q_e = B \ln K_t + B \ln C_e \quad (25)$$

Where q_e is the equilibrium adsorption capacity of the adsorbent, C_e is the concentration of antibiotic in the aqueous phase at equilibrium, q_m (mg/g) and K_L (L/mg) are Langmuir parameters representing the monolayer capacity and adsorption constant, respectively. K_f (mg/g)(L/mg) $^{1/n}$ and n are Freundlich parameters representing the adsorption constant and the sorption coefficient, respectively. K_t (L/g) is the Temkin isotherm equilibrium binding constant and B (J/mol) is the Temkin's heat of adsorption (Can et al., 2016). The adsorption isotherm parameters and R^2 values obtained from fitting the experimental data obtained at 25 ± 1 °C to linear plots of the isotherms values are presented in Table 9 and Table 10.

Table 9: Adsorption isotherm constants and correlation coefficients for adsorption of ciprofloxacin onto FA, kaolinite, perlite, talc and vermiculite.

Adsorbent	Langmuir			1/n	Freundlich		R ²	Temkin	
	K _L (L/mg)	q _m (mg/g)	R ²		K _f (mg/g)(L/mg) ^{1/n}			K _t (L/mg)	B (J/mol)
FA	0.017	3.123	0.989	0.942	0.051	0.960	e ^{-1.494}	0.927	0.694
Talc	0.064	6.083	0.998	0.667	0.436	0.993	e ^{-0.035}	1.215	0.928
Perlite	1.505	0.819	0.978	0.221	0.392	0.953	e ^{-3.478}	0.122	0.997
Kaolinite	0.002	500.0	0.995	0.993	0.778	0.992	e ^{-1.427}	10.460	0.930
Vermiculite	0.039	11.93	0.998	0.901	0.115	0.999	e ^{-0.245}	2.056	0.826

Table 10: Adsorption isotherm constants and correlation coefficients for adsorption of isoniazid onto FA, kaolinite, perlite, talc and vermiculite

Adsorbent	Langmuir			1/n	Freundlich		R ²	Temkin	
	K _L × 10 ⁻³ (L/mg)	q _m (mg/g)	R ²		K _f × 10 ⁻³ (mg/g)(L/mg) ^{1/n}			K _t (L/mg)	B (J/mol)
FA	1.134	7.092	0.999	0.983	9.727	0.998	e ^{-2.245}	0.2752	0.953
Talc	0.879	2.481	0.990	0.998	1.973	0.987	e ^{-2.776}	0.1098	0.917
Perlite	45.910	0.130	0.986	0.319	2.488	0.923	e ^{-0.502}	0.0259	0.948
Kaolinite	18.002	0.824	0.994	0.697	25.991	0.999	e ^{-1.804}	0.1925	0.968
Vermiculite	15.365	0.099	0.908	0.486	0.619	0.775	e ^{-1.557}	0.0183	0.790

In order to quantify the applicability of the isotherm models, the R^2 values were used. These revealed that the Langmuir model was the best fit for the sorption of ciprofloxacin onto FA, talc and kaolinite with $R^2 = 0.989$, 0.998 and 0.995 , respectively. This implies that adsorption was predominantly a monolayer physical process. The Freundlich and Temkin isotherms gave the best fit for the sorption of ciprofloxacin onto vermiculite and perlite with $R^2 = 0.999$ and 0.997 , respectively. The Langmuir isotherm also gave the best fit for the adsorption of isoniazid onto FA, talc, perlite and vermiculite exhibiting R^2 values in the range 0.90 - 0.99 , the Freundlich isotherm was the better fit for the sorption of isoniazid onto kaolinite with $R^2 \geq 0.99$. The Freundlich model mainly describes adsorption onto surfaces with no uniform energy distribution which means the adsorption is heterogeneous (Guan

et al., 2017). However, R^2 values only explain the percentage of variance in the dependent variable which is accounted for by the independent variable. It is, therefore, important to also take into account the isotherm parameters when assessing the applicability of an isotherm to a particular dataset. The Freundlich constant, $1/n$ is a function of the strength of adsorption in the adsorption process and ideally, it should have a value less than one during a normal adsorption process which is an indication that the adsorption sites are homogenous in energy and there is no interaction between the adsorbed species. When $1/n$ assumes a value greater than 1, the interactions become weak and adsorption becomes unfavourable (Can et al., 2016). The values of the Freundlich exponent, $1/n$, range from 0.221 - 0.998, indicating a favourable adsorption of ciprofloxacin and isoniazid onto the adsorbents. The heat of sorption, B , calculated from the Temkin plots has values ranging from 0.018 - 10.460 J/mol, indicating a physical adsorption process (physisorption). Unlike the Langmuir and Freundlich isotherm models, the Temkin isotherm takes into account the interactions between adsorbents and antibiotics in solution, which is based on the assumption that the free energy of adsorption is simply a function of surface coverage (Abdelnaeim et al., 2016). Again, looking at the regression R^2 values obtained (69.4 - 99.7 %), this isotherm is applicable to the description of the majority of the equilibrium data and gave the best fit for the adsorption of ciprofloxacin by perlite.

Comparing the isotherms applied, the Langmuir gave the best fit, followed by the Freundlich isotherm and the least fit was obtained from the Temkin isotherm model. Kaolinite exhibited the greatest removals of both ciprofloxacin and isoniazid from aqueous solutions, achieving removals of up to 99% and 55%, respectively. The results indicate that the adsorption of ciprofloxacin and isoniazid mainly by surface coverage of the adsorbents and this corresponds with the findings from the kinetic studies which revealed that adsorption was mainly controlled external mass transfer.

2.4 Conclusions

This chapter presented the feasibility of adsorption of ciprofloxacin and isoniazid using FA, kaolinite, perlite, talc and vermiculite. The adsorbents were used without any chemical modification and can therefore significantly cut costs for wastewater treatment. All adsorbents except FA removed showed excellent adsorption of ciprofloxacin from aqueous solutions with all of them achieving removals ranging from 80 - 99%. The adsorbents were less efficient in removing isoniazid and kaolinite gave the highest removal of 55 %. The ambient pH plays an important role in the adsorption process by affecting the surface charge of the adsorbent, the degree of ionisation and speciation of the adsorbate. The adsorption of ciprofloxacin and isoniazid by all adsorbents except FA is strongly pH dependent suggesting that electrostatic interactions play a significant role in the adsorption process. The influence of pH on the adsorption of ciprofloxacin and isoniazid onto FA was negligible. Adsorption kinetics data were described by the pseudo-second-order equation and application of the intraparticle diffusion model revealed that the adsorption processes were not only controlled by intraparticle diffusion. Both the Langmuir and Freundlich isotherms were applicable to the equilibrium data, indicating that the adsorption processes were spontaneous and exothermic.

3 CHAPTER 3: MICROBIAL ANALYSIS AND STERILIZATION OF ADSORBENTS

3.1 Introduction

The mining and quarrying sector is a major stakeholder of the South African economy and continues to contribute profoundly to the country's gross domestic product (GDP). The total monetary value of this contribution remained relatively constant and ranged from 227.26 to 245.22 billion ZAR (approximately 14.95 to 16.13 billion USD) annually between 2005 and 2014 (Statistics South Africa, 2015). However, the last few years have seen the suspension of operations and retrenchment of employees by some mines due to the decline in gold prices on the world markets. This has had devastating consequences, particularly in a socio-economic context characterised by high unemployment and an estimated average dependency ratio of 10:1 on each mining sector job i.e. on average one miner financially supports 10 individuals (Harvey, 2016). In other areas, there is potential to develop mining as a source of economic growth and employment. The application of low-cost minerals such as talc, vermiculite, perlite and kaolinite and mineral residues such as FA in the remediation of wastewater will possibly create more mining jobs.

Minerals from natural sources are usually contaminated with microorganisms. The type and quantities of the microorganisms mainly depend on the origin of the mineral (Huang et al., 2005), although handling and storage conditions may also play a role. The microorganisms most frequently found on minerals are *Bacillus*, *Streptococcus*, Gram-negative bacteria and yeasts and moulds (de la Rosa et al., 1995). This is because they have a good tolerance to dryness and basic nutritional requirements. Bacteria can also form biofilms on the surfaces of minerals and utilise the nutrients entrapped in the mineral structure (Mueller, 2015). More importantly, some of these minerals could be contaminated with pathogenic bacteria such as *Staphylococcus aureus*, *Clostridium perfringens* and *Pseudomonas aeruginosa* (Osipov and Turova, 1997). Therefore, mining and quarrying of these minerals pose risks to human and environmental health. The risks arise from potential contamination of surface waters and ground water with human pathogens in mine runoff effluent. Miners are also at a high risk of developing occupational diseases such as Pneumoconiosis resulting from

the inhalation of mine dust (Ji et al., 2016). People living in settlements near the mining and /or quarrying sites, as is often the case in South Africa (Eijsackers et al., 2014), may be exposed to pathogens in mineral dust especially when the settlements are in the direction of the atmospheric currents. Moreover, mixing these minerals in their raw state with antibiotic contaminated wastewater might lead to the proliferation of resistance (Jones et al., 2005). Thus, this chapter provides an assessment of the microbiological quality of kaolinite, talc, perlite, vermiculite and FA in order to ascertain the possible use of dry heat sterilisation as a precautionary measure to eliminate the risks posed by the microorganisms present in the minerals. Furthermore, the energy required to sterilise these materials was also assessed. In this chapter kaolinite, talc, perlite, vermiculite and FA are referred to as adsorbents.

3.2 Materials and methods

3.2.1 Isolation of microorganisms from adsorbents

Unless stated otherwise, all procedures were performed using the standard aseptic technique in an LA-1200 BII Laminar-flow hood (Vivid Air, South Africa). All sterilisations in this study were conducted using the Model RAU-53Bd REX MED autoclave (Hirayama Manufacturing, Tokyo, Japan) at 121 °C for 15 min, unless stated otherwise. All culture media were purchased from Merck Pty. Ltd. (Cape Town/Johannesburg, South Africa). Nutrient broth and Sabouraud dextrose broth (used to extract bacteria and fungi, respectively), MacConkey agar (used to enumerate coliform bacteria), nutrient agar (used to enumerate total bacterial count) and Cetrimide agar base with glycerol addition (referred to as Cetrimide agar in further text was used to enumerate *Pseudomonas spp.*), potato - dextrose agar (used to enumerate total fungi count - yeasts and moulds). Ultrapure water was obtained from a Milli-Q® RiOs™ and Academic A10 Water Purification system (Merck Millipore, Bedford, USA). Sodium chloride (NaCl) (99.5 %) and glycerol were purchased from Merck Pty. Ltd. (Cape Town/Johannesburg, South Africa).

3.2.2 Preparation of samples

The samples were ground using a mortar and pestle and mechanically passed through a 315 µm mesh sieve to ensure uniform particle size.

3.2.3 Extraction of microorganisms from adsorbents

For each adsorbent, three 10 g replicates were weighed using a Radwag WLC 1/A2/C/2 balance (accuracy 0.01 g; Lasec Inc., Cape Town, South Africa) and added to sterile 250 mL Erlenmeyer flasks. Using a sterile measuring cylinder, 50 mL nutrient broth, 50 mL Sabouraud broth and 50 mL 0.9 % NaCl (normal saline) were added to each Erlenmeyer flask, respectively. The contents of the Erlenmeyer flasks were thoroughly mixed using a VM 20 vortex mixer (Scientific Instruments Development UK Ltd., London, Britain). The sample mixed with nutrient broth was incubated at 35 - 37 °C for 24 h in the Labcon incubator, FSIM B (Labmark, South Africa) and the sample mixed with Sabouraud-dextrose broth was incubated at 28 °C for 24 h in the Labcon low-temperature incubator, LTIE 10 (Labmark, South Africa). The Erlenmeyer flasks were shaken every 6 h over the duration of incubation. The Erlenmeyer flask containing the sample and normal saline mixture was shaken at room temperature (25±1 °C) at a speed of 150 rpm for 30 min using a Model TS-520D orbital shaker (Already Enterprise Inc., Taipei, Taiwan). All extractions were done in duplicate for each adsorbent. Three Erlenmeyer flasks containing nutrient broth, Sabouraud dextrose broth and normal saline, respectively and no adsorbent were used as negative controls to assess sterility of the liquid media.

3.2.4 Cultivation and enumeration of microorganisms

Cultivation and enumeration of the microorganisms were performed by the standard spread-plate method (Boczek et al., 2014) as depicted in Fig. 18. MacConkey, Cetrimide and potato-dextrose agar were prepared according to manufacturer's instructions, left to cool to about 50 °C prior to being poured into sterile Petri dishes

(20 - 25 mL per plate). The agar plates were left to set overnight. Seven serial 10-fold dilutions of the supernatant from each sample mixture were prepared. Using an Eppendorf® auto-pipette and tip (Sigma-Aldrich (Pty.) Ltd., Johannesburg, South Africa), a 0.5 mL aliquot of the supernatant was transferred into a glass test tube containing 4.5 mL sterile normal saline and thoroughly mixed by vortexing to make a $1:10^{-1}$ dilution. This solution was used to make a dilution of 10^{-2} by repeating the same step. Further dilutions were done consecutively up to a dilution of 10^{-7} . Using an auto-pipette, 0.1 mL of each dilution was transferred onto an agar surface and spread evenly using a glass spreader. Samples extracted using nutrient broth were spread onto nutrient, MacConkey, and Cetrimide agar and incubated at 35 - 37 °C for 24 h, whereas those extracted using Sabouraud-dextrose broth were spread onto potato-dextrose agar and incubated at 28 °C for 5 - 7 days. All counts were done in duplicate for each dilution of the duplicated adsorbent sample. The total viable cell count was reported as colony forming units per gram of adsorbent dry weight given by the formulae:

$$CFU\ g^{-1} = \frac{nV}{WAD_f} \quad (26)$$

Where n is the number of colonies on the agar surface after incubation, V (mL) is the volume of broth or normal saline used to extract the microorganisms, W (g) is the dry weight of the particulate matter obtained by drying the samples to constant weight at 105 °C, described in detail in chapter 2, A (mL) is the aliquot of sample plated which is usually 0.1 mL and D_f is the dilution factor, a dimensionless number that unambiguously describes the strength of dilution. The Cetrimide agar plates were also viewed under UV light using Spectroline® Model CM-10 Viewing Cabinet (Krackeler Scientific Inc., NY, USA) at 366 nm to check for the presence of a green pigment characteristic of *Pseudomonas aeruginosa*.

3.2.5 Sub-culturing of microorganisms

For each sample, colonies that were dominant and/or exhibited a morphological distinctiveness were selected and transferred as single colonies onto nutrient agar

and potato dextrose agar plates for bacteria and fungi cultures, respectively, and incubated as already described. These steps were repeated at least two times until pure colonies were obtained. Pure colonies of bacteria were transferred onto sterile nutrient agar slants using sterile cotton throat swabs (Spellbound Labs, Port Elizabeth, South Africa). For fungi cultures, a sterile scalpel was used to excise and transfer a small piece of specimen onto sterile potato - dextrose agar slants. The agar slant tubes were loosely capped and the necks were wrapped with parafilm to allow for breathing and prevent contamination. The tubes were stored at 4 °C for further analyses.

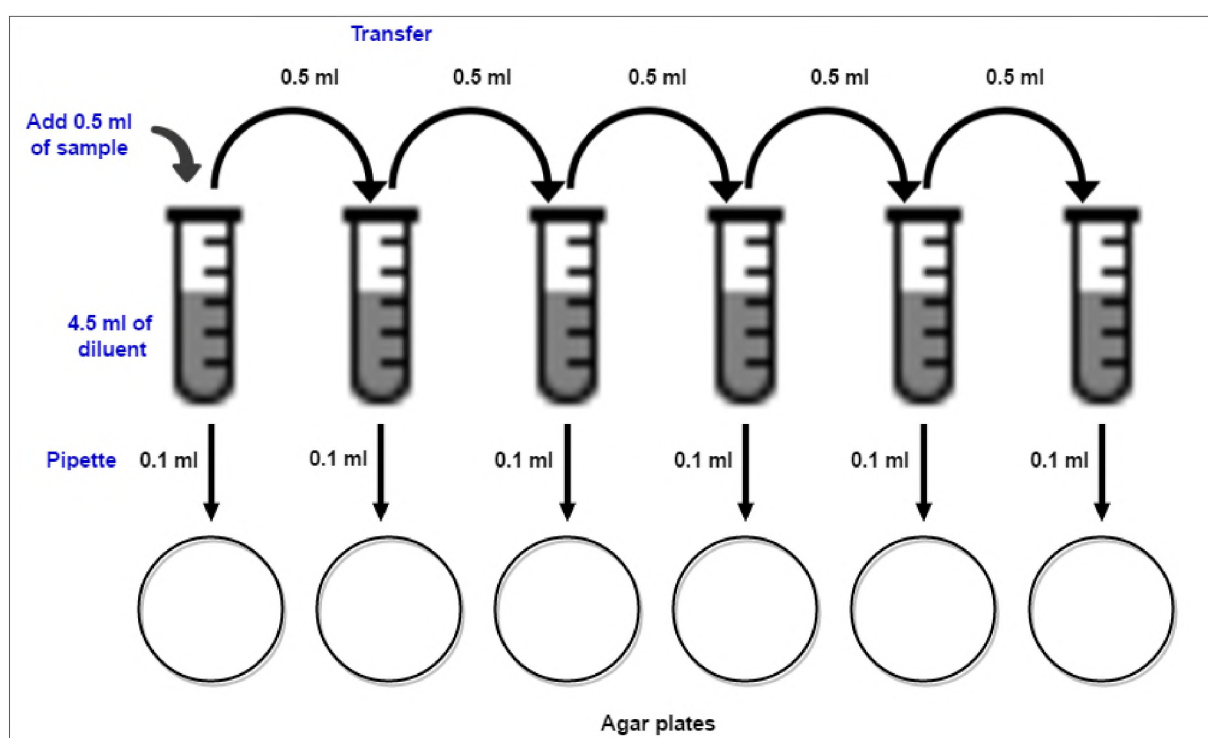


Figure 18: Serial dilution for standard spread-plate technique. (Drawn using Draw.io)

3.2.6 Dry heat inactivation of microorganisms

For each adsorbent, 10 g of the samples were weighed and aseptically added to a marked sterile 250 mL Erlenmeyer flask. The Erlenmeyer flasks were put in a Memmert UFE 700 oven (Mettler, Western Germany) oven at 105 °C for predetermined time intervals ranging from 5 to 2 880 min after which they were taken out covered by sterile aluminum foil and immediately placed in an ice water

bath at a temperature 1-2 °C for 5 min. The microorganisms present in the adsorbent samples were then extracted, cultivated and enumerated as described in section 3.2.3 and 3.2.4. The same procedure was repeated at an oven temperature of 160 °C.

3.2.7 Statistical analysis

Two replications were performed for each sample. The heat-resistance data were analysed using the Paleontological Statistics Software (PAST) package to determine whether there were statistically significant differences among the treatments. The Kruskal - Wallis test was employed to determine significant differences ($P < 0.05$) among means.

3.2.8 Morphological identification of fungi

Identification of fungi was done by the correlation of macroscopic and microscopic examination of characteristic features of different species (Samson et al., 2010). The cultures stored on agar slants were resuscitated by incubating them on potato - dextrose agar at 28 °C for 5 - 7 days. A visual examination of the obverse and reverse sides of the agar plates was performed after incubation. Afterwards, using a sterile pipette, a drop of lactophenol cotton blue stain (Spellbound Labs, Port Elizabeth, South Africa) was placed on a clean microscope glass slide. Using a sterile scalpel, a small piece of fungi specimen was cut from the agar plate and transferred onto the lactophenol cotton blue drop on the slide. Using sterile dissection needles, the fungal cells were teased and flattened. A glass cover slip was gently placed over the drop ensuring that no air bubbles are formed. The micro - morphological characteristics such as shape of spores, structures of conidiophores and appearance of hyphae were examined using an Olympus BX 51 microscope coupled with an SC 30 digital camera with Stream Essentials software (Olympus, Japan) using a high-power objective (40×) for the moulds and an oil immersion objective (100×) for yeasts.

3.2.9 Identification of bacteria

3.2.9.1 Gram stain

All reagents used were purchased from Merck Pty. Ltd., (Cape Town/Johannesburg, South Africa). Bacterial samples from the agar slants were resuscitated on nutrient agar by incubating them at 37 °C for 24 h. Using a pipette, 1 drop of normal saline was placed on a clean microscope glass slide. A colony was picked up from the agar plate and mixed with the drop of saline on the slide. The specimen was air dried and then heat fixed onto the slide by passing over a naked bunsen burner flame several times until the visible liquid dried up. The smear was covered with crystal violet stain and left for 30 seconds before being rinsed gently with water. The smear was then covered with Gram's iodine and left for 1 min before rinsing with ethanol in a dropwise fashion until there was no purple. The smear was then rinsed with deionised water and covered with safranin stain for 1 min. Next, the smear was rinsed with deionised water and blotted dry with filter paper. Lastly, the specimen was examined using a light microscope with an oil-immersion filter under the 100x magnification.

3.2.9.2 KOH string test

A drop of 3 % KOH was placed on a glass slide. Using a sterile inoculation loop (Merck Pty. Ltd., Cape Town/Johannesburg, South Africa), a full loop of culture was scraped from the agar plate and transferred onto a glass slide. The culture was mixed with 3 drops of 3 % (w/v) solution of KOH in Milli-Q and left to react for 2 min. The loop was dipped into the mixture and raised to check the consistency and fibrosity of the mixture. Development of a string would indicate that the KOH hydrolysed the lipids in the outer membrane of Gram-negative bacteria (Moaledj, 1986).

3.2.9.3 API® 20 E

Identification of Gram-negative bacteria was done using the API® 20 E test system and reagents (BioMérieux, Midrand, South Africa) according to manufacturer's instructions. The results were analysed using the Apiweb™ online bacteria database <https://www.apiweb.biomerieux.com/servlet/Identify?action=prepareNew&stripld=1> (BioMérieux, Midrand, South Africa). All procedures were done using the standard aseptic technique.

3.2.10 Genomic Identification of bacteria

3.2.10.1 Materials and chemicals

Ethidium bromide (EtBr) was purchased from Inqaba Biotec (Pretoria, South Africa). PCR tubes and SeaKem® LE Agarose purchased from Lasec Inc. (Port Elizabeth, South Africa). Disodium Ethylenediaminetetraacetic acid dihydrate (EDTA), Glacial Acetic acid (Acetate) and Sodium hydroxide (NaOH) were purchased from Merck (Pty.) Ltd. (Johannesburg, South Africa). Ethanol and Tris - Base were purchased from Sigma-Aldrich (Pty.) Ltd. (Johannesburg, South Africa).

3.2.10.2 Bacterial genome DNA isolation and purification

Bacterial genome extraction and purification were done using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to manufacturer's instructions.

3.2.10.3 Harvesting of bacterial cells

A sterile pipette tip was used to transfer a single colony from the surface of the nutrient agar to a sterile test tube containing 5 mL nutrient broth. The test tube

containing the sample was incubated at 37 °C for 24 h. Using an auto-pipette, 1.2 mL of bacterial suspension was added to a 1.5 mL microcentrifuge tube. The microcentrifuge tube was centrifuged for 10 min at 4 961 ×g using a Mini Spin EL - 122 centrifuge (Eppendorf, Germany). The supernatant was discarded and the pellet of bacterial cell was retained at the bottom of the microcentrifuge tube.

3.2.10.4 Preparation of lysate

The bacterial pellet was resuspended in 100 µL of Digestion Solution, 20 µL of Proteinase K Solution was added and the contents of the microcentrifuge tube were mixed thoroughly by vortexing using a BR - 2000 vortexer (Bio-Rad Laboratories, USA). The microcentrifuge tube was incubated at 56 °C for 30 min in a Single-block Digital Dry Bath (Bio-Rad Laboratories, Taiwan) accompanied by occasional mixing by vortexing throughout the duration. Twenty microliters (200 µL) of RNase A solution was added to the microcentrifuge tube followed by vortexing and then incubation at room temperature for 10 min. Two hundred microliters (200 µL) of Lysis Solution was added and the sample was thoroughly mixed by vortexing for 15 seconds until a uniform suspension was obtained. Four hundred microliters (400 µL) of 50 % ethanol was added and the sample was mixed by vortexing.

3.2.10.5 Purification of bacterial genome DNA

The prepared lysate was transferred to a GeneJET Genomic DNA Purification Column inserted in a 2 mL collection tube and centrifuged at 5 927 ×g for 1 min. The collection tube containing the flow-through solution was discarded and replaced with a new one. Five hundred microliters (500 µL) of Wash Buffer 1 was added to the GeneJET Genomic DNA Purification Column and centrifuged at 7 970 ×g for 1 min. The flow-through in the collecting tube was discarded and the purification column was reinserted into the collection tube. Five hundred microliters (500 µL) of Wash Buffer 2 was added to the GeneJET Genomic DNA Purification Column and centrifuged at 11 866 ×g for 3 min. The collection tube containing the flow-through

solution was discarded and the GeneJET Genomic DNA Purification Column was transferred to a sterile 1.5 mL microcentrifuge tube. Two hundred microliters (200 μ L) of Elution Buffer was added to the centre of the GeneJET Genomic DNA Purification Column. The GeneJET Genomic DNA Purification Column was incubated at room temperature for 2 min and then centrifuged at 7 970 $\times g$ for 1 min.

3.2.11 Purified bacterial genome DNA quantification and analysis

3.2.11.1 Quantification of bacterial genome DNA

The purified DNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) by measuring absorbance at 260 nm of 2 μ L of the sample. The Elution Buffer from the GeneJET Genomic DNA Purification Kit was used as a blank. Measurements for each sample were done in triplicate.

3.2.11.2 Visualising bacterial genome DNA

The DNA products were analysed by electrophoresis (90 V, 60 min) on 0.8 % agarose gel stained with 0.6 μ g/mL ethidium bromide in 1 $\times$ TAE buffer and visualised using a Molecular Imager® ChemiDoc™ XRS+ with Image Lab software (Bio-Rad Laboratories, USA).

3.2.11.3 Buffer preparation

The buffers for the preparation of the agarose gel and subsequent electrophoresis were prepared as follows: Firstly, a 0.5 M Ethylenediaminetetraacetic acid (EDTA) was prepared by adding 11.63 g EDTA disodium dihydrate to 50 mL deionized water (dH₂O); 1.125 g of solid sodium hydroxide (NaOH) was added and the pH was adjusted to using 1 M NaOH solution. The volume was brought up to 62.5 mL with dH₂O. Then, a 50 $\times$ Tris-acetate-EDTA (TAE) buffer solution was prepared by dissolving 24.22 g Tris Base in 60 mL dH₂O and 5.71 mL of 100 % glacial acetic acid and mixed with 10 mL

of the 0.5 M EDTA prior to making up the solution to 100 mL using dH₂O. The 1× TAE running buffer was prepared by adding 980 mL of dH₂O to 20 mL of 50× TAE buffer stock solution. Solutions were sterilised using the Model RAU-53Bd REX MED autoclave (Hirayama Manufacturing, Tokyo, Japan) at 121 °C for 15 min. All pH measurements were done using the Jenway 3510 pH Meter (Lasec Inc., South Africa).

3.2.11.4 Agarose gel preparation

A 0.8 % agarose gel was prepared by adding 0.4 g dehydrated agarose (SeaKem LE Agarose) to 50 mL of dH₂O. The agarose was dissolved by boiling in a microwave oven. The solution was left to cool at to below 50 °C followed by addition of 2.5 µL of 10 mg/L ethidium bromide (EtBr) stock solution to give a final concentration of 0.5 µg/mL. The solution was poured into a casting tray and left to set for 30 min after inserting the comb. All weights were measured using the New Classic MF, MS105 analytical balance (Mettler Toledo, Switzerland) with 0.0001 g accuracy.

3.2.11.5 Electrophoresis and visualisation

The agarose gel was transferred to the buffer well (Bio-Rad Laboratories, USA) followed by addition of 1× TAE buffer until the gel was completely immersed. The comb was carefully removed to create wells on the agarose gel. Two microliters (2 mL) of the GeneRuler DNA Ladder Mix (Thermo Scientific, USA) was loaded into one of the wells to serve as a molecular weight marker. Using a micropipette, 5 µL DNA product was mixed with 1 µL 6× Loading Dye (Thermo Scientific, USA) and carefully loaded into the agarose gel wells. The gel was run at 90 V for 60 min using the PowerPac 300 Electrophoresis Power Supply (Bio-Rad Laboratories, USA) and then visualised using a Molecular Imager® ChemiDoc™ XRS+ with Image Lab software. The DNA products were stored at -20 °C.

3.2.11.6 16S rRNA gene amplification and nucleotide sequencing

The 16S rRNA gene forward and reversed primers were amplified by the PCR technique and the products were visualised using agarose gel electrophoresis.

3.2.11.7 Optimisation of primers and PCR amplification of the 16S rRNA gene

The DNA samples and frozen reagents were freeze-thawed on an ice bath for 60 min. Using an auto-pipette, 2 - 4 μL (depending on concentration) of DNA sample, 10 μL of 2 $\times$ ReadyMix (Kapa Biosystems, South Africa), 1 μL each of universal forward (Fd1) and reverse (rP2) primers (both 10 μM) (Inqaba Biotech, South Africa), were added to a sterile PCR and made up to a final volume of 20 μL using sterile dH_2O . The forward and reverse primers are presented in Table 11. The PCR was performed using the T100[™] Thermo Cycler, (Bio-Rad Laboratories, USA) using the following program: initial denaturation for 1 cycle at 95 °C for 5 min followed by 30 cycles of denaturation for 1 min at 95 °C, annealing for 1.5 min at 65 °C, elongation for 1 min at 72 °C; and lastly 1 cycle for final extension was performed for 5 min at 72 °C which was followed by an infinite hold at 4 °C. The thermocycling parameters are presented in Table 12. The optimum annealing temperature was empirically determined by performing PCR gradient with 7 different temperatures spread across the range 50 - 65 °C.

Table 11: Forward and reverse primers and their respective theoretical annealing temperatures

Primer	Sequence	Temperature (°C)
Fd1	5'-AGAGTTTGATCCTGGCTCAG-3'	55.4
rP2	5'-ACGGCTACCTTGTTACGACTT-3'	56.5

Table 12: Thermocycling parameters during PCR

Step	Temperature (°C)	Time (min)	Cycles
Initial Denaturation	95	5	1
Denaturation	95	1	30
Annealing	65	1.5	
Elongation	72	1	
Extension	72	5	1
Storage	4	Hold	

3.2.11.8 Visualising PCR products

The PCR products were analysed by electrophoresis (90 V, 60 min) on 0.8 % agarose gel stained with 0.6 µg/mL ethidium bromide in 1× TAE buffer and visualised using a Molecular Imager® ChemiDoc™ XRS+ with Image Lab software (Bio-Rad Laboratories, USA).

3.2.11.9 Nucleotide sequencing of the 16S rRNA gene

The PCR products were sent to Inqaba Biotec (Pretoria, South Africa) for Sanger sequencing using the Fd1: 5'-AGAGTTTGATCCTGGCTCAG-3' primer. Inqaba Biotec uses BigDye® Terminator 3.1 cycle sequencing on an ABI 3500 XL sequencer.

3.2.11.10 Analysis of the nucleotide sequences of the 16S rRNA gene

Analysis of the 16S rRNA gene sequence and post-sequencing quality checks such as base calling, trimming and reassembly were performed using Finch TV (<http://www.geospiza.com/ftvdlinfo.html>). The nucleotide sequences were compared to standard databases found at the National Center for Biotechnology Information (NCBI) the Ribosomal Database Project. The NCBI database was accessed

using the Basic Local Alignment Search Tool (BLAST) program and the genus and species were identified based on the lowest E-value.

3.3 Results and discussions

3.3.1 Extraction of microorganisms

The extraction of the microorganisms was done using normal saline, nutrient broth in the case of bacteria and Sabouraud dextrose broth in the case of moulds and fungi. A leachability index (LI) obtained from the results and is described by the expression:

$$LI = \frac{CFU g^{-1}(saline)}{CFU g^{-1}(broth)} \quad (27)$$

The LI gives an indication of the degree by which microorganisms can leach out of the adsorbents into soil, groundwater and adjoining water bodies due to the effects of rain (Kanchinadham et al., 2015). This is particularly important in assessing the possible environmental pollution when these adsorbents are mined and, in the case of FA dumped into ash dams and landfills. It will also indicate the ease of bacterial and fungal release from the material thus, providing an indication of the ease of sterilisation. In the investigations conducted, the viable cell count in the range 30 - 300 CFU per plate was used. Plates with CFU within the prescribed range are more statistically reliable (Davis, 2014). The concentrations of microorganisms extracted using normal saline were below the detection limit. The detection limits ranged from 1.51×10^3 - 1.64×10^3 CFU/g for the different adsorbents and are presented in Table 13. The concentrations of microorganisms extracted using nutrient broth and Sabouraud broth from FA were also below the detection limit. Therefore, no further investigations were performed on FA. However, nutrient broth and Sabouraud broth extracted remarkably high concentrations of microorganisms from the rest of the adsorbents. The concentrations ranged from 5.48×10^5 - 1.19×10^9 CFU/g and 2.13×10^6 - 1.25×10^7 CFU/g for bacteria and fungi, respectively. The tremendous

abundance of bacteria and fungi in these mineral adsorbents arises from their excellent adaptability to new environments, flexible biochemical requirements and ability to sporulate in extreme conditions (Kutateladze et al., 2016).

Table 13: Detection limits for microorganisms present in the adsorbents

Adsorbent	Detection limit (CFU/g)
FA	1.51×10^3
Talc	1.53×10^3
Perlite	1.60×10^3
Kaolinite	1.62×10^3
Vermiculite	1.64×10^3

The LI values obtained are presented in Table 14 and Table 15. The values range from 1.28×10^{-6} - 7.04×10^{-5} and 1.22×10^{-4} - 7.62×10^{-3} for bacteria and fungi, respectively. The small LI obtained indicate that even after agitation with saline the microorganisms are retained on the surfaces of the adsorbents which suggests that strong affinity the microorganisms have for the adsorbents (Rodríguez-Jordá et al., 2010). As mentioned in chapter 2, bacterial cell walls consist of polysaccharides, lipids, nucleic acids and peptides which create a highly heterogeneous outer surface capable of forming various interactions with adsorbents such as hydrogen bonds (Hong et al., 2013). The filamentous structure of fungi and flagella present on some fungi also enable them to penetrate deep within the mineral (Huang et al., 2005). We can, therefore, conclude that the sourcing of these adsorbents does not pose a major threat with regards to contamination of groundwater and other environmental facets with microorganisms because it is highly unlikely that rain will result in the leaching out of the microorganisms down the soil profile. However, the luxuriant growth exhibited by the microorganisms when nutrient broth and Sabouraud - dextrose broth, is a cause for concern. It implies that if provided with a conducive environment, for instance, the lungs after inhalation of dust by a miner, the microorganisms are capable of causing infection.

Table 14: Total bacteria and coliform counts presented as the means and stand deviations (SD). Leachability indexes (LI) of the microorganisms obtained from the adsorbents

Adsorbent	Total bacteria count			Total coliform count		
	n = 4 (CFU/g)	SD (CFU/g)	LI	n = 4 (CFU/g)	SD (CFU/g)	LI
FA	ND	-	-	ND	-	-
Talc	1.19×10^9	2.18×10^8	1.28×10^{-6}	5.78×10^7	1.28×10^7	2.64×10^{-5}
Perlite	6.60×10^7	9.64×10^6	2.43×10^{-5}	2.95×10^6	9.28×10^5	5.42×10^{-4}
Kaolinite	5.27×10^8	6.81×10^7	3.08×10^{-6}	2.31×10^7	4.53×10^6	7.04×10^{-5}
Vermiculite	6.02×10^8	5.24×10^7	2.72×10^{-6}	3.86×10^7	7.41×10^6	4.24×10^{-5}

Table 15: *Pseudomonas aeruginosa* and total fungi counts presented as the means and stand deviations (SD). Leachability indexes (LI) of the microorganisms obtained from the adsorbents

Adsorbent	<i>Pseudomonas aeruginosa</i>			Total fungi count		
	n = 4 (CFU/g)	SD (CFU/g)	LI	n = 4 (CFU/g)	SD (CFU/g)	LI
FA	ND	-	-	ND	-	-
Talc	4.96×10^6	5.48×10^5	3.08×10^{-4}	1.25×10^7	2.28×10^6	1.22×10^{-4}
Perlite	ND	-	-	2.61×10^6	5.71×10^5	6.13×10^{-4}
Kaolinite	ND	-	-	2.13×10^6	7.15×10^5	7.62×10^{-3}
Vermiculite	ND	-	-	1.20×10^7	1.94×10^6	1.37×10^{-4}

3.3.2 Isolation and enumeration of microorganisms

The microorganisms present in the adsorbents were isolated enumerated using the spread-plate method. The spread-plate method is a quantitative technique which employs the use of agar-filled Petri dishes to enumerate microorganisms. The microorganisms are subjected to conditions that promote their growth such as nutrients, incubation temperature, pH and atmosphere for an optimum period of time (Davis, 2014). The spread-plate method has the following advantages over the commonly used pour plate method: (1) Improved visibility and consistency of

colonies, (2) Heat-sensitive organisms are not killed by hot media, (3) The translucence of the media is not essential for colony recognition because all colonies are on the surface and as a result a wider range of non-selective and selective differential medium can be used (Boczek et al., 2014; Cormier and Janes, 2014). The technique is used on the assumption that a single cell will grow into a colony of cells which is visible on the solid agar medium. The acceptable viable cell counts known as colony forming units (CFU) depends on the standard operating procedure followed in accordance with the regulatory guidelines describing the method (Ben-David and Davidson, 2014; Boczek et al., 2014; Thomas et al., 2015). In most cases, the number of bacteria in the sample is very high and thus creating a need for dilution of samples prior to spreading. This is usually achieved by decimal dilutions. Serial dilutions are a sequential set of dilutions in which the stock for each dilution in the series is the working solution from the previous dilution. The aim of the technique is to establish an estimate concentration of a sample by counting CFUs cultured from serial dilutions of the sample, and then do back calculations of the counts to the unknown concentration of the initial sample (Ben-David and Davidson, 2014). In this study, serial dilutions were performed to a concentration of $1:10^{-7}$.

The aliquots of isolated microorganisms were spread on MacConkey, nutrient, Cetrimide and potato dextrose agar. The concentrations of the organisms obtained from the different adsorbents are presented in Table 14 and Table 15. A comparison of the concentrations of the microorganisms is presented in Fig. 19. In general, microorganism concentrations showed a high variability among the different minerals. The concentration of microorganisms is determined by the pH of material, temperature, moisture and aeration. Fungi grow best under acidic conditions whereas bacteria require neutral environments (Kutateladze et al., 2016). Talc was the most contaminated and exhibited the highest diversity of microorganisms. As mentioned earlier, FA was free of any microorganisms whereas the rest of the adsorbents had very high concentrations. This is probably because the microorganisms present in coal were incinerated during coal combustion and the potential recontamination of FA dumped after burning was inhibited by its strong alkaline nature reported in chapter 2. For this reason, FA was considered sterile and was not included in further discussions in this chapter.

MacConkey agar is a differential and selective medium for Gram-negative bacteria. It is capable of differentiating whether Gram-negative rods are able to ferment lactose. The concentrations of coliforms extracted from the adsorbents ranged from 2.95×10^6 - 5.78×10^7 CFU/g. It can be seen in Fig. 19 that the concentration of coliforms present in the adsorbents increases in the order perlite < kaolinite < vermiculite < talc. Interestingly, the order is the same for the total bacteria counts of the adsorbents. This indicates that coliform bacteria represent the majority of bacteria contaminating the adsorbents.

MacConkey agar is made up of six major components; (1) agar, a complex carbohydrate, which like for all other semi-solid media provides structural support for cultures, (2) bile salts and (3) crystal violet which inhibits growth of Gram-positive cells, (4) lactose and (5) peptides which serve as a nutrient source for lactose fermenting bacteria and non-lactose fermenting bacteria, respectively, and (6) neutral red dye which (Hossain, 2014) turns red at lower pH. The metabolic by-products from the fermentation of lactose are acidic and result in a decrease in pH. Therefore, lactose fermenters appear as ink red colonies surrounded by a red halo on MacConkey agar. Red colonies of this nature were observed on all plates spread with extracts from each adsorbent.

The colonies were identified as *E. coli* and *Enterobacter cloacae* by biochemical utilisation tests and molecular identification. On the other hand, the ammonium produced by non-lactose fermenting bacteria such as *Pseudomonas aeruginosa* and *Salmonella* results in an increase in pH and their colonies appear translucent on the MacConkey agar (Galat et al., 2016). These types of colonies were only observed on MacConkey plates spread with extracts from talc samples suggesting that talc was the only adsorbent contaminated with non-lactose fermenting bacteria. There is another special group of Gram-negative bacteria known as slow lactose fermenters. Their colonies may appear red or pink on MacConkey agar, however, the distinct red halo observed around lactose fermenting bacteria is not present (Galat et al., 2016). These types of coliforms were also observed in extracts from talc samples and the bacteria were identified as *Serratia liquefaciens* by biochemical utilisation tests.

Cetrimide agar is a medium selective for *Pseudomonas aeruginosa*. It contains the chemical Cetrimide (cetyl trimethyl ammonium bromide) which inhibits the growth of most types of bacteria except *Pseudomonas aeruginosa*. In the experiments conducted, the only colonies which grew on Cetrimide plates were from talc samples and the concentration was 5.48×10^5 CFU/g. This suggests that talc was the only adsorbent contaminated with *Pseudomonas aeruginosa* and this was confirmed by the translucent colonies on MacConkey agar. The Cetrimide agar and colonies were examined under UV light and they appeared as round white colonies which fluoresced green (Corry, 1995). This is because Cetrimide triggers the *Pseudomonas aeruginosa* to produce two water soluble pigments; pyocyanin and pyoverdine (Corry, 1995). Pyocyanin is a blue phenazine pigment and pyoverdine (fluorescein) is a yellow-green pigment. The combination of the two pigments results in a green pigment characteristic of *Pseudomonas aeruginosa* (Hossain, 2014). The water solubility of the pigment allows it to spread throughout the agar. The glycerol serves as a supplementary carbon source. *Pseudomonas aeruginosa* is a ubiquitous water and soil microorganism. It is an opportunistic pathogen and is responsible for severe life-threatening nosocomial infections in South Africa. It is the most common pathogen isolated from patients who have been in a hospital for more than a week. It is also capable of causing infections in healthy individuals (Sari and Fadaee, 2010).

Potato - dextrose agar is a non-selective medium for the cultivation of yeasts and moulds. Potato - dextrose agar also enhances sporulation and pigmentation and can, therefore, be used to cultivate and differentiate a wide range of pathogenic and non-pathogenic fungi. It consists of dextrose which acts as an energy source and growth stimulant, and potato extract which serves as a nutrient source. The acidic pH 5.6 ± 0.2 inhibits bacterial growth. The minerals were mostly contaminated with moulds. Moulds grow as filamentous colonies of various colours, whereas yeasts grow as moist whitish colonies (Person et al., 2010). The concentrations of the total fungi found in the different adsorbents ranged from 2.13×10^6 - 1.25×10^7 CFU/g. The concentrations are significantly lower in comparison with those of total bacteria and increase in the order kaolinite < perlite < vermiculite < talc.

Nutrient agar is a general-purpose medium used to cultivate a wide range of bacteria. It contains a meat extract and yeast extract which serve as a nutrient source to support microbial growth. It also contains peptone which is the primary

source of organic nitrogen for bacterial growth. Nutrient agar also contains 0.5 % NaCl which makes the conditions of the agar similar to those found in the cytoplasm of most microorganisms (Nishiyama et al., 2012). The total bacteria count obtained in this study ranged from 6.60×10^7 - 1.19×10^9 CFU/g. As seen in Fig. 19, talc had the highest concentration of bacteria about 10 times more than the concentration which was found in perlite.

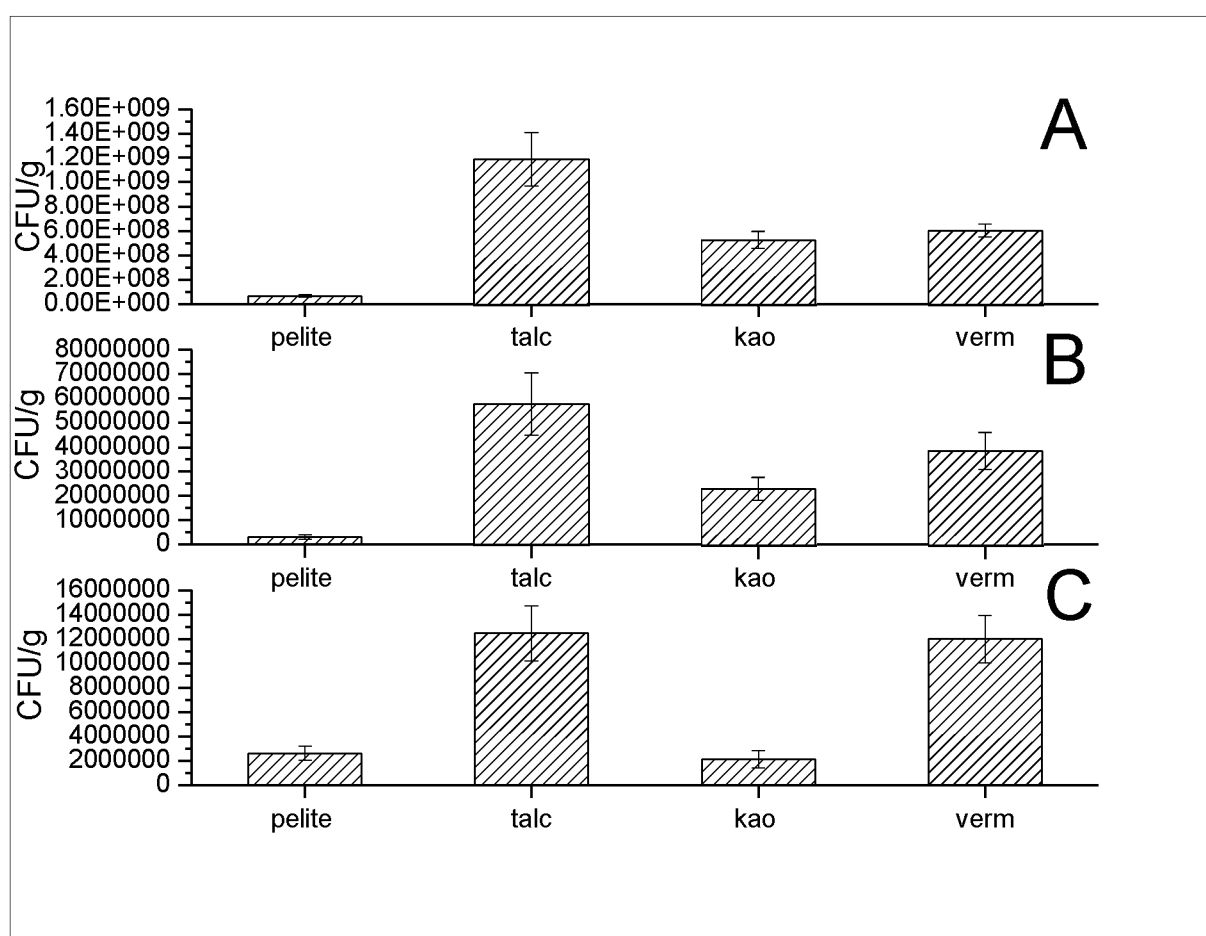


Figure 19: Concentrations of (A) total bacteria, (B) coliforms and (C) fungi extracted from the adsorbents

3.3.3 Heat inactivation

Dry heat sterilisation was attempted by exposing the minerals to temperatures of 105 °C and 160 °C to periods of up to 48 h. The aim was to establish the temperature and time (heat dose) required to eradicate all microorganisms from the mineral

adsorbent samples. The initial concentrations of the indicator microorganisms are presented in Table 14 and Table 15. The microorganism concentrations remained the same after being subjected to dry heat temperature of 105 °C for 48 hours. This indicates a strong resistance to die-off at 105 °C. However, subjecting the microorganisms to dry heat temperature of 160 °C for 15 min reduced all microorganisms to concentrations below the detection limits presented in Table 13. This is probably due to the better penetration of heat into the minerals. The data of the thermal inactivation of the microorganisms at 160 °C is presented in Table 16. The linear plots of $\log_{10}$ surviving cells versus time were used to obtain the decimal reduction times (*D-value*) of the microorganisms. *D-values* were obtained by linear regression analysis of the plots.

D-values are used to define the heat resistance characteristics of microorganisms and are discussed in detail in Chapter 1. The R^2 values obtained from the thermal death curves for all the microorganisms ranged from 0.950 to 0.998 indicating a high degree of linearity. R^2 values greater than 0.90 which achieve at least a 6 log reduction are considered linear (Juneja and Marmer, 1999). Exposing the minerals to dry heat for 15 min eradicated all microorganisms. The *D-values* of total bacteria present in the minerals range from 2.3 - 3.1 min. The bacteria with the greatest heat resistance are in the perlite sample. The results *D-values* for coliform bacteria from all samples are very similar ranging from 1.7-2.2 min. This is an indication of homogeneity in heat resistance by the microorganisms. As mentioned earlier, *Pseudomonas aeruginosa* were only isolated in talc and it had a *D-value* of 2.2 min. The fungi isolated from the samples had *D-values* ranging from 2.1-3.2 min. In general heat resistance by the microorganisms increased in the order coliforms > *Pseudomonas aeruginosa* > total bacteria > total fungi. This heat resistance is most probably due to the presence of thermotolerant bacteria and fungi such as *Bacillus spp.* (Hayrapetyan et al., 2016) and *Aspergillus fumigatus* (Person et al., 2010). These two microorganisms were present in most of the adsorbents. Some yeasts have also been reported to survive in extreme heat as a result of protection from their multi-layered cell walls (Suryanarayanan et al., 2011).

Microorganism resistance to dry heat is described in detail in chapter 1. The *D-values* differ for the same type of microorganisms in different samples. This is probably due

to the different protection rendered by the different minerals. For example, kaolin does not have inner layers and all microorganisms will be found on the surface layers, whereas vermiculite and perlite have inner surface layers where microorganisms can adsorb. The efficiency of the heat inactivation might, therefore, be limited by the heat conduction of the mineral and penetration of heat into different materials. Moisture content and aeration of the minerals might also be contributing factors. Heat resistance by *Bacillus subtilis* var. *niger* spores has been reported to be dependent on the relative humidity before and during treatment by affecting the water content of spores (Byrne et al., 2006). An increase in humidity during dry- heat sterilisation increased heat resistance. It is important to note that the *D-value* of each microorganism is distinctive of each species, and therefore the *D-values* reported in this chapter correspond to the most heat resistant microorganism.

Table 16: *D-values and R^2 values from the thermal destruction curves of the microorganisms at 160 °C*

Adsorbent	Total bacteria		Coliforms		<i>Pseudomonas aeruginosa</i>		Total fungi	
	D_{160} (min)	R^2	D_{160} (min)	R^2	D_{160} (min)	R^2	D_{160} (min)	R^2
Talc	2.3	0.989	1.7	0.981	2.2	0.994	2.1	0.950
Perlite	3.1	0.996	2.2	0.963	ND	ND	3.2	0.954
Kaolinite	2.5	0.968	1.9	0.988	ND	ND	3.2	0.983
Vermiculite	2.3	0.980	1.8	0.982	ND	ND	2.7	0.998

3.3.4 Power consumption

From an economic point of view, the amount of energy required to sterilise the minerals is of primary importance because it gives an indication of whether applying dry heat sterilisation of these minerals at large scale is feasible. Therefore, the power consumption of the UFE 700 oven used in the dry sterilisation experiments was

assessed. The estimation of the power consumption P_s (kW.h.kg⁻¹) of the oven during the sterilisation of 1 kg of mineral dry weight is given by the expression:

$$P_s = \frac{P_r t}{m} \quad (28)$$

Where P_r (W) is the power rating of power consumption of the UFE 700 oven during the sterilisation process, t (h) is the time interval required to reduce all microorganisms to a concentration below the detection limit. m (kg) is the mineral dry weight. According to the manufacture's specifications, the power ratings of the Memmert UFE 700 oven used in the investigations were 2.88 kW and 4.00 kW at room temperature and once heated, respectively.

The P_s values calculated from the experimental data are presented in Table 17. If dry heat sterilisation were to be employed at industrial scale at a temperature of 105 °C, then heat exposure time would have to be more than 48 h. It is assumed that the P_r value will be somewhere in the range 2.88 kW and 4.00 kW during the sterilisation process. The P_s calculated from the experimental data when the value of $P_r = 2.88$ kW and $P_r = 4.00$ kW range from 1.41×10^4 to 1.51×10^4 kW.h.kg⁻¹ and 1.95×10^4 to 2.10×10^4 kW.h.kg⁻¹, respectively, for the different minerals. At a temperature of 160 °C, the heat exposure time required is 0.25 h. Assuming that $P_r = 4.00$, based on the fact that the oven is hot the P_s values obtained for the sterilisation of the minerals ranged from 1.02×10^2 to 1.09×10^2 kW.h.kg⁻¹. It can be seen that the rate of inactivation and the power consumption is significantly decreased when the temperature of 160 °C is used compared 105 °C. It is, therefore, more efficient and economical to dry heat sterilise the adsorbents at 160 °C.

Table 17: Power consumption of the UFE 700 oven during heat treatment of adsorbents at 105 °C and 160 °C

Adsorbent	P_s (kW.h.kg ⁻¹) at 105 °C		P_s (kW.h.kg ⁻¹) at 160 °C
	$P_r = 2.88$ kW	$P_r = 4.00$ kW	$P_r = 4.00$ kW
Talc	1.41×10^4	1.95×10^4	1.02×10^2
Perlite	1.48×10^4	2.05×10^4	1.07×10^2
Kaolinite	1.50×10^4	2.08×10^4	1.08×10^2
Vermiculite	1.51×10^4	2.10×10^4	1.09×10^2

3.3.5 Morphological identification of fungi

The aim of the investigation was to identify the fungal species isolated from talc, vermiculite, talc and kaolinite. The identification was done by morphological characterization by examining the unique structures of the different species based on their macroscopic and microscopic characteristics to species level (Sahu and Padhy, 2014). The different fungi were subcultured on potato - dextrose agar at least two times to ensure purity and left to grow for at least 14 days. Fig. 20 shows some of the fungi that were extracted from the adsorbents. Fig. 21 and Fig. 22 show pure colonies of fungi after sub-culturing.

The species of fungi were deduced from the characteristic structures observed such as micro or macroconidia, spores and hyphae using the Atlas of Clinical Fungi available at <http://www.atlas.clinicalfungi.org/AtlasOnline/> as a morphological guide. The lactophenol blue cotton stain used in mounting the slide consists of phenol which kills the fungi, lactic acid which preserves the structural integrity of the fungus and cotton blue dye which stains the cytoplasm blue in order to improve contrast (Thomas et al., 2008). Thus lactophenol cotton blue serves as both a mounting medium and stain (Thomas et al., 2008). A total of seven species belonging to five genera of fungi were isolated from adsorbents. The fungi isolated from the adsorbents were mostly the moulds; *Penicillium spp.*, *Aspergillus niger*, *Aspergillus*

fumigatus, *Aspergillus flavus*, *Cladosporium spp.* and *Rhizopus oryzae*. One yeast was isolated and was identified as *Candida albicans*. All controls were negative. Fig. 21 and Fig. 22 show the appearance of the isolated species on potato - dextrose agar and under a microscope.

On the obverse side of the plate, *Penicillium spp.* appeared granular with yellow-green round colonies surrounded by a white border (Fig. 21A). The colonies were surrounded by a bright red pigment. The reverse of the plate was entirely red and clearly demonstrated pigmentation of the colony (Fig. 21B). Its microscopic appearance is characterised by branched conidiophores connected to septate hyphae and its phialides have chains of conidia. The phialides appear as elongated finger-like cells (Fig. 21C). This species was isolated from talc and perlite.

The *Aspergillus* genus was represented by three species of which *Aspergillus niger* was the most common, being found in all the samples. *Aspergillus fluvas* was found in kaolinite and talc. *Aspergillus fumigatus* was found in kaolinite, perlite and vermiculite. On the obverse side of the plate, *Aspergillus niger* colonies appeared to be similar to those of *Rhizopus oryzae*. They appeared woolly white/yellow and become “peppery” (Fig. 21D). Under the microscope, it exhibited long smooth conidiophores and characteristic dark brown phialides on the entire vesicle (Fig. 21F).

Aspergillus fumigatus appeared as velvety white colonies which become green/grey with time on agar surfaces (Fig. 21G). The colonies appeared tan underneath (Fig. 21H). Under the microscope, its phialides appeared clear and well defined in columns on the upper two-thirds of the vesicle which stained bright blue. The conidiophores were long and smooth (Fig. 21I).

Aspergillus fluvas also appeared as flat green and velvety colonies on the obverse side of the plate (Fig. 21J) and gold/brown on the reverse side (Fig. 21K). Its microscopic features were similar to those of *Aspergillus fumigatus* (Fig. 21G) except for phialides which were scattered in all directions, staining pale green and coarsely roughened conidiophores. These features distinguish it from *Aspergillus fumigatus*.

Under the microscope, *Rhizopus oryzae* appeared as cyclic structures (sporangia) held by hyphae. The hyphae of *Rhizopus oryzae* were aseptate and the sporangia had many spores on their surfaces (Fig. 22C). The light blue staining indicates that it is an immature sporangium. When sporangium mature, their spores become dark brown they are ready to be discharged during asexual reproduction. On the obverse side of the plate, the colonies appeared woolly white/yellow and became “peppery” black with time. *Rhizopus oryzae* was isolated from all the samples (Fig. 22A).

On examination of the obverse side of the plate *Cladosporium spp.* appeared as black velvety folded circular coliforms with powdered texture and leather-like consistency (Fig. 22D). Under the microscope, it appeared as dark chains of, branched conidiophores (Fig. 22F). This species was only isolated in perlite samples and was absent in the rest of the adsorbents.

Candida albicans appeared as moist and white coloured pasty agar pasty colonies on the obverse side of the plate (Fig. 22G). It is dimorphic yeast and is capable of growing as a yeast and filamentous cells (Thibane et al., 2010). Under the microscope *Candida albicans* produced true germ tubes and chlamydospores with the lactophenol cotton blue stain (Fig. 22I). *Candida albicans* was isolated from all the adsorbents.

The most frequently isolated species were *Aspergillus niger*, *Rhizopus oryzae* and *Candida albicans*. *Aspergillus niger* was the most dominant species. This was expected because its occurrence is widespread with the highest quantities being found in soil (Kutateladze et al., 2016). It is commonly known to cause a disease called black mould on fruits and peanuts but just like *Rhizopus oryzae*, it is a rarely a human pathogen (Person et al., 2010). The rest of the species are all common human pathogens often isolated from clinical samples (Sahu and Padhy, 2014). *Penicillium spp.* and *Aspergillus spp.* are capable of producing secondary metabolites known as mycotoxins which can cause disease and death in humans (Cunha et al., 2014). *Aspergillus fumigatus* is the most common cause of severe invasive Aspergillosis (Person et al., 2010). *Candida albicans* is a very common human pathogen responsible for opportunistic oropharyngeal and genital infections in humans. It is a major threat to human health in South Africa and causes severe

systemic fungal infections in immunocompromised patients (Thibane et al., 2010). *Cladosporium spp.* can cause allergic reactions and skin infections in human beings (Gu et al., 2016).



Figure 20: *Fungi and yeast colonies on potato dextrose agar*

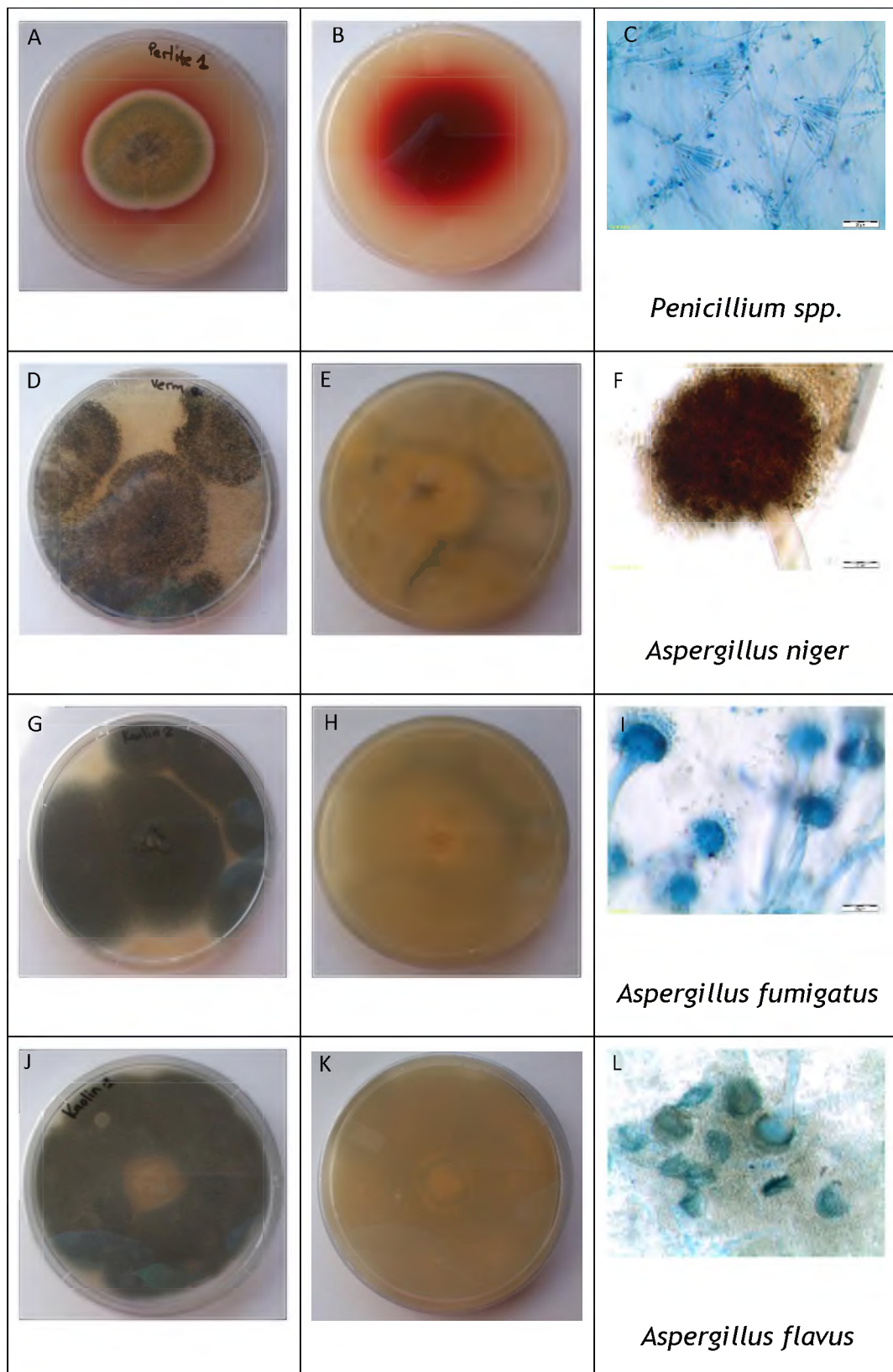


Figure 21: Morphological observation of pure fungal colonies (A) obverse view, (B) reverse view, (C) microscopic view sub-cultured on potato - dextrose at 28 °C for 14 days

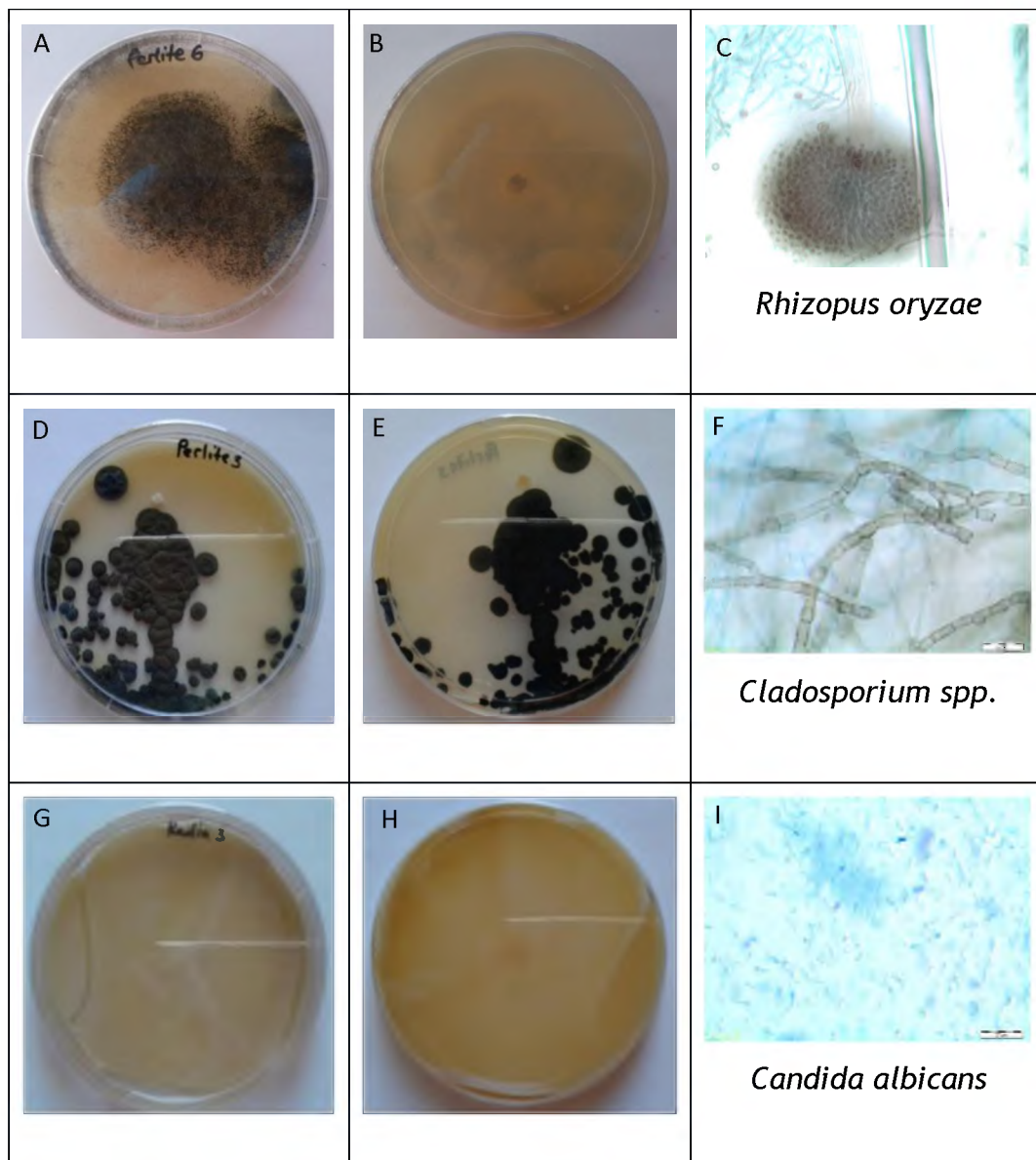


Figure 22: Morphological observation of pure fungal colonies (A) obverse view, (B) reverse view, (C) microscopic view sub - cultured on potato - dextrose at 28 °C for 14 days

3.3.6 Identification of bacteria

3.3.6.1 Gram stain

Gram stain is a well-established test that is usually the first step in the identification of bacterial organism (Saida et al., 2000). Despite the advancement in molecular biology techniques, Gram staining today remains a fundamental step to in bacterial identification (Zywiell et al., 2011). The procedure is used to distinguish bacteria

after application of a sequence of stains based on to their cell wall structure. The cell wall of bacteria is divided into two main categories, either Gram-positive or gram-negative. In Gram-positive bacteria, the outermost layer of the cell wall is the peptidoglycan layer and comprises of polymers of sugars and amino acids. Underneath the peptidoglycan is the cytoplasmic membrane which is predominantly composed of a phospholipid bilayer. The cell wall of Gram-negative bacteria also consists of the layers found in the Gram-positive bacteria cell wall. However, an extra outer coat made up of lipopolysaccharides and protein is present in on the Gram-negative cell wall. The peptidoglycan of Gram-negative bacteria is also thinner than that of Gram-positive bacteria (Yazdankhah et al., 2001).

During Gram staining, the crystal violet added in the first step penetrates and stains all bacteria purple. The iodine added in the second step forms a complex with the crystal violet and the complex is entrapped within the peptidoglycan layer. The alcohol used to rinse the smear of cells alcohol breaks down the outer lipopolysaccharide membrane of gram-negative cells and washes out the crystal violet - iodine complexes from the thinner more porous peptidoglycan layer, unstaining the cells. The peptidoglycan layer of Gram-positive cell walls is much thicker and alcohol can not wash out the crystal violet - iodine complexes from Gram-positive thus retain the crystal violet and remain purple. Finally, the addition of safranin counter stains the Gram-negative cells pink but does not disrupt the Gram-positive cells (Beveridge and Davies, 1983).

Pink and purple rod-shaped cells were observed after Gram staining bacteria were isolated on nutrient agar. This indicated that the adsorbents were contaminated with both Gram-positive and Gram-negative bacteria. Only pink rods were observed in samples isolated on Cetrimide agar and MacConkey agar indication that the bacteria were Gram-negative.

3.3.6.2 KOH string test

The potassium hydroxide (KOH) string test was performed as a confirmatory test for the Gram stain. This is because the Gram stain is not always accurate and some

bacteria exhibit Gram - variable characteristics when subjected to environmental stress, such as the composition of growth medium, temperature, and pH (Saida et al., 2000). For example, some Gram positives such as *Bacillus spp* and *Clostridium spp* often present as difficult species by staining Gram-negative (Beveridge and Davies, 1983). Similar to Gram staining the principle of the KOH is based on the structure of the cell wall of the bacteria. KOH saponifies the lipid-like outer membrane of the Gram-negative bacteria. Gram-negative bacteria tend to lyse when exposed to 3 % KOH resulting from the breaking down of their cell walls. Gram-positive bacteria have thicker peptidoglycan layers and are more resistant to 3 % KOH. When the cell walls of gram-negative bacteria rupture, the inner cellular components are released. The strong alkali nature of the KOH causes the DNA to become more viscous and slimy (Arthi et al., 2003). Hence, stringing when raised. The results obtained from the KOH string test were in correlation with the Gram stain.

3.3.6.3 API® 20E

API® 20E system consists of a series of 20 miniaturised biochemical tests on a single strip and is used to identify *Enterobacteriaceae* and other Gram-negative rods to the species level. The API® 20E test strip is shown in Fig. 23. The normal saline in which the bacterial specimen is suspended rehydrates the desiccated medium in each cell and provides an environment in which the bacteria and the biochemical reagents can react resulting in colour change. Fig. 24. shows colour changes after a test was performed using the API® 20E test strip. The results are assessed against a standard chromogenic chart provided by the manufacturer.

The API® 20E test identified three species of Gram-negative bacteria from the specimens extracted from the adsorbents. The results are presented in Table 18. The identified bacteria were *E. coli*, *Enterobacter cloacae* and *Serratia liquefaciens*. The percentages of identification ranged from 91.8 - 99.8 %, 69.4 - 99.8 %, 61.1 - 85.0 %, respectively. *E. coli* was present in specimens from perlite and vermiculite. *Enterobacter cloacae* were present in specimens from talc and kaolinite. Talc was also contaminated with *Serratia liquefaciens*. Some of the strains extracted from

perlite could not be identified by the API® 20E system and some that were extracted from talc required serological tests.

E. coli is found in human and animal intestines and is an indicator of faecal contamination (Karmali et al., 2010). Most of the isolated strains are not a threat to human health. However, there are some pathogenic strains such as *E. coli* 0157:H7 which can cause serious infections. These pathogenic strains cause infection by producing the verocytotoxin (VTEC) also known as Shiga toxin (STEC) (Karmali et al., 2010). The most common ailments caused by *E. coli* are bloody diarrhoea but in some rare instances, severe complications such as kidney failure may occur (Karmali et al., 2010). *Enterobacter cloacae* is also a human pathogen that responsible for nosocomial infections such as ventilator-associated pneumonia, bacteraemia, and urinary tract infections (Lahlaoui et al., 2012). *Serratia liquefaciens* has been reported to be an opportunistic pathogen which can be a problem for people with lowered immunity (Gray, 2005).

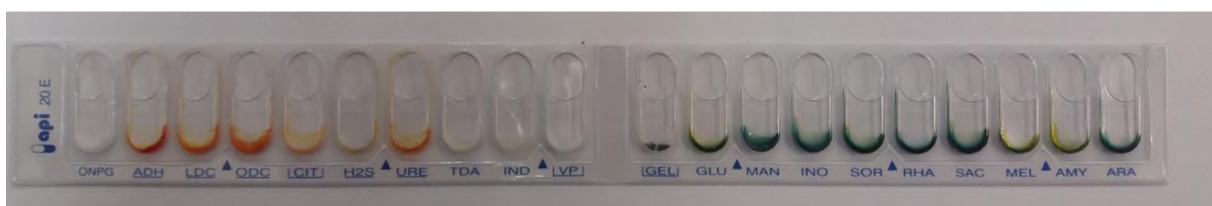


Figure 23: API® 20E test strip



Figure 24: Colour changes after filling the API® 20E test cells inoculated with bacteria specimen

Table 18: API-20E identification of the bacterial strains extracted from (K) kaolinite, (P) perlite, (T) talc, (V) vermiculite and isolated on MacConkey and nutrient agar

Bacterial species	Percentage identification (%)	K	P	T	V
<i>E. coli</i>	91.8 - 99.8		✓		✓
<i>Enterobacter cloacae</i>	69.4 - 99.8	✓		✓	
<i>Serratia liquefaciens</i>	61.1 - 85.0			✓	
Need serological tests	-			✓	
Unidentified spp.	-		✓		

3.3.6.4 DNA extraction, purification and quantification

The Gram-positive specimens and some gram-negative specimens isolated from the adsorbents were identified by DNA sequencing. High-throughput sequencing, particularly 16S rRNA gene sequencing, remains a very important tool in for genetic identification of bacteria in soils and natural environmental samples (Smets et al., 2016). Most sequencing methods require nanograms to micrograms of DNA and this makes DNA extraction a crucial first preparatory step in preparing sequencing libraries. The process involves breaking open the prokaryotic cells to expose the genetic material achieved by mechanical lysis (bead beating and sonicating), chemical lysis (sodium dodecyl sulfate, phenol) and enzymatic lysis (proteinase K, mutanolysin) (Di Bella et al., 2013). In this study, bacterial genome extraction and purification were done using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to manufacturer's instructions. DNA was extracted from bacterial cells and is shown in Fig. 25.

Following extraction, the quality and quantity of the extracted DNA need to be assessed to ensure that an adequate amount of DNA for sequencing is obtained (Hussing et al., 2015). The quantification of nucleic acid concentration is typically done using absorbance methods (Kolitz and Lorsch, 2013). Therefore, to determine the concentration of DNA to be used in PCR a nanodrop was used. The concentrations ranged from 12.9 - 44.5 µg/µL and were sufficient for genotyping.

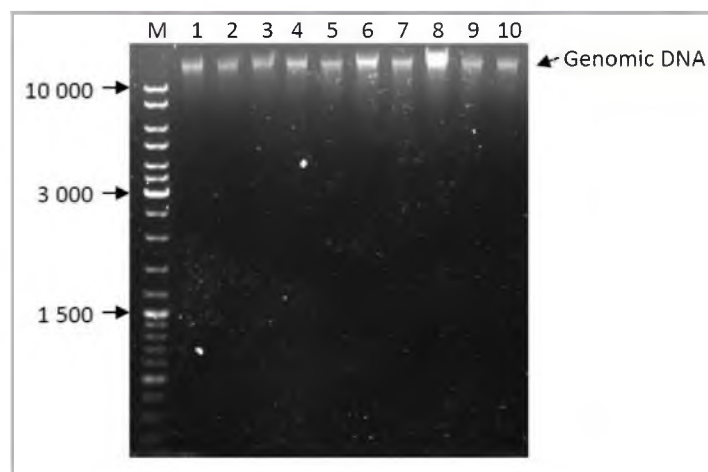


Figure 25: DNA extraction products of the isolated microorganisms

3.3.6.5 Amplification of the 16S rRNA gene by PCR

The 16S ribosomal RNA (16S rRNA) gene is a small subunit ribosomal RNA of prokaryotic cells and is widely used in phylogenetic investigations of microorganisms due to its high stability and low mutation rates throughout the evolution of bacteria, existence in all archaeal and bacterial pedigrees, and a vast amount of variable regions (Kim and Chun, 2014; Zhen et al., 2015). Therefore, universal primers such as the Fd1 and rP2 primers used in this study are usually designed from this highly conserved gene. A primer is a short segment of nucleotides which is complementary to a section of the DNA which is to be amplified in the polymerase chain reaction (PCR) and the sequence produced to taxonomically identify the organisms (Maddocks and Jenkins, 2017).

PCR is an *in vitro* technique used in molecular biology to synthesise multiple copies of a particular sequence of DNA. PCR can be used to achieve many different outcomes such as quantifying RNA and DNA, isolating or mutagenising specific DNA fragments, cloning cDNA and genomic DNA, sequencing and end labelling DNA, and altering sequences to study gene expression. The working principle of the technique is based on the highly sophisticated kinetic interactions, which occur during a series of 20-40 repeated temperature cycles, between the template DNA, product DNA, oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), buffer, and DNA polymerase (Hoy, 2013).

The standard PCR thermocycling involves four steps. Step 1 is an optional activation step, only employed when DNA polymerases that require heat activation are used. It typically consists of a single cycle of heating up to a temperature of about 95 °C, which is held for 10 min. Step 2 is the denaturing step whereby the DNA double strand is separated into single strands by holding the reaction at 90 - 95 °C for 15-20 seconds. The temperature breaks the hydrogen bonds between the complementary base pairs resulting in the unwinding of the DNA. Step 3 involves annealing the primer to the single-stranded target DNA by base pairing and is typically achieved by holding the reaction at 55 - 65 °C for 30 seconds. The cooling allows for the oligonucleotides primers to bind to target sequences. Step 4 is the DNA polymerization step and involves the synthesis of new DNA from the 3' end of the primer achieved by holding the reaction at 72 °C for 1.5 min (Caetano-Anollés, 2013; Whitney et al., 2004). Steps 2 - 4 involve DNA amplification and are repeated at least 25 times yielding an increase in the number of copies of DNA to about 2^n , where n is the number of cycles.

The standard parameters do not always give clean and specific results. Each PCR protocol is unique to a single experiment and thus every new experiment requires optimisation. Optimisation is achieved by adjusting reaction components and cycle parameters to best suit the experiment, particularly the primers, template DNA, buffer, polymerase, dNTPs and incubation, annealing temperature, cycle number, respectively (Hoy, 2013).

3.3.6.6 Optimisation by temperature gradient

The annealing temperature is a very important parameter of PCR that significantly affects the quality of the PCR product. Thus, a preliminary establishment of the appropriate annealing temperature is essential. In this work, the optimum annealing temperature was empirically determined by performing a PCR gradient at 7 different temperatures spread across the range 50 - 65 °C. As seen in Fig. 26, the best results were obtained when the annealing temperature was in the range 59.1 - 65 °C. Lower annealing temperature resulted in smearing due to non-specific amplification. Therefore, based on these preliminary results the annealing temperature of 65 °C

was used for the actual PCR amplification. The annealing duration is also very important and for this study, it was for a period of 1.5 min. Shortening the annealing duration has been reported to reduce products or result in a totally unsuccessful amplification (Saruta et al., 1995).

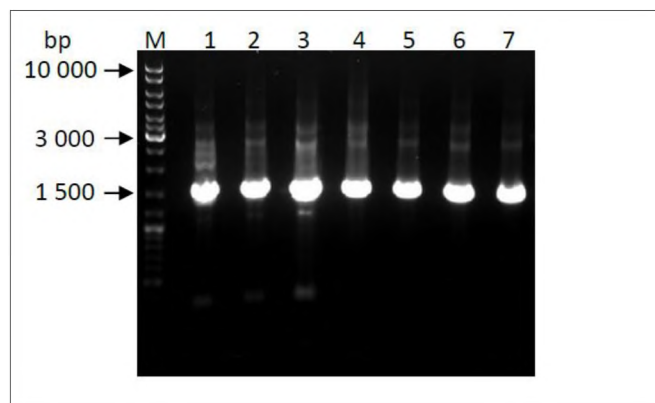


Figure 26: Optimization of annealing temperature by temperature gradient; (1) 50°C, (2) 52.9°C, (3) 55.7 °C, (4) 59.1°C, (5) 62°C, (6) 63.8°C, (7) 65°C

3.3.6.7 PCR

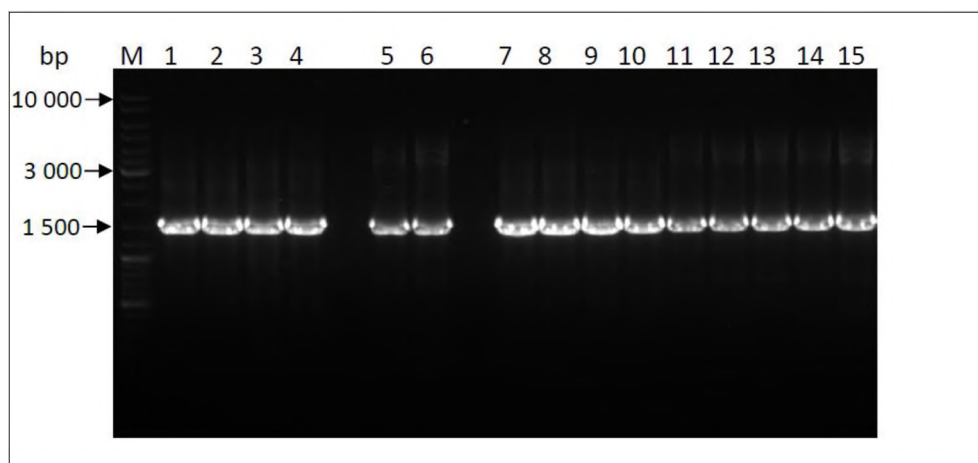


Figure 27: Visualization of final PCR products

Fig. 27 shows some of the final PCR products. The gel electrophoresis results show that very strong bands of the amplified products were detected at the expected size (1500 bp). This indicates a successful amplification of the 16S rRNA which consists of 1500 nucleotides (Saruta et al., 1995).

3.3.6.8 Gene sequencing and comparison with the bioinformatics database

Classification and identification of prokaryotes is mainly accomplished by matching the gene sequences, often 16S rRNA gene sequences, against a database of known sequences. 16S rRNA gene sequences are mostly used to calculate pairwise sequence similarities and phylogenetic analyses after aligning multiple sequences resulting in the species-level identification and genus classification, respectively (Kim and Chun, 2014). At present, many computational methods have been developed and abundant sequencing data is readily available in public databases. Sequence analysis revealed the presence of *Exiguobacterium spp.*, *Pseudomonas aeruginosa*, *Bacillus spp.*, and *E. coli* in the adsorbents. *Bacillus spp.* were identified from specimens isolated from all the adsorbents. *E. coli* was confirmed to be only present in perlite and vermiculite, *Exiguobacterium spp.* and *Pseudomonas aeruginosa* were only present in vermiculite and talc. *Exiguobacterium spp.* are thermophilic bacteria that can be found in hot springs. They are not pathogenic to humans (Sen et al., 2016).

3.4 Conclusions

The experiments described in this chapter showed that kaolinite, talc, perlite and vermiculite were heavily contaminated with microorganisms. The fungi isolated from the adsorbents were mostly the moulds; *Penicillium spp.*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Cladosporium spp.* and *Rhizopus oryzae*. One yeast was isolated and was identified as *Candida albicans*. The bacteria identified were *E. coli*, *Enterobacter cloacae*, *Exiguobacterium spp.*, *Pseudomonas aeruginosa*, *Bacillus spp.* and *Serratia liquefaciens*. Some of these bacteria, moulds and yeasts are pathogenic and pose a threat to human health. The LI values obtained for adsorbents indicated that it is highly unlikely that microorganisms would be leached out of the adsorbents by rain. Heat inactivation of the microorganisms at a 105 °C was totally unsuccessful. However, it was established that a dry heat dose of 160 °C for at least 15 min was sufficient to eradicate all microorganisms present in the adsorbents.

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