Addis Ababa University

School of Graduate Studies

Department of Microbial, Cellular and Molecular Biology

Assessment of Biological and Chemical contaminants of urine-derived struvite fertilizer

By: Nebiyat Nigusie Woldeyohannis

University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbial, Cellular and Molecular Biology (Applied Microbiology)

Addis Ababa University

Addis Ababa, Ethiopia

December 2024

DECLARATION

This is to attest that the thesis prepared by Nebiyat Nigusie Woldeyohannis, entitled *Assessment of Biological and Chemical contaminants of urine-derived struvite fertilizer,* submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy (Applied Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Candidate's Name: Nebiyat Nigusie Woldeyohannis

Signature:---------- Date: ----------

Advisor: Adey Feleke Desta

Signature ---------- Date----------

DEDICATION

This thesis is dedicated to my parents Tiberhi Woldearegay and Nigusie Woldeyohannis (my late father), to my uncle Amanuel Woldearegay, to my siblings Daniel, Yohannis, Eden, Temesgen, Kaleb and Eleni Nigusie and to my friends Estemar Bizuneh and Esrom Aschenaki.

ACKNOWLEDGMENTS

I would love to acknowledge my supervisor Dr Adey Feleke for giving me a continuous and sustained supervision devoting her energy and time. Then my gratitude goes to Dr Tesfaye Alemu for his support to introduce me to Dr Adey when I told him my interest in waste treatment. I would love to acknowledge Mr Elias Yasin and Miss Birtukan Getnet for their support to locate urine collection sites. Then my gratitude goes to Mr Johnny Girma for reactor construction and for small scale process optimization. Then my acknowledgment goes to Mr Gebreab Teklebirhan for giving advice on tools used in locating pathogens in sequence data. Mr Mesfin Diro is acknowledged for his continuous support on consulting in data analysis. Mr Mesele Admassie is then acknowledge for his support in publishing the contents of the thesis data. Dr Haddis Beyene a Psychiatrist is acknowledged for his continuous assistance on consulting me on my mental health throughout the work stress journey of the PhD. At last, but not at least I would love to acknowledge people who supported me to finish my PhD by creating a comfortable environment both at the University and at home. These are Prof Gurja Belay, Senait Gebrehiwot, Mr Melaku Minassae, Mr Aregawi Tsegay, Miss Yemisrach Asefa, Danat and Alula Daniel, Miss Tigist Miraesa, Mr Sisay Alemu, Mr Ayele Gucho, Miss Adey Melesse, Mr Natnael Girma, Miss Hemen Tesfaye, Miss Mesenbet Merkeb, Mr Tedros Amare, Mr Mesfin Tsegaye, Mr Eshetu Yilma and Mr Mesfin Engdawork.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

ACN	acetonitrile
Al	aluminum
AMR	antimicrobial resistance
ARG	antibiotic resistance genes
bp	base pair
BV-RBC	bacterial and viral bioinformatics resource center
cc	common cartridge
CAV	collision cell accelerator voltage
cDNA	copy DNA
Cefo	cefotaxime
Cipro.	Ciprofloxacin
COD	crystallography Open Database
FQs	flouroquinolons
GIZ	Deutsche Gesellschaft für Internationale Zusammenarbeit
HLB	hydrophilic-Lipophilic Balance
HPLC	high performance liquid chromatography
LC-MS/MS	Liquid Chromatography mass spectrometer condition
LOD	limit of detection
LOQ	limit of quantification
m/z	mass to charge ratio
MGRAST	Metagenomic Rapid Annotations using Subsystems Technology
MGS	metagenome-derived genome sequence
MMC	matrix-matched calibration curves
MRM	multiple reaction monitoring
MRSA	methicillin resistant *staphylococcus aureus*
MS/MS	triple-quadrupole mass spectrometer
N50	median and mean length of a set of sequences
NCBI	National Center for Biotechnology Information
OTU	operational taxonomic unit

PCR	polymerase chain reaction
Struvite-K	Potassium struvite
Struvite-NH4	Ammonium struvite
UDDT	urine diversion dry toilets
UDT	urine diversion toilet
UHPLC	ultra-High Performance Liquid Chromatography
WHO	world health organization
WLR	within-laboratory reproducibility

ABSTRACT

Nitrogen (N), phosphorus (P), and potassium (K) are nutrients that are essential for food production and are often referred to as NPK: NPK fertilizers are a crucial part of modern agriculture. Urine is one of the rich sources of nitrogen, phosphorus, and potassium and can be converted to struvite a kind of orthophosphate mineral that is precipitated by Mg, ammonium, and phosphate ions in equal molar ratios.($MgNH_4PO_4.6H_2O$) and its derivative struvite-K, potassium magnesium phosphate hexahydrate ($MgKPO_4{\cdot}6H_2O$) to serve as fertilizer.However, it is commonly contaminated with bacterial pathogens, plasmids, phages, and residual antibiotic drugs which are hazardous to human and animal health. The goal of the current study was to assess the presence of these contaminants and their fate during the optimal process of producing struvite and its derivative K sample is a sample of potassium magnesium phosphate hexahydrate (MgKPO4·6H2O), from composite human urine. To this end, fresh pee, stored urine, and struvite samples throughout the struvite synthesis process were collected for DNA extraction and residual antibiotics analysis for Penicillin G, Cephalosporin, Cefotaxime, Ciprofloxacin, Tetracycline, Oxytetracycline and Doxycycline, Sulfamethoxazole. The community structure of the bacteria (pathogen) was studied with shotgun metagenomic and bioinformatic analysis, whereas the chemical contaminants were analyzed by ultra-high liquid chromatography mass spectrometery. The data showed that the Gram negative, Pseudomonadota (syn, Proteobacteria) was the most dominant bacterial phylum isolated from accounting for 60% and 43% followed by the Gram positive Bacilliota (Firmicutes) with 25% and 39% of the fresh and stored samples, respectively. The phylum Bacilliota (61%), Pseudomonadota (18%), and Bacterodota (12%) dominated the struvite-K sample. Amongest the Phylum Proteobacteria, members of the gamma-Proteobacteria displayed OTU counts of 12,116 on fresh urine samples which was twice (6,155) more than the OTU counts recorded from struvite (stored) samples. The Genome sequence analysis also showed that the genera Streptococcus, Bacillus, and Escherichia phages predominate; in fresh urine, where the Streptococcus phage is almost 50% of the OTU counts. Interestingly, the data showed he stored urine contained higher number of genes for antibiotic resistance than the fresh urine samples. Acinetobacter, Aeromonas, and Enterococcus are the key pathogens analyzed, whereas the top five resistance genes in all three samples were the aminoglycosides, carbapenem, chloramphenicol, erythromycin, and efflux pump. Despite a change in gene families, the

carbapenem, aminoglycoside resistance, and efflux pump families were found to be persistent in struvite. Ciprofloxacin, cefotaxime, oxytetracycline, and tetracycline were among the antimicrobials. The calibration curves showed linearity coefficients (R^2) ranged from 0.9778 to 0.9945. Tetracycline's mean recovery percentage was 92.86 ± 7.26, while sulfomethoxazole's ranged from 71.46 ± 12.62. However, the intra-day precision, which ranged from 5.05 percent for oxytetracycline to 17.66% for sulfomethoxazole, satisfied EPA and EU standards. The potential antibiotics had limits of quantification and detection that varied from (1.47-0.91) µg/kg and (3.03-9.77) µg/kg, respectively. Among the assessed antibiotics none was detected in the product struvite. Based on this study, the presence of pathogen genomes, antibiotic resistance genes and their carriers requires due attention during urine sanitization. The absence of residual antibiotic drugs in struvite indicates its potential safety for agricultural application while further study on the mechanism of disappearance is recommended.

Key words: *struvite, human urine, fertilizer, mobilomes resistomes, pathogens*

CHAPTER ONE

1. INTRODUCTION

Human excreta is the bulk of domestic wastewater, also called sanitary sewage, discharged from homes. In a recent review, Du *et al.* (2024) gave a historical background on the use of human excreta within the context of theoretical interpretations and ecological significance. Thus, human excrement was used as fertilizer in China emerged in the Western Han Dynasty (B.C. 202–A.D. 8), such records did not become prevalent until the Southern Song Dynasty (A.D.1127–1279). Human excrement was so important to agricultural development in China that John Macgowan (A.D. 1835−1922), wrote that "human excrement, affordable and of good quality, was one of the best fertilizers applied by Chinese farmers, and that there would be no China of the day without excrement (Macgowan, 1909). It was even considered "top-class fertilizer" and served as the most essential fertilizer in farmland fertilization.

According to German chemist Justus von Liebig (A.D. 1803-1873), China was a fertile country, the farmland fertility of which kept improving instead of deteriorating after three thousand years of farming. In his 49th "Letter on Chemistry," the chemist highly praised that, without the guidance of any scientific theories, Chinese farmers had found a miracle the recycle and utilization of human excrement (Radkau, 2008) Karl Max (A.D. 1818-1883) also held that the consumption of human excrement was vital to agriculture. While capitalism was wasteful of excrement, people were significantly economical on excrement in Asia, such as southern China (Marx, 1993).

In the early 20th century, on his way to China, American soil scientist Franklin King (A.D. 1848-1911) signed, "We had gone from practices by which three generations had exhausted strong virgin fields, and were coming to others still fertile after thirty centuries of cropping." After his visit, he pointed out explicitly in his world famous Farmers of 40 Centuries; or, Permanent Agriculture in China, Korea, and Japan, "One of the most remarkable agricultural practices adopted by any civilized people is the centuries-long and well-nigh universal conservation and utilization of all human waste in China, Korea, and Japan, turning it to marvelous account in the maintenance of soil fertility and in the production of food" (King, 1911; Song, 2017)

Household wastewater can be divided into three fractions by origin; urine, faeces and grey water. (Aniko, 2015). The authors showed that ddistribution of pollution load of urban wastewater shows that 99% of bacteria originated from faeces; 11% of N-content originated from faeces, 87% from urine and 2% from greywater (GW); 40% of P-content originated from faeces, 50% from urine, 10% from GW; 47% of organic matter content originated from faeces, 12% from urine, 41% from GW. So, 99% of bacteria, 98% of N-content, 90% of P-content and the total amount of drug residues and hormones are in the human excreta, which is less than 2% of the total wastewater volume.

Once human excreta are removed by water from households, then we are not able to regain its original organic material compounds. During flow into the sewer system human excreta already begin to degrade. Urease enzymes hydrolyse the urea content of urine and this reaction generates ammonium ion and CO2 (Diasi *et al.*, 2024). During waste water treatment the ammonium is oxidized to nitrate and the organic phosphorus compounds of human excreta are converted into inorganic phosphates. So, we can say that the valuable organic N and P compounds of human excreta are converted into water pollutants by waste water treatment.

Most people sadly are not aware of the fact that the greatest environmental harm is not this water load, but withdrawal of the very valuable organic matter content of human excreta from the cycle of biosphere (Zseni and Nagy, 2016). This leads to a vicious circle discharging human excreta to freshwater: waste water treatment convert human excreta to water pollutant, while we replace missing nutrients to soils artificially, which lead to the exploitation of soils in the long run.

Urine make up less than 1% of municipal wastewater flows but contains 80% Nitrogen, 56% of phosphorus and 63% of potassium (Randall and Naido, 2018). The largest nutrient and smallest heavy metal contents are found in the urine, which is easily collected separately using a urine-diverting toilet. The urine diversion from wastewater can improve conventional wastewater treatment plants to be energy-efficient and cost-effective, as a considerable quantity of nutrients in wastewater is derived from urine.

Traditionally, nutrients in the solid and liquid wastes are considered as a waste that needs to be removed however the recent concept has been changed to look into nutrient as a resource for recovery and reuse (circularity) rather than as a waste (linearity) (Diaz *et al.*, 2022; Jiménez-Arias *et al.*, 2022).This new concept is driven mainly by two major issues: very high energy and carbon

footprint of the synthetic fertiliser nutrients (mainly nitrogen-based fertiliser) and phosphorus being a finite resource with mine reserves limited to only few countries. The source separated urine is much concentrated form for more effective nutrient recovery and reduces nutrient loading in the WWTP which can help reduce wastewater treatment costs (Zseni and Nagy, 2016). There has been a global wave of efforts to separate urine from sewage and recover nutrients as fertiliser, with initiatives observed in countries such as the United States, Australia, Switzerland, South Africa, and Ethiopia (Sohn *et al.*, 2023). However, urine diversion toilets, which are the first step in this paradigm shift, are still limited to off-grid locations with poor toilet infrastructure and low-income settings.

Pilot projects in Europe and Thekwini, South Africa have shown the limitations of urine diversion toilets, which include being unwieldy, smelly, and unreliable (Sohn *et al.*, 2023). Moreover, it is impractical to retrofit urban settings with separate sewer pipes to transport urine to a central location. It is worth noting that the widespread adoption and success of nutrient recovery from urine would also be contingent upon the socio-economic context of a region or country, including factors such as cultural acceptance and regulatory frameworks to support sustainable practices (Udert *et al.*, 2003).

Struvite can be produced with less microbial load if drying and urine storage are reasonable (Lahr *et al*., 2016; Bischel *et al*., 2014) It is also inconvenient for most microorganisms, since the ammonia in urine kills microorganisms during storage step before struvite making (Lahr *et al.,* 2016). WHO, 2006 also reported that urine stored at 20 ^{0}C for 1 month is safe to fertilize plant foods which are going to be processed further but not eaten raw. However, nothing was reported yet about the fate of antibiotics resistance genes and their carriers together with pharmaceuticals in urine derived fertilizer.

Thus, we hypothesised that struvite can also be a dish of different hazardous biological and chemical contaminants which can be dangerous for human, animals and the environment. If urine sanitization does not consider these two. These biological contaminants are antibiotic resistant genes in which they may distinctly spread to the genomes, plasmids and phages of pathogenic microbes via horizontal gene transfer. These resistance genes may have the ability to enter in to the food chain then to potential pathogens. This gives an advantage to pathogens to be untreatable. On the other hand, genomes of pathogens from struvite may also transform and convert soil

microbial strains. On the other hand, the chemical contaminants are hormones and other pharmaceuticals such as antibiotics taken by human beings to work in their physiology to fight disease. These chemicals are long persistent therefore can affect unimportantly the physiology of other organisms in the environment which are exposed to these (Halling-Sorensen *et al*., 1998). Thus, before applying struvite on soils as fertilizer there is a need to assess its safety from both sources of the contaminants.

Therefore, pertinent information about such contaminants should be gathered along with proper methods that need to be designed and implemented to avoid these hazardous contaminants before applying struvite on soils as a fertilizer.

1.2. Objectives of the study

1.2.1. General Objective

The overall objective of this study is to evaluate the occurrence and fate of pathogenic microorganisms, antibiotic resistant genes and pharmaceuticals in urine-derived struvite fertilizer.

1.2.2. Specific Objectives

The Specific objectives are:

- To investigate the microbiome of composite urine collected for struvite production
- To determine the microbiological safety of struvite produced from urine using optimal process parameters
- To evaluate the presence and fate of mobile genetic elements (MGE) in struvite
- To examine pharmaceuticals in urine and struvite
- To analyse the chemical composition of struvite compound

Hypothesis

H_o: Human urine and its derived struvite contain pathogenic microbes, antibiotic resistant genes and pharmaceuticals which are very dangerous to health and unsafe to the environment.

CHAPTER TWO

2. LITERATURE REVIEW

Human excreta had been used since the past centuries in different parts of the world. Human urine, when separated from the rest of the wastes contains N, K and P in economically feasible amount which recovery can be reasonable. Especially, producing socially acceptable fertilizer from human urine is a paramount though concerns of safety from pathogens, resistance genes and their carriers together with pharmaceuticals consumed by urine donors is there. These concerns are revealed by different works claimed in this section. For example, antibiotic resistant bacteria were observed to migrate from the soil amended with pig manure to phylosphere of the plant observed. On another occasion, on different works, plants were observed to absorb residual antibiotics from soil amended with waste water sludge. This shows the importance of assessing the fate of the contaminants in the human urine derived fertilizer.

2.1. History of reuse of human excreta

Human excreta have been used for crop fertilization in many countries since the earlier times (Du *et al*., 2024). In Japan utilization of human excreta for agriculture is thought to be introduced in the 12th century (Esrey *et al*., 1998; King, 1911; Song, 2017). Urine used to be separated and considered as valuable fertilizer in Japan (Sene, *et al*., 2019). In China, human and animal excreta were composted for thousands of years (Esrey *et al*., 1998; Du *et al*., 2024). Ancient Koreans had the practice of separation of urine and faeces and prevented mixing of water to the excreta (Han and Kim, 2014). They had special containers to transport the urine and faecal matter separately (Figure 1). In Sweden, the fertilizing content of urine was known to be 6 times the faeces since 1867 (Drangert, 1998). In Germany, urine was used to be separated from faeces to produce manageable proportion of fertilizer (Wiedner, *et al*., 2015). Other than its fertilizer uses in ancient Rome, human urine was used for tanning hides and production of gun powder (Heth, 2015). In Denmark, stored human urine was used as a detergent for washing clothes (Drangert, 1998). Similarly in Sweden, human urine was as an agent to dry wounds and as therapy for internal problems via mouth intake (Marshall and Davis, 1914).

In most parts of Africa, use of human urine or faeces for agriculture was not explicitely reported and human excreta is considered to be a taboo (Anderson, 2015; Adewole, *et al.*, 2013; Timmer

and Visker, 1998). Despite this, modern day reports indicate that dried faeces were utilized as fuel after separating from urine by evaporation in Yemen (Esrey, *et al*., 1998) and urine was claimed for its medical and antiseptic purposes in South Africa (Okem, *et al*., 2013).

Figure 1: Manual containers to transport urine (left) and faeces (right) in Korean tradition (Han and Kim, 2014)

2.2. Overview of human urine

Human urine is one of the human excreta rich with different chemical compounds. It is produced as a result of metabolism in human body when body cells produce nitrogenous wastes, various chemicals and water that are removed through the urinary system. Urine is colourless to amber in colour. It contains very small carbohydrate. Human urine is part of the nitrogen cycle. It is with a PH range of 5.5-7 (Richert *et al*., 2010) and specific gravity of 1.003–1.035.

Normal human urine consists of nitrogen, phosphorus, potassium, sodium, calcium, magnesium, sulphur, bicarbonate, chlorine, zinc, iron, manganese, copper, creatinine, urea, uric acid, water, proteins, hormones, metabolites, urobilin (responsible for amber color of urine) and vitamins (B) (Bouatra *et al*., 2013). About 85% of the Nitrogen in urine is found in the form of urea (Lahr, *et al*., 2016). In stored urine the urea get hydrolyzed into ammonia (Lahr, *et al*., 2016). Additionally, urine contains sufficient amount of organic compounds to support microbial growth (Zhang *et al*., 2013). Uric acid and trace levels of heavy metals are found in small amounts under healthy circumstances (Bouatra *et al*., 2013). There is production of organic acids such as acetic, propanoic and butyric acids in stored urine, which are produced by microbial actions (Zhang *et al*., 2013). These acids are responsible for the pungent smell of stored urine together with the ammonia (Zhang *et al*., 2013).

The earlier thought that connotes urine as a sterile fluid is being revised through numerous studies that indicated the presence of various groups of bacteria (Larsen and Monif, 2001). Microbiota-based Studies indicate that urine collected from infants contain *Lactobacillus* (Ackerman and Chai, 2019). Urine collected from individuals of age one till puberty was dominated by diphtheroids, *S. epidermidis*, streptococci, and *E. coli.* Urine collected from individuals at puberty and beyond upto adulthood is expected to be contaminated by *L. acidophilus,* corynebacteria, peptostreptococci, staphylococci, streptococci, and Bacteroides (Larsen and Monif, 2001). In females after menopause, urine was shown to be contaminated with yeasts such as Torulopsis and Candida while the microorganisms in adult male human urine are mainly *Lactobacillus* spp. (Larsen and Monif, 2001). Quantitiative study showed that urine contains as small as 240 microbes if collected clean catch from a healthy adult (Lahr, *et al*., 2016), dominated by the following bacterial orders; *Actinomycetales*, *Bacteriodales*, *Clostridiales, Enterobacteriales, Lactobacillales and Pseudomonadales*, if not contaminated with faecal and vaginal microbiota (Richert *et al*., 2010).

Undiluted urine was observed to contain 3-7g N per liter and around 1g of P per liter (Richert *et al*., 2010 ; Lahr *et al*., 2016) which this makes it a nice N and P source to produce struvite. Thus, urine becomes an option as source of a good quality fertilizer since it is eco-friendly to recycle waste especially it is environmental friendly since it is surplus all the time and its manageability to a slow releasing struvite. It is also a win-win phenomenon to use it since it is also answering sanitation issues.

2.3. Source-separation of human urine

Source separation of urine is an act of separation of urine from its origin and treating separately. Source separation of urine is derived from Terra Preta Sanitation, an effieicent method for the management of human excreta in the ancient Amazonian Civilization (de Gisi, *et al.,* 2014). Currently, Urine Diversion Dry Toilets (UDDT) are the commonly used designs for toilets that separately collect urine and faeces (Mnkeni and Austin, 2009; Münch and Winker, 2009). The urine fraction of the wastewater contains 70-80 % of the nitrogen and 50% of the phosphorous and potassium while in volume, urine constitutes only 1% of the wastewater (Wilsenach and van Loosdrecht, 2003).

A premier quality in urine for making source separation important is that urine contains a very low amount of heavy metals compared to the faecal matter, which is loaded with various heavy metals

in high concentration (Ronteltap *et al*., 2007; Jönsson *et al*., 2000; Jönsson *et al*., 1997; Jönsson *et al*., 1999 ; Johansson *et al*., 2001; Stephanie and Treavor, 2015 and Vinnerås, 2001). It would be a loss if we mix such a solution with large amount of heavy metal content, the other fractions of the waste water. Besides urine separated toilets were observed to save flush water by 80% (Jönsson 2000; Jönsson *et al*., 1997; Jönsson *et al*., 1999 and Johansson *et al*., 2001 and Vinnerås, 2001).

Source separation is found to be more effective in recovering plant nutrients with reports of harvesting 27 times more N, 35 times more phosphorous and 25 times more potassium than the conventional system (Jönsson, *et al.,* 1997; Jönsson, *et al.,* 1999 and Johansson, *et al*., 2001). Source separation of urine saves energy of about 32 MJ/ person and year since it decreases nutrients from waste water system along with urine replaced chemical-fertilizers, which requires 75MJ/ person and year to produce (Larsen and Gujer, 1996 and Vinnerås, 2001). Thus, urine separation saved a total of 63MJ/ person and year since it only requires 44MJ/ person and year for transporting the urine mixture 33 km with a truck to a farm and spread it as fertilizer (Liu *et al.,* 2013).

The potential of urine as Nitrogen source was found to perform as 90% to that of the commercial ammonium-nitrate fertilizer according to studies in Sweden (Johansson *et al*., 2001). The phosphorous effect was found out to be equal to that of the chemical fertilizer (Kirchmann and Petterson, 1995 and Hellström *et al*., 1999). Experimental studies on barley, oat and wheat showed the absence of toxic effect observed from ammonia.

Though source separated human urine has a paramount advantage in agriculture, the presence of pharmaceuticals (such as propranolol, ibuprofen, diclofenac and carbamazepine), hormones (such as estrone, estradiol and ethinylestradiol) require attention due to potential accumulation through long-term usage. In order to alleviate the issues with regard to traces pharmaceutical and biological hazards, pre-treatment with membrane filtration (Prakash *et al*., 2004), electro dialysis with ozonation (Lamichhane and Babcock, 2012) or ion-exchange treatment (Landry and Boyer, 2013) from urine were claimed necessary. But these treatments are not effective, for example nanofiltration can not avoid dalton size pharmacetucal residues and ozonation changes only the chemical nature of pharmaceuticals without total elimination. Besides, the proposed methods are

expensive for low-income countries and cause nutrient loss during treatment. So, according to experiments on the fate of pharmaceuticals scientists have studied (Ronteltap *et al*., 2007) during struvite precipitation and found promising results for future use of struvite for agriculture.

2.4. Struvite as fertilizer

Historically, the term struvite is referred as a type of kidney stone, made of magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6H_2O$). They are formed when urine becomes more alkaline, often due to a urinary tract infection. It was later used to identify a slow-release fertiliser for sustainable phosphorus management (Talboys *et al.*, 2015). It is extracted from recycled sources of phosphorus (P), from wastewater, with a potential to substitute for more soluble manufactured fertilisers and help reduce the long-term threat to food security from dwindling finite reserves of phosphate rock (PR).

Struvite compounds are characterized by the formula A^{+2} B^{+} $PO4^{-3}$ yH_2O, where A^{+2} depicts a bivalent cation like (Mg^{+2}, Mn^{+2}, Ca^{+2}); B^{+} is a monovalent cation such as, Rb^{+}, Mn^{+}, K^{+}, $NH4^{+}$ Na^{+} or Ti^{+} whereas y=6-8 (Banks *et al*., 1975, Mathew *et al*., 1979). Historically, struvite was considered a product of disturbance in wastewater treatment plants (WWTPs) rather than a recoverable product for use as a fertilizer. Struvite usually creates a major maintenance problem by blocking piping and preventing flow in the side stream biological nitrogen and phosphorus removal treatment plants and anaerobic digesters (Le Corre *et al*., 2009). The high concentrations of phosphorus and nitrogen combined with turbulence of flow encourage the spontaneous precipitation of struvite in pipes and pumps along with digesters, thus decreasing the treatment capacity

Struvite crystals or urine derived fertilizer is another option of urine fertilizer. Struvite-NH_4 is a crystal or powder with its average size 2±3.8 mm (Abe, 1995; Etter *et al*., 2011; Wilsenach *et al*., 2007; Liu *et al*., 2008a, 2008b; Antonini *et al*., 2011; Decrey *et al*., 2011 and Winker *et al*., 2009). It is a phosphorus, nitrogen and magnesium rich fertilizer which contain them in 13, 5 and 10% respectively (Rahman *et al*., 2014; Etter *et al*., 2011; Wilsenach *et al*., 2007; Liu *et al*., 2008a, 2008b; Antonini *et al*., 2011; Decrey *et al*., 2011 and Winker *et al*., 2009). Struvite-NH_4 can be made from MgO addition for P recovery through $MgNH_4PO_46H_2O$ or from a mixture of MgO and Na_3PO_4 for maximum P and N recovery through $NH_4H_2PO_4$ (Liu *et al*., 2013).

Struvite-NH_4 is an alternative eco-friendly phosphate fertilizer to maintain agricultural production (Munch and Barr, 2001, Shu *et al*., 2006 and Massey *et al*., 2007). It is also a cheap source of N and P for fertilizer industries. On the contrary rock phosphate is a non-renewable source of phosphate fertilizer. The present consumption of it reaches over one million tonnes yearly (Rahman *et al*., 2011). On the other hand nitrogen fertilizer consumption reaches 3 million tonnes per year (Rahman *et al*., 2011). These amounts of fertilizers are produced from consumable sources and require unreasonably high energy which make these fertilizers non eco- friendly.

Struvite-NH_4 is produced in an equimolecular concentration of Mg^{+2}, NH^{+}_4, PO^{-3} at alkaline condition (7.5-9) (Hao *et al*., 2008 and Bouropoulos and Koutsoukos, 2000). It is made according to the following chemical reaction: $Mg^{+2} + NH^{+}_4 + H_2PO^{-}_4 + 6H_2O \rightarrow MgNH_4PO_4.6H_2O + 2H^{+}$. It is made by addition of Mg to waste water or human urine. It has a molecular weight $245.43 gmol^{-1}$. It is sparingly soluble in neutral and alkaline solutions but readily soluble in acid (Chirmuley, 1994). Its solubility is $0.018g\ 100ml^{-1}$ in water and $0.178g\ 100ml^{-1}$ in 0.01N HCl at 25˚C (Le Corre *et al*., 2009) and its solubility constant is $10^{-13.26}$ (Ohlinger *et al*., 1998). Efficiency of struvite crystallization depends on concentration and molar ratios of $Mg^{+2} + NH^{+}_4 + PO^{-}_4{}^3$, PH, aeration rate, temperature and presence of Ca^{+2} in the reacting mass (Stratful *et al*., 2001, Le Corre *et al*., 2005, Hao *et al*., 2008 and Yetilmezsoy and Zengin, 2009). Struvite-NH_4 is heat sensitive. It should not be heated above 40-50˚C to avoid the risk of ammonia loss (Bhuiyan *et al*., 2008 and Frost *et al*., 2004).

It is a compound that its nutrients not flashed faster by rainfall; this makes it a good fertilizer (Lee *et al*., 2009). It releases its nutrients very slowly in water. Struvite-NH_4 is a slow leaching nutrient fertilizer, unlike the rock phosphate and urea fertilizers which are exposed to fast leaching of N and P, volatilization of NH_3 and denitrification. This leads to surface water contamination which result eutrophication followed by aquatic death (Khan and Ansari, 2005 and Lee *et al*., 2003). In addition, ground water contamination through percolation is another problem with these fertilizers (Liang *et al*., 2007,). Severely, the different forms of nitrogen and phosphorous from the chemical fertilizer; ammonia, nitrite, nitrate, orthophosphate and monophosphate, are toxic to aquatic life (Rahman *et al*., 2014). Thus, using struvite-NH_4 fertilizer is a solution to all the problems related with chemical fertilizers since it is a slow leaching compound so that a lower or no volatilization of nitrogenous compounds (Rahman *et al*., 2014). In addition, using struvite-NH_4 is a win-win

phenomenon to rock the green economy at optimum; using waste as source of fertilizer.

As an additional fertilizer quality of struvite, it increases PH of soil. Struvite-NH_4 addition to acidic soils is helpful since it increases soil PH unlike chemical fertilizers which decrease (Rahman *et al*., 2014). Struvite-NH_4 was found helpful fertilizer in semiarid environment, where, it contains large amount of elemental and ionic substances (Massey *et al*., 2009). Together with this, over application of struvite-NH_4 does not burn roots since it release nutrients slowly (Scope Newsletter, 2003). It also decreases global warming with its slow nutrient release therefore plants use all the N without loss (Nelson, 2000; Lee *et al*., 2009 and Westerman *et al*., 2009). Unlike urea fertilizer its losses nutrients faster and N loss occurs in different forms to contribute the global warming gases (NO, N_2O and CH_4) (Matson *et al*., 1998, Perala *et al*., 2006, Chu *et al*., 2007 and Aguilera *et al*., 2013). About 60 % of the urea is lost always unlike the case in struvite (Liang *et al*., 2007). Generally, when we use urea as fertilizer, we contribute 39% of CH4 and 60% of N_2O of the greenhouse gases (OECD, 2000). We can see how damaging is using urea to the environment but at the same time having a slow nutrient releasing fertilizer; struvite is a success despite the danger we have now.

In addition, struvite-NH_4 was observed to have an increased yield and P uptake by lettuce than phosphate rock fertilizer (González-Ponce *et al*., 2009). This was thought that the Mg incorporated with struvite has a cooperative effect on P uptake (González-Ponce *et al*., 2009). In another experiment also struvite-NH_4 increased yield in ryegrass and bioavailability of phosphrous was higher (Deinert *et al*., 2020) than chemical fertilizer. In the same study phytate consuming microbes were higher in the soil where struvite was added. This shows microbes can also participate in bioavailability of struvite.

Struvite-NH_4 is not only a good quality fertilizer but it has also problems with regard to traces pharmaceuticals and biological hazards. Thus, pre-treatment with membrane filtration (Prakash *et al*., 2004), electro dialysis with ozonation (Lamichhane and Babcock, 2012) or ion exchange treatment (Landry and Boyer, 2013) from urine were claimed neccesarry. But these treatments are not hundred percent effective, for example nanofiltration can not avoid dalton size pharmacetucal residues and also ozonation it changes only chemical nature of pharmaceuticals but it can not avoid in general. Besides, the proposed methods are expensive for developing countries like Ethiopia.

In addition, to strengthen struvite-NH_4 use as fertilizer cheap sources of Mg are being introduced

by different scientists. For example, sea water (Dai *et al*., 2014), wood ash (Sakthivel *et al*., 2012), bittern or brine (Etter *et al*., 2011) and geological reserves in case like Ethiopia are some of the proposed.

Preparation of struvite-NH_4 from urine has another advantage since struvite crystalization also helps to separate nutrients from micropollutants. Especially, the organic micropollutants from natural urine which these have inhibitory effects on photosynthesis of algea (Escher *et al*., 2006). If struvite-NH_4 is used no detected micropollutants will be found on it, this was proofed by studing the toxicity of solutions prepared from struvite-NH_4 on the green unicellular algae called, *Desmosdesmus subspicatus* photosynthesis rate (Escher *et al*., 2006).

2.5. How to prepare struvite from human urine?

Solid fertilizer which contains nitrogen and phosphorous can be prepared using human urine (Battistoni *et al*., 2000 and Kabdasli *et al*., 2006). Solid urine fertilizer is prepared by adding a required amount of magnesium to the human urine. The addition of equimolecular concentration of magnesium at alkaline condition at around 7.5-9 PH (Hao *et al*., 2008 and Bouropoulos and Koutsoukos, 2000) make the phosphorous and nitrogen sources in the urine to make a compound called struvite. Pure struvite-NH_4 or with high struvite-NH_4 content > 95% is produced at neutral PH (7.0-7.5) though at this PH range struvite-NH_4 formation is slower that can take up to 3 months (Hao *et al*., 2013). But at PH (8.0-9.0) the struvite-NH_4 content decreased to 30-70% and at PH range above 9.5 the struvite-NH_4 content decreased to less than 30%. Struvite-NH_4 consists Mg^{+2}, NH^{+}_4, $H_2PO^{-}_4$ and $6H_2O$. The chemical reaction to make struvite-NH_4 is $Mg^{+2} + NH^{+}_4 + H_2PO^{-}_4 + 6H_2O \rightarrow MgNH_4(K^{+}$ or Na^{+2}, etc) $PO_4.6H_2O + 2H^{+}$. The solid struvite-NH_4 is made when magnesium is added to the urine. Struvite-NH_4 is collected solid after the chemical reaction.

For struvite-NH_4 precipitation the kinetics is divided it to two; these are nucleation and growth. Nucleation is when ions combine to form crystal embryo which is a foundation of growth. Nucleation occurs when supersaturation (higher amount of ion exist) (Ohlinger *et al.,* 1999). In case of nucleation in urine it happens heterogeneous nucleation where this nucleation happens when there are impurities in solution and there is no highly supersaturation of constituent ions (Sohnel and Garside, 1992). In solutions the driving force for nucleation is the difference in chemical potential of the super saturated (μ_1) solution and solid states (μ_2): $\Delta\mu = \mu_2 - \mu_1$. However, the induction time for heterogeneous nucleation is:

Log t_{ind} = $A/(\log S_a)^2$ –B, where $A= \beta(\gamma^s)^3 v^2 f(\theta)/(2.3\ K_B\ T)\ v^2$; B = Ω_{het} = if $f(\theta) < 1$.
$f(\theta)$ = correction factor for heterogeneous nucleation; θ = wetting angle of solid phase by liquid; Ω_{het} = heterogeneous pre-exponential factor; v = molecular volume; γ^s = surface energy; S_a = Activity based supersaturation ratio; T = absolute temperature; K_B = Boltzmann constant; β = geometric factor (Nielsen, 1955; Packter 1968; Gunn and Murthy, 1972 and Sohnel and Handlirova, 1984) cited in (Ohlinger *et al.,* 1999).

On the other hand growth is another precipitation kinetics. It is the process of incorporation of constituent ions to a preexisting embryo to form a visible crystal. After the time elapsed between mixing of constituent and the appearance of measurable precipitate (induction time) crystal growth continues until equilibrium is reached. Efficiency of struvite-NH_4 crystallization depends on concentration and molar ratios of Mg^{+2} + NH^{+}_4 + $PO^{-}_4{}^3$, degree of supersaturation, PH, aeration rate, temperature and presence of Ca^{+2} in the reacting mass (Yetilmezsoy and Zengin, 2009). Struvite-NH_4 is not restricted to urine but it can be prepared from any waste which contains phosphorus and nitrogen source. Struvite-NH_4 can be solidified as fine solid or large crystals in regular and irregular forms by using different technologies. Even a struvite-NH_4 is in the market these days as a label called enhanced struvite-NH_4; it is a struvite-NH_4 treated with phosphoric acid to achieve a modified form of DAP (diammonium phospate), one of the most used fertilizer in the world (Gaterell *et al*., 2000).

In the present time different countries around the world develop legislation for minimum phosphorous content of treated waste water that joins rivers, that is 0.1mg/L (Clean Water Services, USA). To reach this amount people have developed technologies to recover struvite-NH_4 from waste water. In different countries struvite-NH_4 is being recovered using fluidized bed reactors for example in Japan (PHOSNIX), Canada (Ostara), Netherland (PHOSPAQ™, AirPrex), and Germany (Seaborne, AirPrex) at industrial level.

One of the technologies to recover struvite-NH_4 is Ostara's Pearl Nutrient Recovery Process which this process is found cost effective than avoiding phosphorous using microbes which this may expose for formation of struvite-NH_4 causing blockage of the system. This will lead for extra cost to clean systems. The ostara technology is recovering struvite-NH_4 as fertilizer by the name of crystal green which this sustainable fertilizer closes the nutrient loop (Ostara, 2010). Ostara's Pearl Nutrient Recovery Process uses fluidized bed reactors which recovers N and P from sludge

dewatering centrate and produce high quality N, P and Mg fertilizer.

In addition, Ostara and other the like technologies are big hope in the future to recover nutrients from source separated urine to make waste management easier. As it can recover phosphorous and nitrogen from source separated urine, which is 1% of the sewage but contains the largest amount of N and P content would be cost effective (Larsen and Gujer, 1996).

Separating urine from source is cost effective and less treatment intensive where it becomes easier in waste water management, water conservation and using urine as source of phosphorous and nitrogen (Lahr *et al*., 2016). Though a big challenge to use urine as fertilizer is its nutrients are diluted in big amount of water. Thus, to avoid the large amount of water different solutions were suggested such as evaporation, reverse osmosis, freeze-thaw, electrodialysis and dehydrating urine using wood ash (Ek *et al*., 2006, Ganrot *et al*., 2007 and Senecal and Vinneras, 2017).

Experiments were run in different times to recover nutrients from source separated human urine. As an example, in South Africa in eThekwini people have recovered struvite-NH_4 using automated reactors (Grau *et al*., 2015). Besides they measured the efficiency of their reactors in recovery. It is not only in South Africa but people around the world has awaken for urine for example in Nepal, Vietnam and Durban (Bischel *et al*., 2016). In another instance in Netherlands (Wilsenach, *et al.,* 2007) they have found that urine has a much higher phosphate concentration than sludge reject water. Thus, magnesium ammonium phosphate as well as potassium magnesium phosphate are the two types of struvite's successfully precipitated. The fluidized bed reactor used was also successful to remove the two types of struvite's.

2.6. Fertilizer potential of struvite

Struvite-NH_4 is a compound that its nutrients not flashed faster by rainfall; this makes it a good fertilizer (Lee *et al*., 2009). It releases its nutrients very slowly. Thus, using struvite-NH_4 fertilizer is a solution to all the problems related with chemical fertilizers since it is a slow leaching compound so that a lower or no volatilization of nitrogenous compounds (Rahman *et al*., 2014).

As an additional fertilizer quality of struvite-NH_4 it increases PH of soil. Struvite-NH_4 addition to acidic soils is helpful since it increases soil PH unlike chemical fertilizers which decrease (Rahman *et al*., 2014). Struvite-NH_4 was found helpful fertilizer in semiarid environment, where there is high amount of salts and ions (Massey *et al*., 2009). Together with this over application of struvite-

NH_4 does not burn roots since it release nutrients slowly (Scope Newsletter, 2003). It also decrease global warming with its slow nutrient release therefore plants use all the N without loss (Westerman *et al*., 2009).

In addition, struvite-NH_4 use as fertilizer is cheap as it is made from continously available resource, urine. Together with this the cheap sources of Mg are being introduced by different scientists for example sea water (Dai *et al*., 2014), wood ash (Sakthivel *et al*., 2012) and bittern or brine (Etter *et al*., 2011). But incase of Ethiopia we have a dolomite Ministry of Mines and Petroleum) in nothern, western and southern Ethiopia. Together, magnesite reserve in nature as magnisum source for instance we have in Adola, Harar, Wolloga, Sidamo, Moyalle and Assosa in economically fisible amount (Ministry of Mines and Petroleum).

Our work is worthwhile to do since the 95% of Ethiopian population that is the farmers are poor to afford chemical fertilizers and our farm lands are in short of nitrogen and phosphorous. As a result, this big society needs a cheap solution. In addition, urine is cheap to find and using human urine as a source of struvite-NH_4 resolves contamination of aquatic environments by nitrogen and phosphorus.

2.7. Struvite-K nature, preparation and its potential as fertilizer

Magnesium potassium phosphate hexahydrate ($MgKPO_4.6H_2O$), also known as struvite-K, is an isomorphous crystalline compound of struvite with the replacement of NH_4 ion with K ion (Banks *et al*., 1975). Struvite-K has crystallographic properties similar to those of struvite and Studies have reported its potential application as an efficient fertilizer like struvite (Xu, *et al*., 2015). As a result, struvite-K was a mineral approved for fertilizer application in 2003 by the Commission on New Minerals and Mineral Names, International Mineralogical Association (Burke and Ferraris, 2004).

Struvite-K is a phosphate fertilizer to maintain agricultural production (Munch and Barr, 2001; Shu *et al*., 2006 and Massey *et al*., 2007). It is also a cheap source of K and P for fertilizer industries. On the contrary rock phosphate is a non-renewable source of phosphate fertilizer (Rahman *et al*., 2011).

Struvite-K is a compound that is not flashed by rainfall making it an eco-friendly fertilizer (Rhaman *et al*., 2014). It releases its nutrients very slowly in water. Struvite-K is a slow leaching

nutrient fertilizer, unlike the rock phosphate and K fertilizers which are exposed to fast leaching of K and P. This leads to surface water contamination which result eutrophication followed by aquatic death (Khan and Ansari, 2005 and Lee *et al*., 2003). In addition, ground water contamination through percolation is another problem with these fertilizers (Liang *et al*., 2007,). In addition, using struvite-K is a win-win phenomenon to rock the green economy at optimum; using waste as source of fertilizer.

Preparation of $KMgPO_4.6H_2O$ from urine has an advantage since struvite-K crystallization separates nutrients from the majority of the chemical micropollutants (Escher *et al*., 2006) as it was shown by experiments on the chemically analogous compound, struvite.

The substitution of NH4+ with K+ in struvite-K causes a slight difference in crystal structure as the ionic radius of K+ is analogous to that of NH4+ (Chauhan *et al*., 2011). However, it has a higher density (1.864g/cm3) than struvite-NH_4^+ (1.711g/cm3.) (Ohlinger *et al*., 1998). Optimal PH for struvite-K production is 10 and 11 (Xu *et al*., 2011 and Wilsenach *et al*., 2007). It is formed by the following chemical formula at PH 10; $Mg^{+2}+ K^+ +HPO_4^{-2} + 6H_2O \rightarrow MgKPO_4.6H_2O+H^+$ (Wilsenach *et al*., 2007).

The recovery of struvite-K is affected by pH, proportion of Mg: P: K and presence of competitive ions such as sodium and calcium (Kabdaşh, *et al*., 2022). Its solubility product was found to be between 10.77 and 12.22 (Kabdaşh *et al*., 2009, Yang *et al*., 2019 and Lothenbach *et al*., 2019). It exhibits a difference with struvite that is between 12.6 and 13.26 (Xu *et al*., 2015). The presence of ammonia and calcium prevents the formation of struvite-K. In urine, struvite-K co-precipitates with $MgNaPO_4.7H2O$. Thus, impurity is another issue for struvite-K precipitation. Different modelling studies involved in preparation of optimum production conditions from various types of waste-water revealed that the molar ratio of 2:1:2 of Mg:K:P as optimal for optimal yield of struvite (Xu *et al*., 2015, Yang *et al*., 2019 and Lee *et al*., 2016).

Similar to struvite, struvite-K consists of tetrahedra PO4-3, octahedral [Mg (H2O)] +2 and K+ and these are connected with hydrogen bonds (Mathew *et al*., 1979; Ohlinger *et al.,* 1998, and Shih *et al.,* 2016). Each K+ is bound to penta oxygen atoms which one comes from phosphate tetrahedra and the other from water molecules (Mathew *et al.,* 1979). Thus, struvite-K has an orthorhombic needle like crystalline structure. It is also transparent to whitish in appearance (Wilsenach *et al.,* 2007: Graeser *et al.,* 2008: Shih *et al.,* 2016).

Struvite-K has a potential to supplement and replace the supply of inorganic phosphate and potassium reserves (Kabdaşh *et al*., 2019, Kuşcuoğlu, 2008 and Hidayat and Harada, 2021). This struvite family is sparingly soluble in water (Kabdaşlı, *et al*., 2022), though the exact solubility is yet to be known. Besides, struvite-K is heavier than struvite (Ohlinger *et al*., 1998) which makes it insoluble during application in floody areas (Rahman *et al*., 2014). Particularly, the low solubility permits for a slow nutrient release during plants growing season therefore, avoiding overloading of soil and plants with nutrients. This prevents burning of root of fertilized plants (Gaterell *et al*., 2011 and Dunseth *et al*., 1970). In addition, as a slow release fertilizer, its nutrients are effectively absorbed by plants hence leaching, drainage and percolation are avoided, thus preventing aquatic pollution (Gaterell *et al*., 2011, Dunseth *et al*., 1970 and Liu *et al*., 2011). Additionally, the thermal dissociation of struvite-K starts above 75 ^{o}C and thus, it dries up within a short period of time (Chauhan *et al*., 2011). This property has an advantage in large-scale production of the fertilizer.

Application of K-struvite to acidic soils helps amend the soil. Moreover, the fertilizing value of struvite-K was compared against struvite on ornamental plants, vegetables, turfgrass (El Diwani *et al*., 2007, Ryu *et al*., 2012, Uysal *et al*., 2010, Uysal *et al*., 2014, Siciliano 2016 and Liu *et al*., 2011) and crops (Hidayat and Harada 2021). In a study of Ryu *et al*., 2012, struvite-K was better fertilizer than struvite as a result of its richness in potassium. Like struvite, its crystals do not carry or react with heavy metals, thus its safety from heavy metal threats (Ryu *et al*., 2012).

2.8. Economic factors to use human urine derived fertilizers

The cost is in production of urine diversion toilets in every house hold. The starting installation construction cost is high but less than the conventional toilets (Larsen *et al*., 2001). Both the construction and maintenance costs were observed in the USA. Studies in South Africa and Niger also indicate subsistence farmers have profited from the diverting toilet than using chemical fertilizer and using no fertilizer (Richert *et al*., 2010). Even these farmers could cover the eco-toilet installation cost within less than 2 years after selling their urine fertilized plant products and selling urine respectively. This shows installation cost is not comparable with the profit from the fertilization benefit from urine stored in diverting toilets. But installation of urine diversion toilets could remain unprofitable in urban areas unless people sell the urine to some organizations who prepare urine fertilizer.

In another instance in Switzerland people have studied the efficiency of source separating toilets they call them NoMix technology, it was observed to be successful to collect about 70-75% of what was expected (Rossi *et al*., 2009). Together with this in Germany, people have used UDT for about three years at the main office of GIZ. The people found out that more must be done on the efficiency of water and urine separation of the toilets. In addition, cleaning and maintenance instructions were claimed required before using them widely (Blume and Winker, 2011).

On the other hand, water used to wash struvite will not be economical for developing countries since there is no a trend to reuse water. As a result, consumption of water to clean struvite from heavy metal and micro-pollutants is another economical limitation in Southern gobe. (Fry *et al*., 2008). Thus, struvite should be accessed for chemical hazards unwashed just to measure how heavy is the burden to use unwashed struvite.

2.9. Constrains to use human urine derived struvite

Generally, the issues summarized into four categories come on the way to wide-scale application of urine-derived struvite from humans. These are; pathogenic microorganisms, antimicrobial resistance genes (ARGs), pharmaceuticals as well as social acceptance. Pathogenic microorganisms may survive carrying virulence genes and/or antimicrobial resistance genes with the ability to transform into other microorganisms. Antibiotic resistant genes can be carried and transported by integrons, plasmids, phages and transposon genes. Pharmaceuticals which they are low molecular weight chemical compounds (Dalton size: 200-500/1000Da where 1Da= $1.66x10^{-27}$kg) (Pynnönen and Tuhkanen, 2012) after uptake and excreted through urine after consumption either partially or non-metabolized. These four issues are detailed in the subsequent subsections with the need to be addressed through continuous research and data-driven policies.

2.9.1. Pathogenic microorganisms in source-separated urine

In apparently healthy individuals, urine is thought to contain non-pathogenic microbes derived from the urethra (Shaw, 2010). But if infections happen due to *Escherichia coli* (Andreu, 2005), *Salmonella typhi, Leptospira interrogans, Salmonella paratyphi, Schistosoma haematobium* (Faeacham *et al*., 1983), *Mycobacterium tuberculosis* and *M. bovis* (Grange and Yates, 1992), polyomaviruses (Bofill-Mas *et al*., 2000) and Hepatitis B virus (Knutsson and Kidd-Ljunggren, 2000), the urine carries any of the above-mentioned microbes/ova from infected individuals. Thus, transmission can be due to either direct contact with the urine during application, or via the food

products during consumption. Contamination by faeceal matter happens in the diverting toilet or urinal if there is misuse of separating toilets (Schonning *et al*., 2002) and if there is lack of occasional maintenance to such toiletes (Kraft, 2010). Thus, a large number of bacteria, viruses, protozoal and helminth ova can contaminate urine through faecal crossover (Fane, 2004 and Prüss *et al*., 2002). A study in South Africa revealed that urine was found contaminated with *Aeromonas spp*., *Shigella spp*., *E.coli* O157:H7, *Clostridium perfringens* and viruses like polyomavirus, rotavirus and adenovirus (Bischel *et al*., 2015).

The common pathogenic viruses detected in faeces are rotavirus, enteric adenovirus, Norwalk-like virus (Tauxe and Cohen 1995), reoviruses, hepatitis A virus (Feachem, 1983). Most enteric viruses are resistant to both low (3.5) and high pH (10) (Snowdon *et al*., 1989a and Gerba, 1996). On the other hand, examples of helminths that can contaminate human urine are hookworms (ancylostomiasis) and whipworms (trichriasis). Ascaris ova were recovered from soil after several years of applying human excreta (Snowdon *et al*., 1989a; Lewis-Jones and Winkler 1991). Possibly helminth ova and enteric viruses are the resistant pathogens that can contaminate urine and soil (Bischel *et al*., 2016). Ascaris ova are among the most resistant of all the helminth ova as a result of their multi-layered chitin and lipid (Wheeler and Carroll, 1989).

The vegetative stages of most of the above-mentioned pathogens have low survival outside the host after excretion due to environmental stress (Ahmed *et al*., 2017). Viruses, spores of protozoa and helminth ova are exceptions to survive and cause disease in humans (Ahmed *et al*., 2017 and Decrey *et al*., 2011). For example, enteric viruses are the major causes of gastrointestinal infections in humans in the middle- to high-income countries (Mead *et al*., 1999). Therefore, the process of converting urine to struvite need to be optimized in a way to minimize pathogens transmission. Ahmed and colleagues (2017) studied the persistence of *E.coli* and MS2 phage in urine from 15˚C to 37 ˚C and reported that *E. coli* persisted for 5 days at at 35˚C while MS2 Phage persisted for 10 days at 25˚C respectively. Other studies also reported on the persistence of ΦX174 and the eggs of *Ascaris suum* in source-separated urine stored for 37-47 days and the subsequent struvite (Decrey, *et al*., 2011). The results indicated that both the phage and ova persisted for upto 10 days and also were found retained on the struvite product, with ascaris ova showing accumulation in the struvite (Decrey, *et al*., 2011). They also found that air-drying the struvite for several days did not inhibit both the phage and the ova from being infective (Decrey, *et al*., 2011). A decrease in

number of ova occurred at drying conditions with increased temperature (35˚C) and decreased relative humidity (Decrey, *et al*., 2011).

Viruses need very low viral load to contaminate urine (Höglund *et al*., 1998). The probability of high persistence of enteric viruses was reported by (Höglund *et al*., 2002). In their report, rotavirus and phage 28B took 35 and 71 days to decrease significantly in urine at 20˚C and pH 9. Together with this, bacterial persistence was observed on struvite product at lower humidity and a temperature of 35˚C (Bischel *et al*., 2016). Increasing the relative humidity and simultaneous heating to 35˚C was shown to decrease the bacterial count (Bischel *et al*., 2016). In another study by (Makaya, *et al*., 2014), the bacteria *Clostridium perfringens* and *Enterococcus faecalis* were observed to be persistent in treatments of pH 8.6 and temperature 35 ˚C and 42˚C in urine stored up to 30 days. Various species of the genus *Enterococcus* was found resistant at 10 ˚C and 42 ˚C at pH 9.6 and at salt concentration of 6.5% (Kenner, 1978; WHO, 1993; Cooper and Olivieri, 1998). The studies show that using insufficiently treated urine for struvite production may result in pathogens capable of being infective. As a message, it is important to make struvite with low public health risk before applying into soil.

2.9.2. Resistome and mobilome in source separated-urine derived from different bacterial Species

A myriad of antimicrobial resistance genes present in a sample/specimen is termed as resistome. Urine collected for fertilizer production purpose are of composite nature, from various individuals at various time and space and thus it is assumed to contain a various types of antimicrobial resistance genes of chromosomal and extrachromosal origin, hence the term resistome is used in this study. Antimicrobial resistance (AMR) is reported to increase from time to time all over the world mainly as a result of anthropogenic pressure, rather than spontaneous mutation (Ali, *et al*., 2018). Ultimately, around 50,000 lives per year are reported to be lost every year in the USA and Europe (Simlai *et al*., 2016). If AMR is left unimpeded, it will become the cause of 10 million deaths annually by 2050 (WHO, 2019).

Antimicrobial resistant genes are naturally carried in chromosomes flanked by integron gene cassette or on plasmids, phages and on transposons. Most of the time resistant genes are found naturally in bacteria which produce antibiotics as a result of evolution. These genes can be transferred in biofilms of bacteria either to phylogenetically related or distant bacterial species through mobile genetic elements such as integrons, plasmids, conjugative transposons, phages and chromosomal islands.

Rapid spread of antimicrobial resistance happens during horizontal transfer of antimicrobial resistance genes via plasmids and integrons among individuals, species, and bacterial kingdoms. (Hall and Barlow, 2004). In human gut, horizontal gene transfer through conjugation and transduction under normal circumstances, thus making the human gut microbiota a reservoir of antibiotic resistant genes (Forslund *et al*., 2013 and Hu *et al*., 2013).

Intrinsic antimicrobial resistance is the result of inherent structural or functional behaviors while acquired resistance is the result of mutation on chromosomal gene or through horizontal gene transfer. The two mechanisms of intrinsic resistance are reduced permeability of plasma membrane and increased efflux. Reduction of permeability in gram negative bacteria was achieved by limiting entrance of antibiotics by down regulation of the expression of the non-specific channels called porins or by replacing them with more specific channels (Baroud, *et al*., 2013 and Wozniak, 2012). Members of the family *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp. exhibit intrinsic resistance through reduced permeability by downregulating porins to resist newer drugs such as carbapenems and cephalosporins (Baroud, *et al*., 2013 and Wozniak, 2012). The increased efflux is a mechanism of pumping out what enters into the cell. These proteins reside along the plasma membrane. It is the result of upregulation of the expression of specific or non-specific efflux proteins which causes resistance. Specific efflux proteins pump specific type of antibiotic drugs structurally similar antibiotic drugs with the prominat example of the protein encoded by the *Tet* genes (Huang *et al*., 2022). On the other hand, non-specific efflux proteins pump out dissimilar antibiotic drugs, thus called multidrug efflux pumps (Hu *et al*., 2012). Clinically important multidrug resistance efflux pumps recently are product of the resistance gene *Mde* in *Streptococcous mutans*, *FuaABC* in *Stenotrophomonas maltophilia*, *KexD* in *Klebsiella pneumoniae* and *LmrS* in *Staphylococcus aureus* (Floyd *et al*., 2010; Hu *et al*., 2012 and Ogawa,

et al., 2012). Genes encode for efflux pumps can be transferred through plasmids despite their presence in most bacteria (Dolejska *et al*., 2013).

The acquired resistance mechanisms are: Changes in antibiotic targets by mutation, modification (and protection) of antibiotics targets, inactivation of antibiotics by hydrolysis and inactivation of antibiotic by transfer of a chemical. The mechanism of changes in antibiotic targets by mutation is a change in the structure of an antibiotics target. This prevents efficient antibiotics binding but still the target is enabled to undergo normal function. For example, linezolid is an antibiotic used against Gram positive bacteria, targeting the 23S rRNA ribosomal subunit. A point mutation occurred on one of the genes of the 23S rRNA changes the structure of this subunit and resistance occurred (Eliopoulos, *et al*., 2004). Another example of target change resistance is in methicillin-resistant *S. aureus* (MRSA) (Katayama *et al*., 2000), which is acquisition of gene homologous to original target penicillin binding protein. The homologous gene which acquires mutation is penicillin binding protein 2, which functions similar to the former protein when it is inhibited by antibiotics. This is carried by staphylococcal cassette chromosome mec (SCCmec) element. It carries a penicillin binding protein 2 gene which produces beta-lactam insensitive protein (Liu *et al*., 2016).

The mechanism of modification (and protection) of antibiotic target sites is not mutation of genes rather binding of a group of chemicals into targets to alter target patterns. For example, the erythromycin ribosome methylase (*erm*) family of genes adds methyl group into 16S rRNA and alter the drug-binding site. As a result, it prevents the binding of macrolides, lincosamines and streptogramins (Kumar, *et al*., 2014). Another example is the protective action of pentapeptide repeat proteins which prevent binding to and protect topoisomerase IV and DNA gyrase from the lethal action of quinolones by interacting drug-target complex and promote release of antibiotics (Vetting *et al*., 2011). On the other hand, colistin resistance occours in bacteria due to addition of phosphoethanolamine to lipid A; this causes the reduction of negative charge on colistin target lipopolysacharide which results in failure to bind to colistin (Beceiro *et al*., 2011).

The mechanism of inactivation of antibiotics by hydrolysis is an act of hydrolyzing the antibiotics by enzymes. The powerful enzymes are extended spectrum beta lactamases, carbapenemases, including imipenemase, Verona integron encoded metallo β-lactamase, carbapenemase, oxacillinase and the NDM enzymes in Gram-negative bacteria such as *K. pneumoniae*, *E. coli*, *P.*

aeruginosa and *A. baumannii*, that have had caused the emergence of isolates that are resistant to all beta lactam antibiotics (Voulgari *et al*., 2013, Johnson and Woodford 2013, Lynch *et al*., 2013). For example, the CTX-M14 and CTX-M15 enzymes are the most widely isolated extended spectrum beta lactamase worldwide, especially in cephalosporin-resistant *E. coli* and *K. pneumoniae* (Poirel, *et al*., 2012 and Zhao *et al*., 2013).

Most importantly, horizontal gene transfer of antibiotic resistant gene pre-dates the human use of antibiotics as it was seen in OXA-type beta-lactamases which have already been carried on plasmids and these were transferred between bacteria for millions of years (Barlow, 2002). A near time history, the presence of *tetQ* on plasmid in the gut microbes before the use of tetracycline in human is another evidence. The strictly anaerobic human gut microbes such as *Bacteriodes* transfer antibiotic resistant genes to either a closely related bacteria or unrelated bacteria which might be an opportunistic pathogen (Barlow, 2012). These bacteria can have access to urine derived fertilizer and may cause public health issues.

On the other hand, antibiotic misuse is being reported to increase transfer of genetic materials containing resistant genes (Waters and Salyers, 2013; Jernberg *et al*., 2007 and Girardi *et al*., 2011). Due to different mechanisms such as biofilm formation favouring (Cargill and Upton, 2009; Kaplan *et al*., 2011; Rachid *et al*., 2000; Schadow *et al*., 1988; Dunne 1990; Wang *et al*., 2010; Qu *et al*., 2010; Linares *et al*., 2006; Hoffman *et al*., 2005). The concentration at which resistant genes give protection to their host is below the minimum inhibitory concentration established in clinical settings (Singer *et al*., 2016). Antibiotics are found to increase biofilm formation if they are introduced in sub-inhibitory concentration by upregulating carbohydrate synthesis and downregulating protein synthesis (Ferrer, *et al*., 2016). The carbohydrates colanic acid and glycogen used as thick networks and coverages in biofilm of bacteria (Sailer *et al*., 2003 and Siva *et al*., 2014). These biofilms which is a group of bacteria stick to a place and producing a polymeric substance (EPS), which is a matrix made up of proteins, polysaccharides, lipids, and nucleic acids (Tetz *et al*., 2009 and Sutherland, 2001). This polymeric matrix gives protection against sub-inhibitory concentration of antibiotics by protecting penetration (Smith, 2005). Biofilm can be favourable for conjugation, transposition, transduction and transformation, which leads to the spread of antibiotic resistance genes (ARG) in biofilm since it allows the presence of high density cells in high proximity (Krol *et al*., 2011; Aminov, 2011 and Salcedoa *et al*., 2014).

Sub-inhibitory concentrations of antibiotics also found to trigger error-prone SOS response and error correcting repair systems (Baharoglu and Mazel, 2014). The way the antibiotics (beta-lactams, quinolones, and aminoglycosides) trigger these responses is by favouring formation of free radicals of oxygen species, which are mutagens (Kohanski *et al*., 2010). Thus, the induction of these DNA damage and repair cascade by low concentration of antibiotics leads to an increase in mutation rates, which can result in the emergence of multi-drug resistance (Kohanski *et al*., 2010). The SOS response not only cause mutations but also trigger horizontal gene transfer, thereby providing a mechanism for the spread of antibiotics resistant genes from antibiotics exposed previously (Beaber *et al*., 2004). Thus, in countries where antibiotics use is not well regulated and policy enforced; misuse and abuse are high, thus high AMR frequency.

Resistant genes are found to be more spread in areas where there is no sufficient sanitary conditions in addition to a wide spread use of antibiotics (Pehrsson *et al*., 2016). Such areas are wide spread in low-income countries. In these areas there is no a good record of the gene's distribution and there is no sufficient treatment for infections caused by multidrug resistant bacteria. So, most of the case death is the ultimate fate of large number of people. In addition, in the low to middle income countries, abuse and misuse of antibiotics occur since the sale of antibiotics and supply chain is unregulated and purchase is without prescription (Ayukekbong *et al*., 2017).

The presence of antimicrobial resistance genes in human excreta was indicated in a study in South Africa where people found the existence of sulfonamide resistant gene (*sul1*) in urine sample collected from eThekiwi province (Bischel *et al*., 2015). It has been observed that the plasmid mediated resistance (*mcr1*) was detected in faecal samples of a cohort in China (Gündoğdu, 2016). If not properly managed, the presence of resistant genes in human urine and the application of its product in agriculture can be a means of spreading these hazards in soil organisms.

The mechanisms that resistance genes transferred from one bacterium to another are via conjugation, transformation, and transduction and vertically from an ancestor to progeny. The mobile genetic elements ruled by these are; phages, plasmids, genomic islands or integrative conjugative elements (ICEs), transposons and integrons (conjugative or carried by phage). Transposons and integrons can be transferred through conjugation. The difference between integrons and transposons is the earlier contain integrase and the later contain transposase to integrate them in the chromosome. Genomic islands are either mobile or sometimes can be

transferred by transformation, conjugation or transduction with the ability of excision and integration. They are different in GC content from the recipient's chromosome. Thus, they are different materials than the chromosome of the recipient. Integrative conjugative elements are a combination of plasmid and composite transposons (composed of more than one insertion sequence). These have the ability to excise, integrate to chromosome and transfer but unlike plasmids they can not replicate autonomously (Wozniak and Waldor, 2010). These are an intermediate state between plasmids and transposons. For example, some excise from the chromosome and form an intermediate circular DNA then transfer (Salyers *et al*., 1995).

On the other hand, phages can be used as vehicles to distribute resistance genes. As they are relatively persistent in different adverse extracellular environments (Muniesa *et al*., 2013a, 2013b). Together, their ability of infecting many bacterial genera also contributes to their higher ability to resistance gene transfer (Evans *et al*., 2010; Petrovski *et al*., 2011; Souza *et al*., 1972). Thus, the contribution of phages is important in the environment (American Academy of Microbiology, 2009). Studies show the involvement of ARGs in viral particles collected from contaminated water bodies (Balcazar, 2014; Colomer-Lluch *et al*., 2011a, 2011b, 2014; Muniesa *et al*., 2004).

Identified phages responsible to transport ARG are P1-like phages that were reported to carry β-lactamase genes in *E. coli* (Billard-Pomares *et al.,* 2014). On another instance, P22 phages were reported to carry β-lactamase genes in Salmonella (Schmieger and Schicklmaier, 1999). Transduction of different resistant genes in *Streptococcous* was observed for tetracycline resistance, acquisition of multiple resistance to chloramphenicol, macrolide antibiotics, lincomycin and clindamycin or erythromycin (Hyder and Streitfeld, 1978; Ubukata *et al.,* 1975). In addition, the prophage Wβ was observed to carry fosfomycin resistance in *Bacillus anthracis* (Schuch and Fischetti, 2006).

Bacteriophages can undergo either specialized, generalized or lateral transduction (Bushman, 2002). Specialized transduction is packing of only a few specific genes while generalized transduction is mobilization of any fragment of the bacterial genome. The bacteriophages λ and T7 undergo specialized mobilization by packing DNA by the cos mechanism. In this mechanism the phage pack the adjacent bacterial genome piece with the viral genome. In contrast, the Bacteriophages; P22, P1, T4 pack DNA by generalized transduction depending on pac site homology that this site is recognized mistakenly by the viral machinery. In this case the bacterial

genome piece is packed with out the viral genome. A few Bacteriophages appear in either circular or linear plasmid for example the phage ExPΦs in *S. aureus* was reported to mobilize ARGs and virulence genes (Utter *et al*., 2014).

In general, transduction phages were observed to mobilize parts of plasmids (Zinder and Lederberg, 1952). In *Pseudomonas aeruginosa*, a conjugative phage which was able to transfer imipenem, aztreonam and ceftazidime resistance genes both by transduction and conjugation of phages AP-1 and AP-12 was reported by (Blahová *et al*., 1993). On the other hand, a phage which carries a transposon was observed in *Actinobacillus actinomycetemcomitans* and *streptococcous pyogens.* The phages both transduce and move along bacterial genome like transposons containing ARGs (Willi *et al*., 1997 and Banks *et al*., 2003). Phages were also observed to mobilize genomic islands in *S. aureus* (Mašlaňová *et al*., 2013). The staphylococcal cassette chromosome mec (SCCmec) was observed packaged in phages.

Lateral transduction is another type of transduction in virus (Davidson, 2018). It was discovered in the temperate phages of *S. aureus* (Chen *et al*., 2018). This is another means of bacterial genetic material transporting means. In this transduction the viral machinery recognizes the pac site and excise around 7 head full of the adjacent genes of the bacteria starting from the viral genome and packs it in the viral head. After cutting by terminases in to pieces of headfull size. This transduction does not happen by mistake rather it is the viruse's natural life cycle does. This transfers bacterial gene 1000-fold more than the other two transduction means which they happen because of erorr. Thus, mobilomes such as phages and plasmids are worth attention taking to study in struvite. Therefore, this phenomenon is a big concern when we plan to use human urine derived fertilizer. Since while using, we can distribute resistant genes and make soil microflora to harbour resistance and may resistance reaches human pathogens. As a result, freeing of this fertilizer from resistant genes should be a major work.

2.9.3. Possible hazards of pharmaceuticals in urine-derived fertilizer

Though urine can be a good source of struvite it can also be a dish of different problems to human health and the ecosystem. This is as a result of involvement of different naturally excreted hormones and anthropogenic pharmaceuticals (Bischel *et al*., 2015). Since, most of the pharmaceuticals consumed are excreted via urine as a parent compound and as metabolite though

there are small portion via faeces (Jjemba, 2006; Jones *et al*., 2007 and Kümmerer, 2009). These chemicals since they are designed to affect biochemical and physiological conditions in human at the same time, they can bring biochemical and physiological changes in microbes in soil, aquatic animals and plants. Especially, the chemical cocktail of the pharmaceuticals in drinking water is under fear by scientists specifically to children, child bearing age women, elderly and to people who are immune compromised (Darlymple *et al*., 2007). The fear is strong as they are continuously introduced into the environment (Chatzitakis *et al*., 2008). In more detail (Kolpin *et al*., 2002) had summarized the fear types as pharmaceuticals may cause: abnormal physiological processes and reproductive impairment, development of antibiotic-resistant bacteria, potential increased toxicity of chemical mixtures, increased incidences of cancer and potential effects on humans and the aquatic environment roughly understood.

The main concern of scientists to study the fate of pharmaceuticals is as a result of different reasons. These are; pharmaceuticals are lipophilic so that they can pass membranes, and they are persistent so that they don't get inactivated before they give cure like xenobiotics (Halling-Sorensen *et al*., 1998). Thus, pharmaceuticals have bioaccumulation effect in both aquatic and terrestrial ecosystems (Kayode-Afolayan *et al*., 2022). Even experimentally, (Hektoen *et al*., 1995) has proofed the persistence of anti-bacterial; oxytetracycline, oxolinic, flumequine, sarafloxacin, florfenicol, sulfadiazine and trimethroprim in marine sediments without losing their antibacterial effect. In addition, some of these substances were observed to adsorb into marine sediments (Hektoen *et al*., 1995).

Amazingly, it is not only necessary to be concerned about the parent pharmaceutical rather of the metabolites resulted after metabolism. As an example, Ibuprofen after its metabolism when it exits the body it became more reactive and more toxic than the parent substance (Halling-Sorensen *et al*., 1998). This is as a result of change in chemical and physical nature of the parent chemical to more water soluble (Halling-Sorensen *et al*., 1998). In general, the anthropogenic pharmaceuticals got changed into polar water soluble during metabolism this is in order that excreted through kidneys (Sheldon *et al*., 1986 cited in Larsen *et al*., 2004). Thus, different former works has shown the adverse effects of pharmaceuticals and their metabolites to different groups of organisms both in aquatic and terrestrial. The effect of micro-pollutants on microorganisms was reported by

(Elvers and Wright, 1995). They reported that Ibuprofen was observed to be anti-dermatophyte fungi, and gram-positive bacteria at concentration of > 150µg/ml at lower PH.

On the other hand, the example of hormones found to cause endocrine disruption even at nanogram per liter levels are estrogens which are excreted naturally from female human and after taken the synthetic once for contraceptive purpose (Lamichhane and Babcock, 2012). These hormones observed to cause feminization of fish in rivers (Rodgers-Gray *et al*., 2000). In addition, other hormones affect gene expressions in living organisms when they get the access to cells (Länge and Dietrich, 2002).

Cyclosporine and staurosporin inhibit P-glycoprotein which is a member of the ABC family of transport proteins, in fresh water mussels (*D. polymorpha* and *C. fluminea*) (Länge and Dietrich, 2002). And this causes reduction of excretion of reactive metabolic products. Thus, this causes self- intoxication and accumulation of other xenobiotics in the body. Other compounds found to have P-glycoprotein inhibiting effect are calcium channel blockers, calmodulin antagonists, anti-hypertensives, vinca alkaloids, steroids, anti-arrhythmics, anti-parasitics and anti-estrogens (Epel and Smital, 2001).

In phytoplanktons the effect of pharmaceuticals and their metabolites was reported by (Harrass *et al*., 1985). These people have revealed the inhibition of six blue green algae species at concentration of 0.09 -0.86 mg/l. According to another study by (Escher *et al*., 2006) not only pharmaceuticals spiked in urine had toxicity to photosynthesis rate of the green unicellular algae, *Desmosdesmus subspicatus* but also human urine itself. This is, as a result of the presence of large concentration (> 0.3mM) of phytotoxic organic compounds with lower hydrophobicity than many pharmaceuticals. In this work the cocktail of pharmaceuticals spiked in urine which had toxic effect on the algae by reducing the rate of photosynthesis were carbamazepine, diclofenac, E_2, EE_2, ibuprofen, propranolol, and sulfamethoxazole at concentration of 0.5mM as cocktail.This shows the pharmaceutical-cocktail which joins the environment can have adverse effects on the organisms in water. In one instance in Israel on micro estuarins, ibuprofen, carbamazepine and caffeine were observed to have negative risks on fish, crustaceans and algae (Topaz *et al*., 2020).

On the other hand the effect of pharmaceuticals and their metabolites on plants, where a study showed that pinto beans were very sensitive to antibiotics; chlortetracycline and oxytetracycline (Batchelder, 1981:1982). In addition, these antibiotics had varying effects on different species of

plants. On another instance other antibiotics were found to be up-taken by plants from soil and joining the food chain of human (Kumar *et al*., 2005 and Tanoue *et al*., 2012). These chemicals are hazardous to human health since they select resistant bacteria against the non-resistant once and promote antibiotic resistance. This disrupts the normal distribution of normal flora in human body. Besides, bioaccumulation of pharmaceuticals was witnessed in tomato fruits where plants were fertilized by human urine derived fertilizer spiked with pharmaceuticals (de Boer *et al*., 2018). This shows the danger of pharmaceuticals can reach to human and animals through the food chain.

When we see crustaceans/copopods (Macri *et al*., 1988) identified the acute toxicity of furazolidone 3-[(Nitrofurfurylidene) amino]-2-oxazolidione on *Dapnia magna*. On the other hand, another scientist had shown raising *Temora turbinate* in pharmaceutical waste concentration above 1ppm, which he observed smaller adult size, reduced egg production rates and abnormal growth pattern (Halling-Sorensen *et al*., 1998). In addition, on another study on marine amphipod *Amphitoe valida* which chronically exposed to pharmaceutical waste concentration above 1% had lower survival rates and low fertility when compared to control group (Lee and Arnold, 1983).

Together with these, anthelmintics taken up by animals had been observed having adverse effect on non-targeted organisms found in dung (Blume *et al*., 1976 cited in Halling-Sorensen *et al*., 1998). In this study degradation of dung was observed delayed as a result of the claimed pharmaceutical presence.

On another occasion where the effect of pharmaceuticals was studied drug resistance is one phenomenon. Thus, different people have reported the occurrence of resistance to antibiotics in fish farms (Nygaard *et al*., 1992). The resistance can be as a result of mutation of some genes, transfer of resistance genes and due to selective pressure of inhibiting non-resistant and favouring resistant once.

The other phenomenon related with adverse effect of pharmaceuticals is mutagenicity to organisms. One report showed the mutagenicity of urine of patients in therapeutic treatment with tinidazol (Espinosa-Aquirre *et al*., 1996) and metronidazole to bacteria (Connor *et al*., 1977). These therapeutic agents are prescribed against protozoal infections.

Antibiotics are mostly prescribed pharmaceuticals in Ethiopia to diseases related to hygine such as for typhoid fever. But these and other antibiotics have never been studied for their effect to the environment and to human health after metabolism. Besides their distribution in the waste is not yet assessed. But we discuss the effect of antibiotics on nature and human as keeping in mind the potential risks of the antibiotics we use in the country.

Some antibiotics are found to get metabolized in to more toxic to human than the parent compound in human and animals' body. For example, sulfamethoxazole is acetylated to be more toxic (Kümmerer, 2009). If this join drinking water its effect could be harsh. On the other hand, according to studies in Germany about 70% of the antibiotics administered in the body of human and animals is excreted as parental compounds which are active in their activity (Kümmerer and Henninger, 2003).

Bio-accumulation and bio-transformation of the antibiotics; sulfadiazine, sulfamethoxazole, trimethoprim, enrofloxacin, ofloxacin, clarithromycin and azithromycin were assessed in sea cucumber (Apostichopus japonicus) (Zhu *et al*., 2020). The antibiotics were observed to accumulate in the Echinoderms's tissues at higher concentrations. It was also found out that the antibiotics had bio transformation possibility since they were also located in the digestive tract. Different tests have shown that manure which contains antibiotics when applied on soil, it was observed different plants take up into their tissues the antibiotics. For example, vegetables were found taking up such as carrot roots (tubers), lettuce leaves (Boxall *et al*., 2006), green onion (*Allium cepa* L.), and cabbage (*Brassica oleracea L., Capitata* group) absorbed chlortetracycline (Kumar *et al*., 2005 and Grote *et al*., 2007). And from crop plants corn was observed to take up chlortetracycline (Kumar *et al*., 2005). The antibiotics sulfamethazine was also observed to be taken up by corn, lettuce and potato (Dolliver *et al*., 2007). Though there is also an observation that not all antibiotics taken up by all plants but different plants absorb different antibiotics sometimes (Boxall *et al*., 2006). Boxall *et al*., 2006 in his work on carrot roots and lettuce leaves showed that the lettuces taken up only florfenicol, levamisole and trimethoprim but the carrot roots were found taking up diazinon, enrofloxacin, florfenicol, and trimethoprim.

In addition, long persistence of FQs, macrolides (human antibiotics) in soil were observed after amending it by sewage sludge which contained these antibiotics. The soil was found containing the antibiotics for several months after application (Golet *et al*., 2002 and Giger *et al*., 2003a). This

means the antibiotics are active to kill microbes in the soil which this can lead to disturbance of the soil ecology and hinder nutrient cycle. The same is true for struvite application which may contain antibiotics.

Phyllosphere microflora was found changed in aquatic plants after long time exposure of them to antibiotics (Rico *et al*., 2014). In addition, onion, cabbage and corn plants were observed taking up the antibiotics chlortetracycline (Dolliver *et al*., 2007). In another instance sulfamethazine was observed to be taken up by corn, lettuce and potato (Dolliver *et al*., 2007). Wheat plant also was observed to take up chlortetracycline on grain (Tasho and Cho, 2016). In another study also lettuces and carrot were observed taking up trimethoprim (Boxall *et al*., 2006). Sub-inhibitory concentrations of antibiotics are found to have affects in plants on seed germination and root elongation (Pan and Chu 2016).

Antibiotics were observed also to have adverse effects on aquatic organisms. According to works of (Macrì *et al*., 1988; Migliore *et al*., 1993, 1997; Brambilla *et al*., 1994 and Wollenberger *et al*., 2000), the hatching rate of *Artemia* sp. cysts significantly slowed down, high mortality rate for naupli and toxic effects on reproduction of *Daphnia magna* was recorded in the presence of antibiotics. In another work by (Macrì *et al*., 1988) the antibacterial, furazolidone caused toxicity on *Culex pipiens* molestus larvae, *Daphnia magna* and *Artemia salina*. Also, antibiotics were observed changing the behaviour of aquatic organisms. For example, the phototaxis property of *Daphnia magna* was observed to be changed because of antibiotics (Dojmi di Delupis *et al*., 1992 and Brambilla *et al*., 1994). In an additional study by (Kim *et al*., 2007) acute toxicity of sulfamathoxazole, sulphachlorpyridazine, sulfathiazole, sulphamethazine, sulphadimethoxine, and trimethoprim was observed on a marine bacterium (*Vibrio fischeri*), a freshwater invertebrate (*Daphnia magna*), and the Japanese medaka fish (*Oryzias latipes*). Again, another work on aquatic organisms' susceptibility to antibiotics was (Park and Choi, 2008). In this work the antibiotics neomycin was observed to be the most toxic for acute exposure to the aquatic organisms, *Vibrio fischeri*, *Daphnia magna*, *Moina macrocopa*, and *Oryzias latipes*, toxicity followed by trimethoprim, sulfamathoxazole and enrofloxacin. Sulphamethazine, oxytetracycline, chlortetracycline, sulphadimethoxine and sulfathiazole had intermediate toxicity, while ampicillin and amoxicillin were the least toxic. In chronic toxicity neomycin affected adversely adult survival and reproduction of *D. magna* and *M. macrocopa* with low mg L^{-1} levels exposure. Another acute

toxicity of antibiotics was tested by (Yamashita *et al*., 2006). The antibiotics, levofloxacin and clarithromycin were found highly inhibiting for the growth of microalgae. The authors claimed the phyotoxicity of clarithromycin was 100-fold higher than that of levofloxacin. In addition, on toxicity tests of the antibiotics' on Daphnia reproduction the authors showed that both of the antibiotics have been found with toxicity.

Together with this, different studies have revealed the toxicity of some pharmaceuticals to the aquatic organisms. For example (Lienert *et al*., 2007 and Sanderson *et al*., 2004) have found that the drugs prescribed to hepatitis B, C, HIV and Ibuprofen are found toxic to aquatic animals. They are predicted to cause reduced growth and fertility together with another lethal physiological effect according to a model formed from information on experimental data found on another drugs (using (Q)SAR modeling of 3000 different compounds).

The problem with pharmaceuticals is even bigger in developing countries since there is no proper sanitation (Pynnönen and Tuhkanen, 2012). Besides there is no any information on the amounts of substances excreted and mixed with drinking water and their effect to the environment (Pynnönen and Tuhkanen, 2012). However, there are indications that people are consuming large amounts of antiamoebiasis, antiprotozoal and drugs designed to treat HIV infections these include antiviral, antiretroviral, cocktail of antibacterial and anti-tuberculosis drugs with drinking water in Sub Saharan (Pynnönen and Tuhkanen, 2012). Together with these, the authors indicated that there is accumulation of micro-pollutants in soil and in the aquatic environment. Though these all problems are there with respect to micro-pollutants to humans and the ecosystem there is no any information on the presence of all most taken micro-pollutants in the ecosystem and their harm to both human and the ecosystem in Ethiopia. For example, typhoid is a common problem in Ethiopia so the medication to treat it may have a big abundance in the environment.

Though, struvite is a sustainable means to collect nutrients with small contamination with the existing pharmaceuticals and other natural chemicals in urine as it was described elsewhere. However, there are inconvienences to wash away pharmaceuticals in water stressed areas like Ethiopia. A study of (Ronteltap *et al*., 2007) showed that it was possible to separate struvite from pharmaceuticals in urine up to more than 98% if we wash it with clean water. This study also shows that the pharmaceuticals never precipitate in the struvite crystal rather when the struvite is made they remain in solution and stick on the surface. Though, washing is not a promising step

for areas like Africa where water is a scarce resource. As a result, the extent of pharmaceuticlas should be known in an washed struvite. So that, to devise another means to address the issue of cleaning up of struvite from pharmaceuticals.

Besides to flourish the green economy in Ethiopia recycling urine as fertilizer is a great success but considering its safe use from micro pollutants. Together, it is a win-win phenomenon to recycle waste since it is also cleaning the cites from a big nusance which is urine along every street of the cities. But most of all Ethiopia is a country with millions of poor farmers who cannot afford chemical fertilizer so that, this study is a big promise of the future to use human urine and struvite as eco-friendly, acceptable and cheap fertilizer.

2.9.4. Social acceptance

Social perception is another obstacle to use human urine as fertilizer. Especially in the developing world utilizing human waste for growing food cannot be resisted as a result of disgust, cultural norms and taboos, considering it as spiritual pollutant, personal prejudice, as a result of ethical issues and fear of disease (Mugivhisa and Olowoyo, 2015; Adewole *et al*., 2013, and Andersson, 2015). Together with these the other obstacle in the developing world is people resist using latrines (Timmer and Visker, 1998). They do prefer bushes (Timmer and Visker, 1998).

Fast growing population and increasing water shortage are problems in Africa, thus these hinder to provide quality sanitation and sewerage connections (Moe and Rheingans, 2006). Utilizing urine in agriculture helps to reduce both environmental and health risks related with poor sanitation (Jackson, 2005). Besides, utilizing nutrients from urine improves food availability in Ethiopia, even in Africa. As a result of food prices are getting higher as a result of exhaustion of phosphorus supply in the world (Cordell and White, 2006). Though to apply urine-based fertilizer in agriculture, in Africa needs a big effort to create awareness through trainings. For example, in case of Ethiopians there is a big despise for toilet use in agriculture as a result of different traditional beliefs just like other African countries.

An attitude study in South Africa (Mugivhisa and Olowoyo, 2015) on human urine fertilizer on about 225 people who are staff and Limpopo university students shows 82.7% and 81.1% would not eat spinach and maize fertilized with urine respectively. Only 38.3% said they would eat vegetables fertilized with animal urine making it more tolerable as compared to human urine. Health reasons were given as the main reasons why respondents were unwilling to eat crops

fertilized with human urine. However, 69.9% of the respondents were positive to eat human urine fertilized foods if they get good information on the safety of using it for agricultural purpose. On another study in Ghana, Nigeria and Uganda (Cofie *et al*., 2010; Adewole *et al*., 2013 and Andersson, 2014) on farmers; they do not want to use urine as a result of cultural norms. And the others never want to use because of religious objections. On another study in Nepal on 201 people indicates 68% of the people are not interested to use urine as fertilizer and 38 % of them believe urine will not be accepted as fertilizer.

A study in Nigeria reveals that lack of awareness to fertilizing values of human urine had been seen to reduce interest of using urine as fertilizer by participants. But, after awareness was created participants of 80% became willing to use urine fertilizers (Sridhar *et al*., 2005). With the rest of these participants the main obstacle not to accept urine as fertilizer is as a result of fear of health risks. In another study it was found in Ghana and Nigeria teaching people focusing on the quality of urine grown agricultural product in relation to health will increase confidence and acceptance of the biological fertilizer (Cofe *et al*., 2010).

Another study again, in rural areas of eThekwini municipality, South Africa the acceptance of urine was assessed among samples of the society. In this study, disgust and unpleasant odour, fear of disease and fear of social stigma were the biggest reasons not to accept human urine-based fertilizers (Okem *et al*., 2013). Only to a few extent religion affected the acceptance of the fertilizers. In another study also, urine-based fertilizers acceptance was hindered as a result of health issues (Beler Baykal *et al*., 2011) and as a result of unpleasant smell (Mariwah and Drangert, 2011).

From an experience in Kenya awareness works on human urine derived fertilizers, Ethiopian researchers and policy makers can learn a lot. Approachable awareness works show changes in different societies towards urine-based fertilizers (Uddin, *et al*., 2012). In Kenya of one district called Nyanza, people were interviewed and their attitudes were collected by different approaches. About 99% of studied groups appreciated using urine diversion dehydration toilets (UDDT) and products of these toilets for agriculture (Uddin, *et al*., 2012). In addition, among the participant group of UDDT non users, around 88% supported use of DDDT products in agriculture (Uddin, *et al*., 2012). This result in attitude change is gotten by movements of different responsible groups in Kenya. The main role players were mass media, NGO and mainly two women associations who

mobilized the society in that district (Uddin, *et al*., 2012). This is a promising beginning in Africa as a whole especially in Ethiopia where there are a lot of despising for toilet contents. From this experience, training the society of the rural farmer approaching through respected elders of women and men could be a promising way in Ethiopia.

In another experience in Uganda also traditional beliefs and taboos not to use human waste were broken as a result of practical researches and discussions with the rural farmers (Anderson, 2015). It is a success story in Uganda from participatory action research. Farmers got satisfied from fertilization of their farmland with human urine. In this study farmers and researchers planted maize in different plots using different fertilization and they have gotten that human urine fertilization yielded a good result in maize yield. The farmers changed their attitudes towards urine through discussion and practical exemplary works of the study. The farmers already adopted to utilize urine for fertilization these times and spread the knowledge to surrounding neighbouring farmers around 80 in number.

Generally, awareness development on the safety of using human urine is very essential. Works must be there to change perception of reusing nutrient rich wastes like urine. In addition, teaching societies sustainable development; being eco-friendly is helpful for societies to accept urine as fertilizer. Since this will make them aware what is happening to the world as a result of Global warming and lack of nutrients like P and consumption of unreasonably large amount of energy. As a big solution, including waste recycling concepts in school books, influencing religious units and using mass media which can play a big role in this situation to convince people showing different experiences from Europe, China, North America and Japan on nutrient recycling.

A survey at Addis Ababa University on acceptance of urine fertilized and struvite fertilized agricultural products showed that about 60% of the participants agreed struvite as fertilizer than simply being urine (Unpublished data). Thus, this shows struvite is acceptable among a good percentage of participants.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Urine collection and yield-based process optimization

Fresh urine sample (90 L) was collected from Piazza where a variety of people (men passer-bys) can be found on March 22, 2018. It was stored for 20 days in a seald container in the laboratory. Then, the amount of phosphate was measured in order to calculate the amount of Mg to be added. To measure the phospate amount the palintest of phosphate (Wagtech,UK) was used. The amount of Phosphate was measured following vanadomolybdate method (Nelson, 1954) where it is made by Wagtech WTD method where all the reagents required are provided in the form of test tablets. The test was carried out simply by adding a single tablet to urine sample. A supplementary tablet was used for removal of Silica interference. In this test Phosphate reacted with ammonium molybdate in the presence of ammonium vanadate, to form the yellow phosphovanadomolybdate. The colour intensity is equivalent to the amount of Phosphate. The colour extent was measured by Wagtech WTD automatic wavelength selection photometer, UK. Our actual experiment was as follows. Test tube was filled with urine sample to the 10 ml mark. To avoid silica contamination phosphate SR tablet was crushed and mixed to dissolve. One phosphate HR tablet was crushed and mixed to dissolve. Mixture was incubated for 10 minutes to allow full colour development Phot 29 was selected on the photometer and reading was recorded after blanking with urine.

3.2. Struvite Production

Struvite production was following a method in (Nebiyat and Adey, 2023). At first the amount of PO_4^{-3} was measured using photometer (Phot 29) (Wagtech). Then, an equimolecular amount of $MgCl_2$ to PO_4^{-3} was prepared to mix in the 20 days stored urine. The pH of the urine was adjusted to 10 by 5M NaOH. Then the calculated amount of $MgCl_2$ mass was added to the pH adjusted urine which was put in a batch reactor (30 L). The mixture was incubated at ambient temperature with stirring by hand for 10 min. The struvite powder was allowed to settle for 30 min followed by decanting to separate the struvite from the urine. Then, struvite was dried into powder in the refrigirator by leaving the container open at 8 ^oC.

3.3. Genomic DNA Extraction from Struvite

DNA extraction was done by NucleoSpin Soil kit (Macherey-Nagel) following manufacturer's recommendations. 250 mg of struvite was added to a MN Bead Tube Type A containing ceramic beads. Then 700 µL of Buffer SL1 was added. There after 150 µL Enhancer SX was added. Then sample was vortexed at full speed at room temperature (25 °C) for 5min. Then sample was centrifuged for 2 min at 11,000 x g to eliminate the foam caused by the detergent in Buffer SL1. Then 150 µL of Buffer SL3 was added to sample followed by vortexing for 5 s. This was then incubated for 5 min at 4 °C to facilitate contaminant precipitation. Then sample was centrifuged for 1 min at 11,000 x g. Lysate was filtered by Placing a NucleoSpin Inhibitor Removal Column in a Collection Tube (2 mL). The 700 µL clear supernatant was then pipetted onto the filter. Then, this was centrifuged for 1 min at 11,000 x g. Then, flow through was collected and the NucleoSpin Inhibitor Removal Column was removed. Thereafter, 250 µL Buffer SB was added followed by vortexing for 5s. Then a NucleoSpin Soil Column was placed in a Collection Tube (2 mL) followed by loading of 550 µL sample onto the column. This was then centrifuged for 1 min at 11,000 x g. The flow through was discarded and the column was placed back into the collection tube. The remaining sample was loaded onto the column followed by centrifugation for 1 min at 11,000 x g. Flow through was discarded and the column was placed back into the collection tube. Then Bound DNA was washed four times. In the first wash, 500 µL Buffer SB was pipetted to the NucleoSpin Soil Column. This was followed by centrifugation for 30 s at 11,000 x g. Then flow through was discarded and the column was placed back into the collection tube. The same procedures followed by three successive washes with same procedures except increasing the volumes of buffers SW1, SW2. Then, at this stage the membrane which carried the DNA was dried by spinning the column for 2 min at 11, 000x g. Here the NucleoSpin Soil Column was placed in a new microcentrifuge tube. Then 50 µL of Buffer SE was pipetted to the column. Without closing the lid, column was incubated for 1 min at room temperature (25 °C). Then closing the lid, column was centrifuged for 30 s at 11,000 x g to collect the eluted DNA.

3.4. Bacterial DNA extraction from fresh and stored urine

Both the fresh and stored urine samples stored at -20 ºC were thawed at 8ºC to extract DNA following the methods described by Tsai and Olson, (1991); Silva *et al*., (2013); Geissler *et al*., (2012) with slight modifications. Briefly, urine samples were shaken before use to mix in case they developed solids. About 5 ml of each of the urine samples were poured into the falcon tubes. Samples of urine were kept on ice, and 2 ml of buffer (0.15M NaCl, 0.1M Na2 EDTA, pH 8), which was on ice, was added (Tsai and Olson, 1991). Then, samples were ultrasonicated using an ultrasonicator (Bandelin Sonopuls, Germany), putting samples on ice at 2 x 10^{-4} Hz for 45 s every15 min (Geissler et al., 2012). Before that, the ultrasonicator was cleaned using sodium hypochlorite and washed using UV-treated autoclaved distilled water. Samples were left on ice, and 2 ml of the second solution (0.1M NaCl, 0.5M Tris, 10% SDS, pH 8, where it was adjusted by HCl) was added to each (Tsai and Olson, 1991). The mixtures were immediately transferred to ambient temperatures. Then 40 µl of proteinase K (20mg/ml) that was on ice was added to each of the mixtures (Silva *et al*., 2013). Then the mixtures were incubated for 1 hour at 60ºC. The incubated mixtures were then mixed with a 2 ml phenol:chloroform:isoamyl (24:25:1) mixture from Sigma, Germany. Then the samples were centrifuged at 5000 rpm for12 min. The 3 ml upper aqueous part from each was taken into a new falcon, and these were mixed with 2.5ml) of chloroform, followed by spinning at 5000 rpm for 12 min. The above aqueous part (3 ml) in each was transferred to new falcons. The aqueous part of each of the samples was mixed with 2 ml of cold isopropanol (-20 ºC) from ISLAMPUR, India, followed by spinning at 5000 rpm for 12 min. The supernatant was removed, and pellets were rinsed with 2 ml of cold 70% ETOH. This was then spun at 5000 rpm for 12 min. Each of the falcons was blotted with tissue paper. Pellets of the two samples were then air dried in a safety cabinet for 1 h. Pellet was then dissolved with sterile UV-treated 50 µl Mili-Q (Applichem, Germany) water, followed by incubating mixtures at ambient temperature for 12 min, pipetting up and down. Then extracted DNA was transferred into an eppenddorf tube, followed by measuring the quality of DNA using a nanodrop (Thermo Scientific, USA). Finally, crude DNA was stored at -20 ºC before sequencing.

3.5. Viral genome (gRNA) extraction from struvite

Inorder to extract gRNA from virus TRIzol Reagent was used. TRIzol Reagent is a monophasic solution of phenol and guanidine isothiocyanate. TRIzol reagent lyses viral particles and maintains the integrity of the RNA by being highly effective inhibitor of RNase activity while disrupting cells and dissolving cell components during sample homogenization. In this experiment 250 mg of struvite was first dissolved with 2.5 ml of Trizol (Invitrogen) and homogenized. Chloroform of 0.5 mL was added and incubated for 3 minutes. Then lysate was centrifuged for 15 minutes at 12,000 × g at 4°C. The RNA in aqueous upper layer was transferred to a new tube and precipitated from the aqueous layer with isopropanol of about 1.25 mL. This was incubated for 10 minutes. Then it was centrifuged for 10 minutes at 12,000 × g at 4°C. After centrifugation RNA precipitate was observed forming a white gel-like pellet at the bottom of the tube. The supernatant was then discarded. Pellet was resuspended in 1 mL of 75% ethanol. It was vortexed briefly followed by centrifugation for 5 minutes at 7500 × g at 4°C. Supernatant was discarded and pellet was vacuum dried for 5 minutes. Then pellet was resuspended in 20 µL of RNase-free water, 0.1 mM EDTA. Followed by incubating in a water bath at 55–60°C for 10–15 minutes.

3.6. Purification of viral genome (gRNA) obtained from Trizol extraction

RNA in the lysate was purified using RNeasy plus mini kit (Qiagen). The homogenized lysate was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube. This was centrifuged for 30 s at 10,000 rpm. The column was discarded, and the flow-through was saved. At this step the gDNA was removed by the column in collaboration with high salted buffer. One volume of 70 % ethanol was added to the flow-through, and mixed by pipetting. Ethanol provides suitable binding conditions for gRNA. Up to 700 µl of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube. This was centrifuged for 15 s at 10,000 rpm. Flow-through was discarded. To wash the column membrane, 700 µl Buffer RW1 was added to the RNeasy spin column. This was then centrifuged for 15 s at 10,000 rpm and the flow-through was discarded. After discarding the flow-through, theRNeasy spin column membrane was treated with 500 µl Buffer RPE, for another wash and centrifuged 10,000 rpm for 15 s and repeated with the same for another wash with a 2 min centrifugation. Then, the RNeasy spin column was placed in a new 1.5 ml collection tube followed by adding 30 µl RNase-free water directly to the spin column membrane. This was then centrifuged for 1 min at 10,000 rpm to elute the RNA.

3.7. Shotgun Sequencing of DNA

3.7.1. Quantification of dsDNA before library preparation of gDNA of bacterial

To quantify dsDNA before library preparation the Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific) was used with the Qubit Fluorometer (Thermo Fisher Scientific). In this assay, dyes bind selectively to dsDNA. When they bind to dsDNA they emit florescence. The kit provides concentrated assay reagent, dilution buffer, and 2 DNA standards. The reagent was diluted using the buffer provided by 1:200 dilution. Then 200 µL of working solution for each standard and sample was prepared. In each tube, either 190 µL of diluted solution was added with 10 µL of standard or 198 µL of diluted solution was added with 2 µL of sample, then the solution was vortexed for 3 sec. The solutions were then incubated for 2min at room temperature. Thereafter, the concentration was read using the Qubit Fluorometer 4.0. Qubit Fluorometer detects fluorescent signal emitted only as a result of the fluorescent dye binding specifically to dsDNA.

3.7.2. Control of dsDNA ‘s integrity before library preparation

Integrity of gDNA was checked using Tapestation from Agilent. Reagents were allowed to equilibrate at room temperature for 30 min before use. It was followed by Vortex mixing for 10sec. Ladder was prepared by mixing 10 µL Genomic DNA Sample Buffer with 1 µL Genomic DNA Ladder. Sample was prepared by mixing 10 µL Genomic DNA Sample Buffer with 1 µL genomic DNA sample (10 ng/µL). Then it was spinned down, then vortexed using IKA vortexer and adaptor at 2000 rpm for 1 min. There after it was spinned down to position the sample at the bottom of the tube.

The 2200 TapeStation Controller Software was then launched. There after Genomic DNA ScreenTape device was loaded and tips were loaded into the 2200 TapeStation instrument. Then samples and ladder were loaded into the 2200 TapeStation instrument. Then the required samples were selected on the 2200 TapeStation Controller Software. Each lane of the ScreenTape can be recognized by the software since there are barcodes in each lane of the screenTape device. Thereafter Start was clicked to load sample on screenTape device, electrophorese the samples and analyse picture by the algorithm. This took about 2 min. Then the algorithm produces a gel image, as well as the electropherogram to show the size of gDNA.

3.7.3. Shearing of gDNA from bacteria and virus

DNA (200ng and 55µl) was sheared to around 500 bp Covaris LE220 ultrasonicator following the manufacturer's protocol. The condition was: Peak Power-450 (the peak electrical power used to drive the ultrasound transducer), Duty Factor-15 (ratio between the on-time of the transducer and total operation time), Cycles per Burst-200 (the number of electrical signals used in each acoustic "burst" of the transducer). The sound transducer works like a loud speaker which converts electrical signals into focused acoustic energy. It works to make electrical energy to create mechanical vibration to disturb the surrounding liquid producing sound.

3.7.4. Library Preparation from gDNA of viral and bacteria

The sequencing library were prepared using KAPA HyperPrep Kit (Kapa Biosystems) following manufacturer's recommendation. In brief, 200ng of input DNA was sheared by Covaris LE220 ultrasonicator. Then it was end-repaired (blunted) by addition of nucleotides to overhangs of both 3' and 5′. Then it was followed by 3'-dA tailed (non-templated deoxyadenosine 5′-monophosphate (dAMP) was added to 3' blunted DNA side. Adapter (dual-indexed) was then ligated with complementary 3'-dTMP (dT) overhangs to the DNA, and ligated product was PCR amplified and cleaned up using SPRIselect Reagent (Beckman Coulter).

3.7.5. PCR amplification of adapter ligated libraries derived from gDNA of bacteria and virus

Adapter ligated libraries were amplified following KAPA hyperprep protocol recommendations. That includes, initial denaturation at 98°C for 45 sec, denaturation at 98°C for 15 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 1 min. This program was run for 6 cycles for 50ng initial concentration of libraries and 0.5 µM amount of primers.

3.7.6. Size selection of libraries from gDNA (viral) and gDNA (bacterial) and cleaning up using Solid phase reversible immobilization (SPRIselect)

At high concentration of (20% polyethylene (PEG) and 2.5 M NaCl final concentrations) DNA binds to the surface of carboxyl and iron coated paramagnetic (magnetic only around magnetic field) particles with size 1uM. In this chemistry DNA's negatively charged phosphate will be shielded by Na+. The water hungry hydrophilic PEG, which precipitates DNA by disrupting the water cloud around DNA. At this point the DNA precipitates and aggregate to one another and bind to the negatively charged beads. Because DNA is away from the water it precipitates. Then,

the Na+ (positively) shielded DNA aggregate then binds to a negatively charged pellet. But during elution or addition of water after removing the bead solution, the negatively charged DNA and the beads repel to one another and DNA remains soluble in water. The size selection by beads depends on the concentration of PEG. Higher molecular mass of DNA precipitates at lower PEG concentrations rather lower molecular mass DNA precipitates at higher PEG concentration. This is as a result that DNA fragment size affects the total charge within molecule. The larger the DNA size the larger are the charges. These charges promote an electrostatic interaction with the beads and displace smaller fragments.

For the libraries of bacterial and viral the double size selection was performed by SPRIselect Reagent (Beckman Coulter) following manufacturer's recommendations. First, the right size selection was done as follows.

The SPRIselect was mixed thoroughly to re-suspend the SPRI beads. Then the required volume of SPRIselect for the desired ratio (SPRI beads volume per sample volume) to the sample. Volume of sample * ratio = volume of SPRIselect. The total reaction volume was mixed by pipetting 10 times and it was incubated at ambient temperature for 1 minute. The reaction vessel was put on magnetic stand and the SPRI beads were allowed to settle to the magnet. Here, 0.5x ratio removes most of the library fragments >1000bp. Decreasing the ratio of SPRIselect volume to sample volume will increase the efficiency of binding larger fragments at the right size selection. Then, the clear supernatant, was transferred to a new reaction vessel since it contains the right side (small fragments) selected DNA libraries. The reaction vessel with the remaining beads (larger fragments) was put to discard the larger fragments. To the right side selected sizes of DNA containing supernatant, the required volume of SPRIselect was added using the calculation below to undergo left size selection, Sample Volume µL () * (Left size ratio (0.12x) - Right size ratio (0.5x)) = volume of SPRIselect.

The total reaction volume was mixed by pipetting 10 times and incubated at ambient temperature for 1 minute. The reaction vessel was then put on magnetic stand and the SPRI beads were allowed to settle to the magnet. Here, DNA fragments >400bp were bound on the beads by using 0.7x ratio. In principle, the larger the ratio of SPRIselect bead volume to DNA solution volume, the larger the efficiency of binding smaller fragments. Then the clear supernatant was removed and discarded. With the reaction vessel still on the magnet, 180 µL of 85% ethanol (non-denatured)

was added. Followed by incubation at ambient temperature for 30 seconds. The ethanol supernatant was removed and discarded. The reaction vessel was removed from the magnet and 50 µL of molecular biology grade water was added to reaction vessel to elute. The total elution volume was mixed by pipetting 10 times to re-suspend the beads and incubated at ambient temperature for 1 minute. The reaction vessel was put on the magnetic stand and allowed the SPRI beads to settle to the magnet. Finally, the eluate (size selected libraries) was transferred to an appropriate storage vessel.

3.7.7. Viral gRNA sample quantity measurement

The quantity of gRNA was measured by RNA qubit. The Qubit working solution was prepared by diluting the Qubit RNA HS Reagent to a ratio of 1:200 in Qubit RNA HS Buffer. Distribute the diluted working solution into the 2 of standard tubes (190 µL) and sample tube (198 µL). Then 10 µL of standard RNA was added into each standard tubes and 2 µL into sample tubes. Each mixture was vortexed for 3 seconds and incubated for 2 minutes at room temperature. The Qubit Fluorometer 4.0 was calibrated using the standard solutions. Thereafter, the concentration of sample was read by the calibrated instrument.

3.7.8. Control of Integrity (quality) of gRNA

Quality of gRNA was checked using RNA Tapestation (Agilent). Reagents were allowed to equilibrate at room temperature for 30 min. Reagents were vortex mixed. Followed by sample RNA thawing on ice. Ladder was then prepared by mixing 5 µL RNA Sample Buffer with 1 µL RNA Ladder. Sample was prepared by mixing 5 µL RNA Sample Buffer with 1 µL RNA sample. Sample and ladder were spinned down and then vortexed using IKA vortexer and adaptor at 2000 rpm for 1 min. Then both get spinned down to position the RNA at the bottom of the tube. Then denaturation of ladder and sample followed. Ladder and sample were heated at 72 °C for 3 min. Then ladder and sample were put on ice for 2 min. This was followed by spinning down to position RNA at the bottom of the tube. Then, sample, ScreenTape device and loading tips were loaded into the 2200 TapeStation instrument. Thereafter, the 2200 TapeStation Controller software was launched. RNA protocol was selected and Start was clicked to undergo the automated process to continue.

3.7.9. Library preparation for gRNA

Library was prepared from gRNA following Library prep, SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian. 50ng of input gRNA was used to produce high quality libraries. cDNA synthesis proceeds without fragmentation of gRNA since the gRNA was highly degraded therefore, we followed a protocol suited for degraded gRNA. At first, 8 µl RNA (50ng) and 1 µl SMART Pico Oligos Mix v2 were mixed on ice. The tube was incubated at 72°C in a preheated, hot-lid thermal cycler for exactly 3 min. Then, immediately the sample was put on an ice-cold PCR chiller rack for 2 min. To undergo cDNA synthesis 4 µl 5X First-Strand Buffer, 4.5 µl SMART TSO Mix v2, 9 µl RNA (50ng) from above, 0.5 µl RNase Inhibitor and 2 µl SMARTScribe Reverse Transcriptase were mixed on ice. This was followed by Mixing by vortexing for 2 sec, then spinning the tube to collect the contents at the bottom. The tube was incubated in a preheated hot-lid thermal cycler with the following program: 42°C for 90min, 70°C for 10 min. This program produced the cDNA for the downstream process.

At this next step, adapters were bound to the cDNA from above by amplification as follows. A PCR master mix was prepared for the reaction by combining 2 µl Nuclease-Free Water, 25 µl SeqAmp CB PCR Buffer (2X) and 1 µl SeqAmp DNA polymerase. This was mixed and spinned in a microcentrifuge. This master mix was mixed with the cDNA from the above. Then, 1 µl of each 5' and 3' PCR Primer HT was added to the mixture. Mixture was mixed by vortexing and was spinned down. The tubes carrying the mixture was placed in a preheated hot-lid thermal cycler to perform pcr. The program for the pcr was: 94°C for 1 min and for 13 cycles by the following program. 98°C for 15 sec, 55°C for 15 sec, 68°C for 30 sec and 68°C for 2 min. After this step the libraries were ready for cleaning, that was the next step.

At this step, purification of the amplified RNA-seq libraries was undergone by using AMPure beads. First, AMPure beads were equilibrated at room temperature for 30 min before use. Then, 40 µl of AMPure beads were added to library sample. This was mixed by pipetting followed by incubating at room temperature for 8 min. This allowed the libraries to bind the beads. Then, sample was spinned to collect liquid at the bottom. Sample was then placed on a magnetic separation device for some minutes until the solution became clear. Once the solutions became clear, the supernatant was pipetted out and discarded, while the sample was on the magnetic device. Already kept sample on the magnetic device, 200 µl of freshly diluted 80% ethanol was added to

sample without disturbing the beads. Then sample was kept for 30 sec and supernatant was pipetted out and discarded. Here the cDNA remained bound to the beads and the contaminants were washed away by the ethanol. The washing step was done twice. Then, the sample-tube was removed from the magnetic device. Followed by spinning at 2000xg for 1 min to collect the left ethanol at the bottom of the tube. Here, the tube was placed back on the magnetic device for 30 sec. Without disturbing the beads, pipette out the remaining ethanol. To dry the pellet, the tube was left opened for 5 min, the sample-tube, while resting on the magnetic device. Then, sample-tube was removed from magnetic device and 30 µl of nuclease free water was added. This was mixed thoroughly by pipetting up and down. At this step the beads were released from the libraries and suspended free. In order to settle the unbound beads at the bottom, the sample-tube was put on the magnetic device. Here the libraries remained in the supernatant. So, libraries were collected pipetting out supernatant and putting it in a fresh tube. Here, we have the purified libraries in the fresh tube.

3.7.10. Sequencing by Synthesis

For sequencing by synthesis illumina hiseq X machine was used. The flow cell contained oligonucleotides which were complementary to the adapter region. Each library, which was made single strand by NaOH (company's confidential), bound by hybridization into the oligonucleotides on the flow cell during washing of with adapter solution. Then, clustering occurred threfore, before sequencing, single-molecule of DNA templates were bridge amplified to form clonal clusters inside the flow cell. For cluster formation repeated thermocycling (pcr) process occurred. At each step of amplification, all the new strands were maintained on the flow cell. At last, all the reverse strands were washed off from flow cell. Only the forward strands remained intact to the flow cell. This step was to create millions of individuals, dense clonal clusters containing approximately 2,000 molecules. This is followed by blocking of the 3'-ends of the DNA strands and flow cell-bound oligonucleotides to prevent interference with the sequencing reaction. The flow cell contained greater than 200 million clusters with approximately 1,000 molecules/cluster, and it was ready for sequencing. Then the sequencing was proceeded. A primer was attached to the forward strands adapter primer binding site, on the unbound ends of the templates in the clusters. Then a fluorescently tagged dNTP was added by a high fidelity DNA polymerase to the DNA strand. Only one base was added per round due to the fluorophore acting as a blocking group; though, the blocking group was reversible. Each of the four bases had a unique emission of color, and after each round, the machine recorded which base was added. Once the color was recorded the

fluorophore was washed away and another dNTP was added to the continuing strand and the process was repeated until a required sequencing coverage (20 million reads per sample for viral and bacterial gDNA and 40 million read per sample for viral gRNA).

3.8. Sequence data Analysis

Quality contol of sequence data was using FastQC (V 0.11.7) by using Phred score of 30. Adapters were trimmed using trimmomatic trimmomatic (V 0.33). Trimmed sequence data was assembled using velvet (V 1.2.9) using values N50, q25. Antibiotic resistance genes were predicted by abricate under 97% cutoff values. Clinically important microbs were predicted by binning from the metagenomic data base of bacterial and viral bioinformatics resource center (BV-RBC). Plasmids were predicted from genoms of the clinically important genoms by a machine learning tool called RF plasmid (van der Graaf-van Bloois et al., 2021). From the clinically important viruses antibiotic resistance genes were predicted by ResFinder under selected %ID (percentage identity) threshold is 50 and selected minimum length for ResFinder 60 (Kleinheinz et al., 2014). Inference organisms for plasmids carring was searched on ncbi. In order to annotate metagenomic sequence data was submitted to MGRAST (Metagenomics Rapid Annotation using Subsystems Technology). The data from MGRAST was cleaned using openrefine (3.2 version).

3.9. Spectrometry Sample preparation and Analysis

3.9.1. Analytical Standards, Chemicals, Reagents and Equipments

Certified reference standards of the highest purity grades from eight antimicrobial types from six classes including B-lactams (Penicillin G (89.5%)), Cephalosporin (Cefotaxime (95%)), Fluoroquinolones (Ciprofloxacin (98 %)), Tetracycline (Tetracycline (97.5%), Oxytetracycline (91.3%) and Doxycycline (85.7%)), Sulfonamides (Sulfamethoxazole (purity)). They were from United States Pharmacopoeia and Sigma Aldrich.

Chemicals like ACN (acetonitrile) and MeOH (methanol), the HPLC grade were supplied from Sisco Research laboratories (SRL) (Maharashtra, India). anhydrous citric acid (99%), disodium EDTA dihydrate (99%), anhydrous dibasic sodium phosphate ($Na_2HPO_4.2H_2O$ (99%), formic acid (FA) (99%) was acquired from (Val de Reuil, France). Deionized water of resistivity 18.2 MΩ cm^{-1} was produced in-house using Barnstead GenPure Pro UV – TOC/UF water purification system

from Thermo scientific (Langenselbold, Germany). Sample extraction cartridges were from Waters (Milford, Massachusetts, USA) Oasis Hydrophilic-Lipophilic Balance (HLB) cartridges (6cc, 200 mg and sorbent type Oasis® HLB, 30µm).

In this study, different types of sample processing apparatus were used. The main instruments which, were used in sample preparations include, sample cleaner and concentrator. Sample cleanup and concentration steps were used using vacuum manifold (Supelco, Germany) and nitrogen sample concentrator (MultiVap 54 Lab Tech, USA) respectively. Analytical microbalance (± 0.01mg, Sartorius Lab instruments Goettingen, Germany), pH meter (HANNA pH-ORP, HI11310; USA). Discovery of analytes of interest was performed with an Agilent 1290 Infinity II Ultra-High Performance Liquid Chromatography (UHPLC) system. It was equipped with a reversed phase (RP) Phenomenex® Synergi hydro- (4.6mm × 150 mm; 4 µm) analytical column through security guard cartridge system (4x3.0 mm) for chromatographic separation. The LC system was coupled with an Agilent 6470 LC/TQ/ triple quad mass spectrometer (Agilent Technologies Ltd., Singapore) via an electrospray ionization source which was operating with MassHunter software.

3.9.2. Preparation of mobile phase and standard solutions

Aqueous mobile phase or mobile phase (Water with 0.1% formic acid (FA)): 1.0 mL of FA was added to a half-filled volumetric flask of 1.0-liter capacity, then filled to 1L with deionized water. The flask was degassed offline using Sonicator for 10 minutes, and then transferred to the aqueous reservoir of the machine. Mobile phase B or organic mobile phase (acetonitrile or ACN with 0.1% FA) was prepared by mixing 1.0 mL of FA pipetted into a 1 L volumetric flask and completed to 1L using ACN.

In order to prepare standard solutions, the stock solutions were prepared at concentrations corresponding to 1.0 mg/mL (1000µg/ml) in appropriate solvent. Standard solutions were prepared separately by transferring 10.0 mg equivalent of the base materials quantitatively in to 10.0 ml volumetric flasks. Oxytetracycline, ciprofloxacin and tetracycline were dissolved in methanol. Howevere, penicillin-G, cefotaxime, sulfamethoxazole and doxycycline were prepared in deionized water and diluted to the volume accordingly.

Intermediate standard solutions for oxytetracycline, erythromycine, ciprofloxacin and tetracycline were prepared by pipetting 400µl aliquot of stock and diluting in a 10 ml volumetric flask with methanol to 40ng/µl. Intermediate standard solution for penicillin-G, cefotaxime, sulfamethoxazole and doxycycline (20 ng/µl) was prepared by transferring 200µl stock of each followed by diluting to 10 ml final volume with water. All intermediate stocks were stored in amber vials at ≤ -20 ^{0}C. A working standard (WS) was made by pipetting 1.0 ml of each of intermediate solutions into a 10 ml volumetric flask and diluting to the mark with diluent (80:20 water/Acetonitrile) giving final concentrations of 4 ng/µl and 2 ng/µl respectively.

3.9.3. Solid Phase Sample Extraction and Clean up Procedures

Solid phase extraction of antibiotics was performed following Jin *et al*., 2009 by centrifugation speed modification. The extraction was performed from urine sample which was collected from piazza using frame shift urinals, struvite solution and from the control urine with known history of no antibiotics added but spiked with antibiotics. About 10 ml of each urine sample was taken in to conical centrifugation tubes and these were spinned at 4500 rpm for 15 min. The supernatant was transferred to a new conical centrifugation tube followed by addition of 1 ml of EDTA 5% (m/m) and PH was adjusted 4 by HCl. The mixture was shaken for 5 min followed by solid phase extraction. Then, the concentrated residues were reconstituted with 1 ml initial mobile phase, recapped and vortexed for 30 seconds followed by centrifugation for 15 min at 4500 rpm at 4 ^{0}C. Then, very clear supernatant supposed to contain antimicrobial residues of interest, were transferred into autosampler vials and closed tightly to make it ready for injection. Finally, a volume of 10 µl injection was injected from each preparations in to the UHPLC-MS/MS system.

3.9.4. Analysis method

Analysis was performed by UHPLC of an Agilent 1290 Infinity II system (Agilent Technologies Ltd., USA) interfaced to an Agilent 6470 LC/TQ/ triple-quadrupole mass spectrometer (MS/MS) equipped with Agilent jet stream electrospray ionization source. It was operated in positive mode (AJS-ESI +) and controlled by MassHunter software. Antimicrobials separation was chromatographically achieved on Phenomenex Synergi hydro-RP, (4.6 mm × 150 mm; 4 µm, 80 A° dimensions) column with guard cartridge system (4 x 3 mm). The mobile phase was a teritiary gradient mobile phase with flow rate of 1 ml/min for a total run time of 22 min (Table 1).

Table 1: Mobile phase gradient profile

Steps	Time	Mobile phase -A	Mobile phase -B	Mobile phase -C
1	5.50 min	90.00 %	5.00 %	5.00 %
2	5.51 min	80.00 %	10.00 %	10.00 %
3	11.50 min	80.00 %	10.00 %	10.00 %
4	20.00 min	40.00 %	50.00 %	10.00 %
5	22.00 min	90.00 %	5.00 %	5.00 %

3.9.5. Liquid Chromatography mass spectrometer condition (LC-MS/MS)

The column compartment was operated at 30 °C, rather, the auto-sampler temperature was set at 10 °C. The injection volume was 10 µL. The auto sampler was rinsed after each injection using a solution of H_2O:MeOH (50:50, v/v). The system was conditioned with a mobile phase for more than an hour prior to actual analysis. Gradient elution of mobile phase was used for separation of multi-class antimicrobials.

The Electrospray ion source in +ve modes specific to Agilent company (AJS-ESI +) was used with data acquisition in multiple reaction monitoring (MRM) mode. This was analyzed using MassHunter software. The triple quad mass spectrometer parameters were adjusted and the source parameters were set as follows: gas temperature, 350 °C; gas flow rate, 12 L/min; sheath gas temperature, 250 °C; sheath gas flow, 11 L/min; nebulizer pressure, 40 psi; capillary voltage, 4 KV; nozzle voltage, 500 V.

Test of specificity, accuracy, linearity and precision of method was undergone using the aquired data from spiked standards. A test on the method specificity and confirmation on the absence of potential interfering compounds around the retention time of each antimicrobials was performed. About 20 blank extract of the matrix and 20 spiked extracts were analyzed. Then were checked for the presence of significantly interfering peak at each mass transition of targeted antimicrobials within 2.5 % margin of the retention time. (EPA, 2007, EU, 2000)

Linearity of the method was verified by constructing a matrix-matched calibration curves (MMC) of aliquots obtained from samples spiked with antimicrobial standards of interest. Calibration standards were prepared by spiking urine matrics with a known quantity of target analytes of

calibration range comprising the MRLs. The spiked concentrations were 50, 100 and 150ppb times of the corresponding MRLs, each in triplicates, with expected concentration equidistantly ranging from 25 to 300 µg/L. The plot of the MMC for each compound was based on the peak areas of each analyte at various concentrations.

The accuracy, expressed in terms of recovery was calculated by dividing the mean measured or calculated concentration of the analyte to respective spiked level to express the result as a percentage. However, precision of the method was assessed for each analyte using aliquots of a blank matrix fortified in triplicates at concentrations corresponding 0.5, 1 and 1.5 times (US-Environmental Protection Agency, 2007). Precision of the procedure was evaluated in terms of within-laboratory reproducibility (WLR). Precision of intra-day variation was calculated as the relative standard deviation:

$$\text{Precision} = \frac{\text{Standard deviation} \times 100}{\text{Mean}}$$

At one hand, the limit of detection (LOD) is the lowest concentration of antimicrobials that can be detected at a specified level of confidence. In order to determine LOD, a concentration of 10 µg/L (one tenth of MRLs) of matrix-matched samples of the six mixed standards were prepared in ten different replicates. Then independent measurements of each samples were taken ten times and their standard deviation (Stdv.) were calculated and the LOD determined as LOD = 3 X Stdv. On the other hand, to know the limit of quantification (LOQ), which is "the lowest concentration at which the performance of a method is acceptable for a specified use", calculated as LOQ = 10 X Stdv. (Magnusson and Örnemark, 2014).

3.10. X-ray Diffraction Experiment to Analyze the Content of struvite family derived from urine

Powdered form of struvite was delivered to chemistry laboratory at Addis Ababa University. It was run on X-ray diffraction spectroscopy (Mini-flex 600 PXRD). The measurement mode was continuous and scan axis was $2\theta = 10 \sim 90°$, step width was 0.02°, scan speed was 4° / min (20 min.). To visualize the graph OriginLab2024 was used. In order to make peak matching profile of the graph it was visualized by Origin2024 a trial Match software (version) was used, where this soft ware refers the XRD database called COD (Crystallography Open Database).

3.11. Statistical Analysis

Statistical analysis and visualization of data was done using R 4.2.2 programing language and packages such as ggplot2, reshape2, tibble, phyloseq, dplyr and linux built in Libre calc were used.

CHAPTER FOUR

4. RESULTS

4.1. X-ray diffraction analysis of struvite

Matching peak profile by Match software resulted that the XRD scanned fertilizer contained struvite-K. Evidently, different physical characteristics such as crystal system (shape) was orthorhombic and the cell parameters are a= 6.87300 Å b= 6.16000 Å c= 11.08700 Å. These cell parameters are the angles between edges of the cell of struvite-K which are a, b and c and they are specific to it. The diffraction pattern below in Fig. 2 is a combination of diffraction patterns of the fertilizer at different phase of the fertilizer at different angles. The intensity of the diffracted rays scattered at different angles of struvite-K are plotted to display in Fig. 2. Each phase of the struvite-K below is a unique diffraction pattern due to the fertilizer's specific chemistry and atomic arrangement.

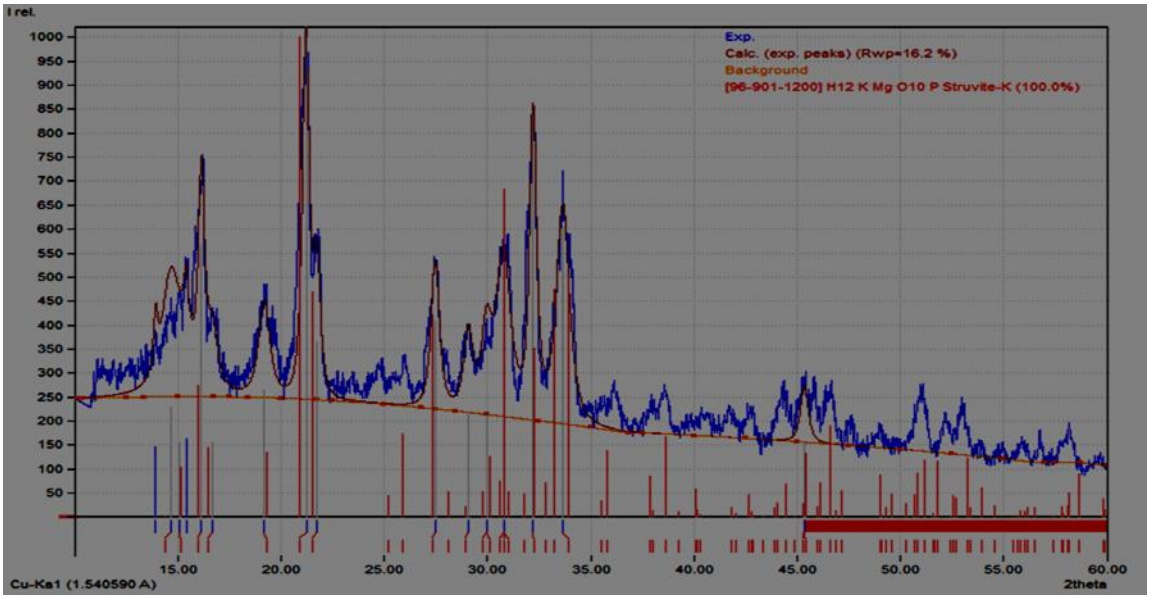


Figure 2 matching peak profile of experimental XRD result (blue) with a standard struvite-K (red) origin of standard from Mathew and Schroeder, 1979

4.2. Microbial community composition and abundance through struvite making

In the process of struvite making the distribution and structure of microbial community was assessed at different taxa level. The distribution of microbial community and its structures were distnict in the three different samples (Fig. 3, 4 & 5). The taxonomic levels which were assessed were phyla, class, family, and genus. Moreover, measurement of richness and coverage of microbial dynamics were assessed by different alpha diversity indicies (Fig. 4 & 5).

Using a paired-end format and an average insert size of 150 bases, a total of 20.16 giga bases (Gb) with 4.7, 6.8, and 4.0 Gb reads were produced for the fresh, preserved stored urine and struvite samples, respectively. For the fresh, preserved stored urine and struvite samples, the average GC content for the readings was 37, 57, and 40%, respectively. The fresh, preserve stored urine and struvite samples had 16, 22, and 13 million readings per sample, respectively.

To make the graphs of OTU abundance in each sample a data subsating was done by selecting the most dominant taxa. The most dominant taxa were only if they are among the top 10% of taxa in at least half of the struvite, fresh, and preserved urine dataset samples.

4.2.1. Microbial composition in fresh urine, stored urine and struvite at phyla level

Bacterial composition of fresh urine, stored urine and struvite was accessed through struvite production process. A total OTUs of 201,132 from fresh urine, 316,150 from stored urine, and 246,769 from struvite were counted. (Table 2 and Fig. 3).

Table 2 OTU percent composition of the most prevalent phyla in the three samples were represented by *Pseudomonadota*, *Bacillota*, and *Bacteriodota*.

Sample	*Pseudomonadota* (Protobacteria) (% composition)	*Bacillota* (Firmicutes) (% composition)	*Bacteriodota (Bacteroidetes)* (% composition)
Fresh urine	60	26	3
Stored urine	17	39	9
Struvite	45	61	11

The most dominant bacterial phyla in fresh and stored urine were *Pseudomonadota* with 120,925 otus (60 %) in fresh urine and 143,409 otus (45 %) in stored urine. On the other hand, the dominant phylum was *Bacillota* 60 % (150,200 otus) in struvite. This was followed by the next abundant phyla from fresh urine comprising 26 % *Bacillota* (52090 otus), 7% *Actinomycetota* (13264 otus), 3 % *Bacteroidota* (6880 otus), and 2 % *Campylobacterota* (3478 otus) among the phyla which were counted less that 1 percent. The next abundant phyla in stored urine were *Bacillota* 39 % (124273 otus), 9 % *Bacteroidota* (29078 otus), *Actinomycetota* 2% (5126 otus), *Thermodesulfobacteriota* 1 % (2774) and *Fusobacteria* 1 % (2767 otus) among other phyla which represented less than one percent. However, in struvite the next dominant bacterial phyla were *Pseudomonadota* 17 % (44012 otus), *Bacteroidota* 11% (28334), *Thermdesulfobacteriota* 2% (5932 otus), *Actinomycetota* 2 % (4015 otus), *Fusobacteriota* 2 % (4008 otus) and *Thermotoga* 1% (1340 otus) among others. In the process of struvite preparation from fresh urine to struvite production, the phylum of *Pseudomonadota* decreased from 60 % to 17 %, while the phylum *Bacillota* increased from 26 % to 60 %. On the other hand, the phylum of *Actinomycetota* decresed from 7 % to 2 %. Together, the phylum *Bacteroidota* increased from 3 % to 11 %. The phylum *Campylobacterota* decreased from 2 % to less than one percent. However, the phyla *Thermdesulfobacteriota*, *Fusobacteriota* and *Thermotoga* increased from less than one in to 2% and 1% respectively.

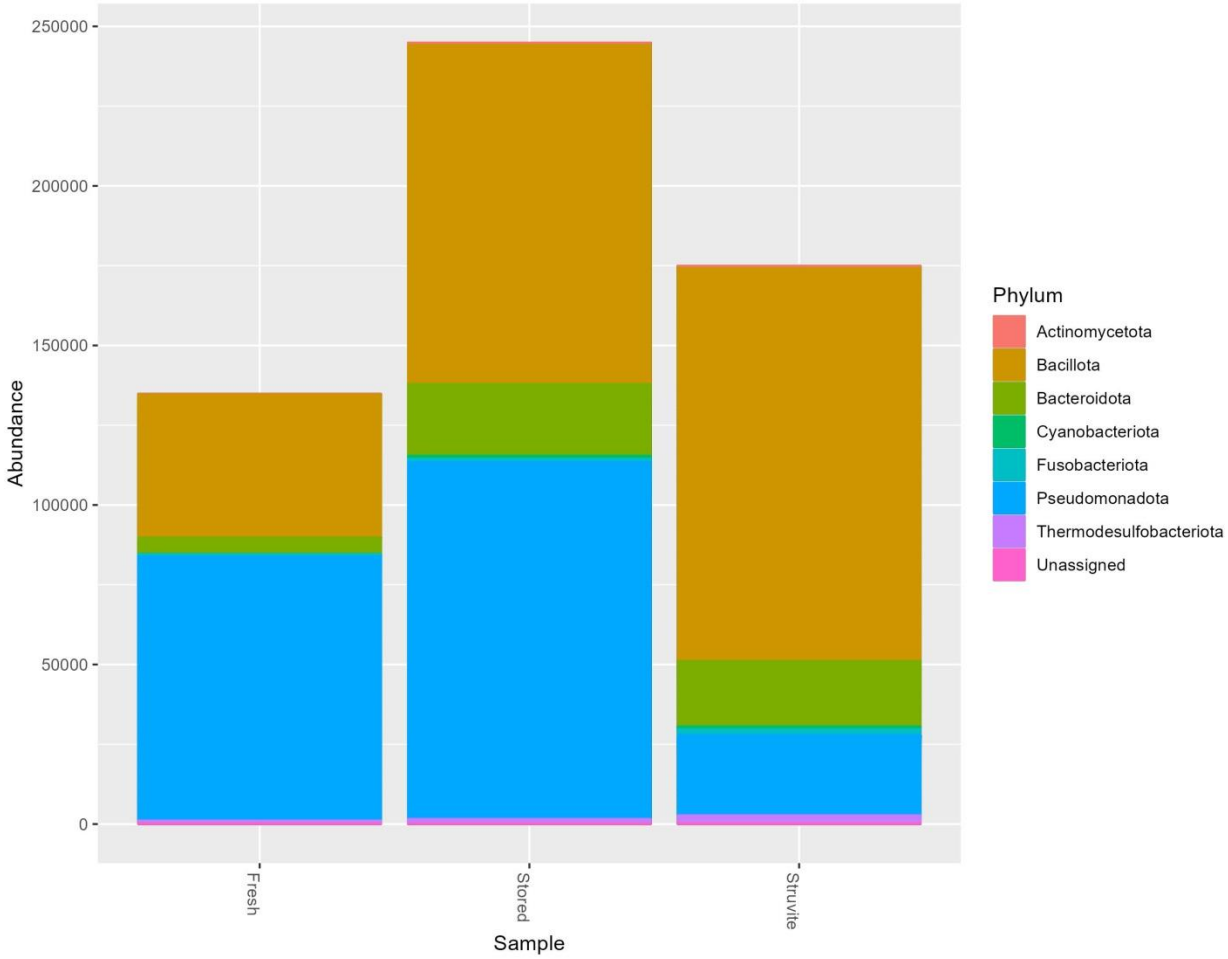


Figure 3: Distribution of bacterial phyla found in fresh urine, stored urine and struvite

4.2.2 Microbial representation in fresh urine, stored urine and struvite-K at class level

The data also showed a pattern in class distribution of microbes across during the process of the making of struvite. *Alphaproteobacteria* was the dominant class in fresh urine with OTU count of 60,773 (30 %) and in stored urine 98,064 (31 %), while this class was only 1 % (2823) in struvite (Fig. 4). Rather, in struvite the abundant class of bacteria was *Clostridia* 92, 166 otus (37 %) which this class was only 5 % (9, 794 otus) and 15 % (46, 352 otus) in fresh urine and stored urine respectively. The second abundant class in fresh urine was *gammaproteobacteria* 26 % (52, 815 otus) but it decreased into 8 % (23, 804 otus) and 10 % (23, 495 otus) in stored urine and struvite respectively. *Bacilli* was the next abundant class in fresh urine with otu counts of 41025 (20 %), this class was found out to increase in stored urine in to 63, 443 otus (20 %) but in struvite with a

slight decrease 42,405 otus (17%). *Betaproteobacteria* was found to increase from 3 % (6,588 otus) in fresh urine to 7 % (20,658 otus) and 6 % (13671) in stored urine and struvite respectively. *Flavobacteria* shown a decrease in stored urine to 3158 otus (1%) from 2% (4712 otus) in fresh urine but increased to 2 % (4,884 otus) in struvite. *Epsilonproteobacteria* was seen to decrease to less than 1 % in stored urine and struvite from 2 % in fresh urine. Among the class taxa of the bacteria the unassigned in fresh urine was 1 % but then decreased in to less than one percent in stored urine and struvite. *Bacterodia* was observed to increase from 1% in fresh urine to 7 % in stored and in struvite to 8 %. The same is true for the *Erysipelotrichia* increased in stored urine from less than one in fresh urine in to 2 % and 1 % in struvite. Similarly, *Tissierellia* increased from less than one in fresh urine to 2 % in stored urine and 5% in struvite. As a similar trend *Fusobacteria* and *Desulfobacteria* incereased to 1 % in stored urine. Rather, it increased to 1 % and 2 % in struvite respectively.

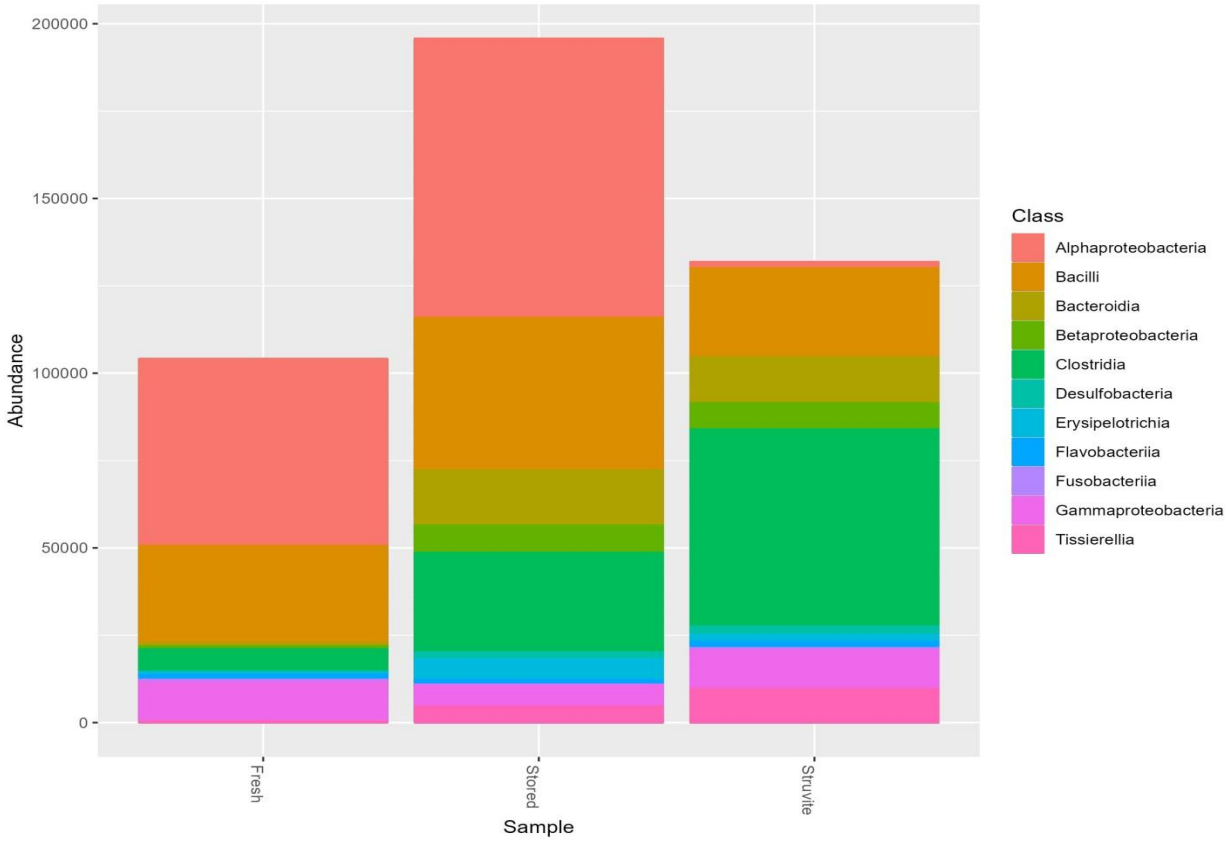


Figure 4: Bacterial classes found in fresh urine, stored urine and struvite

4.2.3. Valuation of richness and coverage of microbial dynamics through struvite-K making

Different measures of alpha diversity were applied to the otu abundance data which was used in this paper. The indices Shannon, Choa 1 and ACE (abundance-based coverage estimator) showed that the fresh urine sample had more individual number of species (richer) than stored urine and the struvite sample (Fig. 5). Thus, fresh urine had more richness than the two. Richness increased in struvite than stored urine. However, the decanted struvite could be a comfortable place for some microbes to grow as the ammonia evaporates in the cooler place open struvite container. The measures of evenness such as Simpson and InvSimpson used in this study showed that the stored urine was the least in the uniformity of microbial community composition compared to the fresh urine and the struvite samples. But still, the individual species in struvite were less in number compared to the fresh urine. The fresh urine community is the most even. However, struvite was more even than the stored urine. Therefore, fresh urine had the highest diversity than stored urine and struvite.

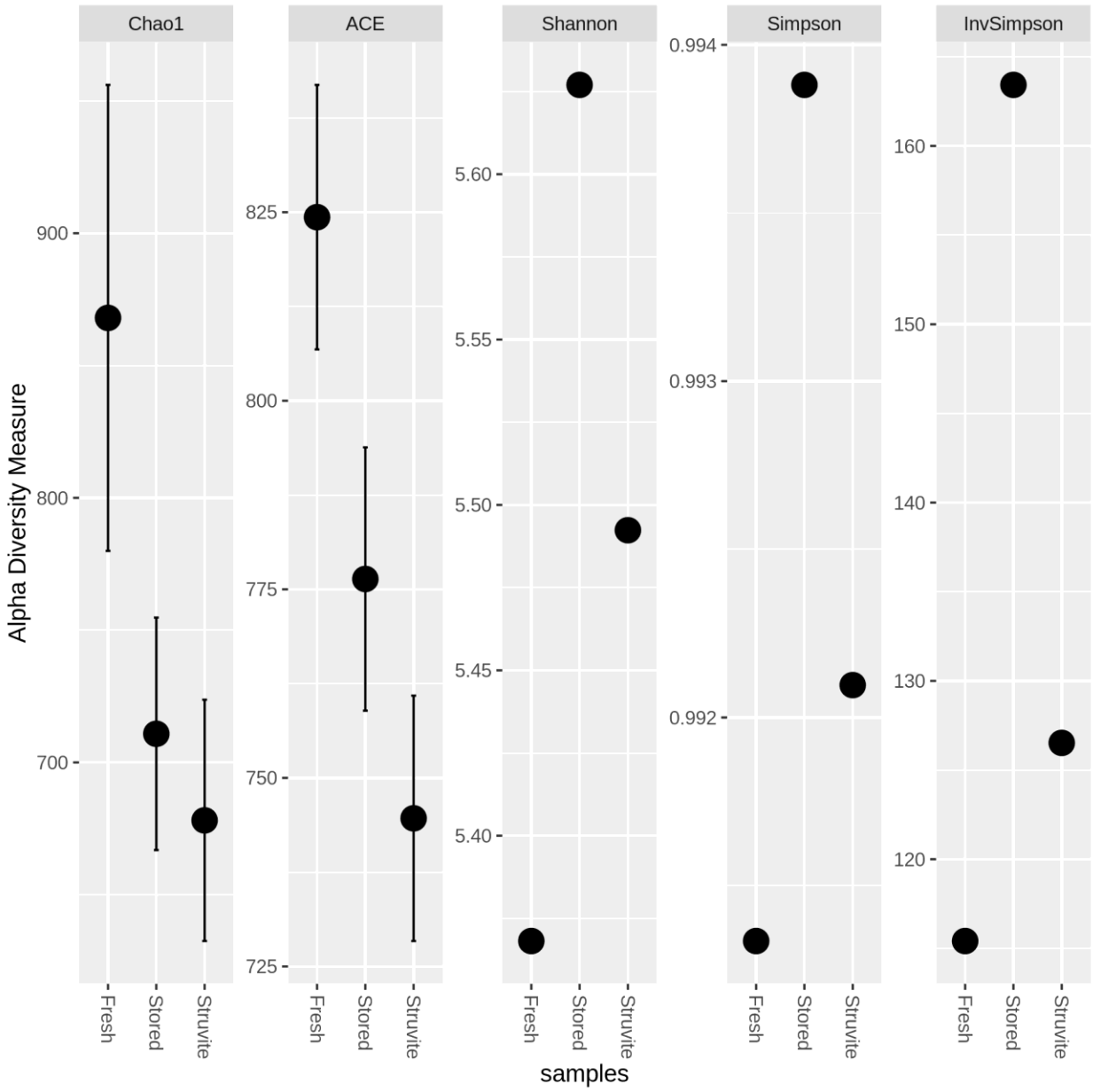


Figure 5: Alpha diversity measures for microbes found in fresh urine, stored urine and struvite

4.2.4. Shared genera between the samples of struvite, fresh and stored urine

A total of 568 genera were derived from the metagenome sequence from struvite, fresh and stored urine. Out of the total, around 96 % (546) of the genera were shared by the three struvite, fresh and stored urine samples (Fig. 6). However, all of the genera found in fresh urine also found in stored urine and struvite. About 0.5 % (3) of the genera were shared only by fresh urine and stored urine only. About 1.1 % (6) of the genera was detected only in stored urine only. Around 0.5 % (3) of the genera were shared between fresh urine and struvite. Around 0.7 % (4) of the genera were however shared between struvite and stored urine. About 1.1 % (6) were contained in struvite only.

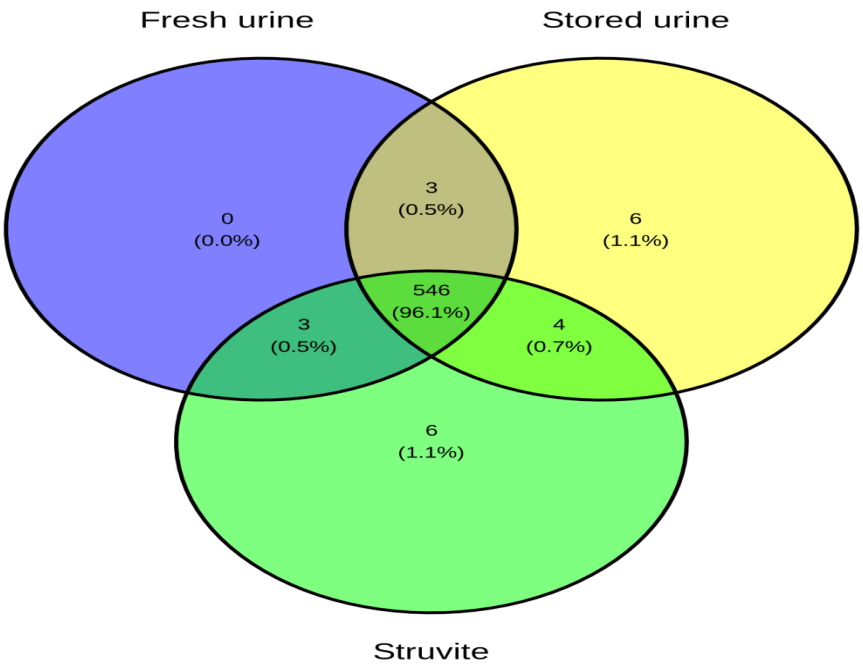


Figure 6: Number and percentages of shared genera in the three samples fresh urine stored urine and struvite

4.2.5. Coverage and distribution of OTUs over the samples

The coverage graph indicated that in all the three samples the number of reads keep on increasing as the number of species increase, indicating that the sampling has not yet represented the rare groups of the community (Fig. 7).

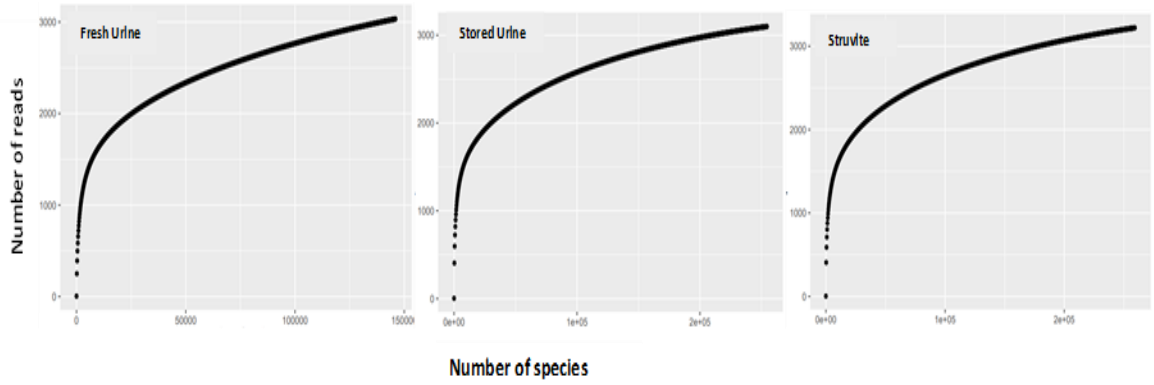


Figure 7: Rarefaction curve indicating the sample coverage and sequencing depth of the fresh, stored and struvite samples

4.3. Pathogenic bacteria through struvite formation

Pathogenic microorganisms are one of the main contaminants in human urine derived struvite. As there is a probability of contamination with microbs from the digestive tract or from diseased individuals. In our study we discovered the presence of different clinically important microorganisms derived from struvite.

4.3.1. Gamma proteobacteria distribution at family level through struvite making from human urine

Gammaproteobacteria is a class of *Proteobacteria* that are medically important and all Gram-negative consisting of several groups of aerobic or facultative pathogenic bacteria (Rizzatti *et al*., 2017: Vázquez-Rosas-Landa *et al*., 2017) as a result we focused on their classification in fresh urine, stored urine and struvite. From a total of 201,129 otus families, in fresh urine about 52,730 (26 %) of the otus were among the *gammaproteobacteria* (Fig. 8 and Appendix 2). Among these, *Morganellaceae* was 31 % (16,304 otus), *Pseudomonadaceae* (12,647 otus) 24 %, *Moraxellaceae* (6,874 otus) 13 %, *Enterobacteriace* 7 % (3,555), *Yersiniaceae* 5 % (2375 otus), *Aeromonadaceae*

4 % (2,297 otus). All the *Vibrionaceae* (1,164 otus), *Pasteurellaceae* (950 otus), *Shewanellaceae* (793 otus) were 2 % each. The others such as *Erwiniaceae*, *Chromatiaceae*, *Alteromonadaceae*, *Ectothorhodospiraceae* were each 1 % among the others.

In stored urine the total otu of the family *gammaproteobacteria* decreased into 23, 800 otus (14 %). Among the left otus 27 % (6,423 otus) of them were *Pseudomonadaceae*, *Moraxellaceae* 8 % (1981 otus), *Xanthomonadaceae* (1,595 otus) and *Enterobactereaceae* (1,556 otus) 7 % each. *Pasteurellaceae* (1,137 otus) and *Morganellaceae* (1,132 otus) were each 5 %. *Vibrionaceae* (1,044 otus), *Aeromonadaceae* (926 otus), *Shewanellaceae* (877 otus), *Ectothiorhodspiraceae* (845 otus) counted 4 % each. *Yersiniaceae* and *Altreromonadaceae* were observed to be 3 %. *Chromatiaceae*, *Pseudoaltromonadaceae* and *Pectobacteriaceae* were observed to be 2 %. Rather, *Methylococcaceae*, *Halomonadaceae*, *Erwiniaceae*, *Hahellaceae*, *Halomonadaceae*, *Cellvibrionaceae*, Unassigned families, *Thiobacillaceae*, *Legionellaceae*, *Cardiobacteriaceae*, *Francisellaceae*, *Piscirickllesiaceae* and *Psychromonadaceae* were observed to be 1 % each.

Different members of the family *gammaproteobacteria* that resisted the higher pH and high amount of ammonia in stored urine also persisted in struvite resisting the low water activity. The number of otus showed a slight decrease to 23, 339 otus (12 %) from the number of otus in stored urine. But from the fresh urine number of otus showed a decrease by more than a half. *Morganellaceae* was observed to have a significant decrease to 2 % (402 otus) and the same was true with *Yersiniaceae* (387 otus), *Xanthomonadaceae* (366 otus) and *Ectothiorhodospiraceae* (351). Rather, the *Pseudomonadaceae* was observed to increase almost by half to 50 % (11,716 otus). The most dominating species were *P. aeroguinosa*. The *Enterobacteraceae* (951 otus) and *Pasteurellaceae* (841 otus) were observed to decrease to 4 %. On the other hand the *Aeromonadaceae* (1223 otus) and *Vibrionaceae* (1069 otus) were observed to a slight increase in to 5 %. *Pectobacteriaceae* and *Pseudoaltromonadaceae* each decreased into 1 %; similarly, *Thiobacillaceae* and unassigned families were decreased to less than one percent. *Erwiniaceae*, *Altromonadaceae*, *Halomonadaceae*, *Hahellaceae*, *Alcanivoraceae*, *Cellvibrionaceae*, *Legionallaceae*, *Psychromonadaceae* and *Methylococcous* remained the same. Only *Piscirickettsiaceae* was observed to vanish totally in struvite.

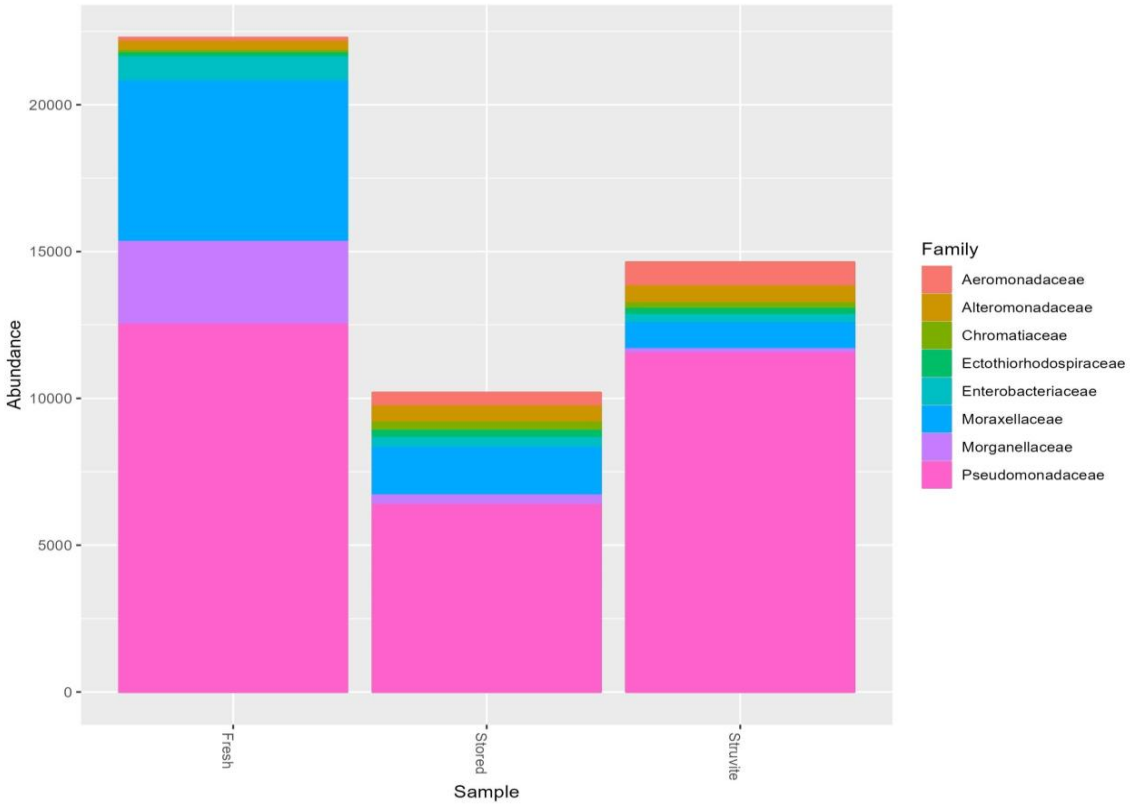


Figure 8: Gamma proteobacteria families found through struvite making

4.3.2. Gamma proteobacteria identified through struvite formation at genus level

The number of otus at genus level decreased from fresh urine to struvite from 52, 730 to 23800 to 23,339 (Fig. 9 and Appendix 2). Thus, roughly by half a decrease was observed. Among these, the genera *Pseudomonas* was dominant in both the samples of fresh urine, stored urine and struvite at proportions of 23 % (12,116 otus), 26 % (6,155 otus) and 47 % (10,955 otus) respectively. The second dominating genera in struvite were *Shewanella* (855 otus) and *Treponema* (943) 4% each. *Shewanella* was observed with a slight difference from otu count 877 in stored urine to in struvite. On the contrary, *Treponema* in stored urine counted (548 otus) but increased in struvite. The third dominating genera were *Acinetobacter*, *Vibrio*, *Azotobacter* and *Marinobacter* each measure 3 % with otus count 589, 757, 761 and 660 respectively. *Acinetobacter* was observed to decrease from the otus count in fresh urine (3079 otus) into stored urine (988 otus) and in struvite (589 otus). Vibrio showed a decrease from the count in fresh urine 861 otus to 758 in stored urine. *Azotobacter* was observed neither increased nor decreased in the three samples. *Marinobacter* increased in stored urine to 3 % from 1 % but remained the same in struvite-K. The next dominating genera

were *Psychrobacter* (495 otus) and *Escherichia* (358 otus) counted 2 % each. *Psychrobacter* counted 7 % (3646 otus) in fresh urine and decreased in stored urine in to 4 % (895 otus) and following a decrement in struvite. In case of *Escherichia*, in fresh urine counted 2 % (974 otus) otherwise, in stored urine decreased int to 2% (452 otus) and continued decreasing in struvite. Among others *Salmonella* was found out to count 1 % (152 otus) in struvite but in fresh urine 1 % 573 (otus) and in stored urine 1 % (212 otus).

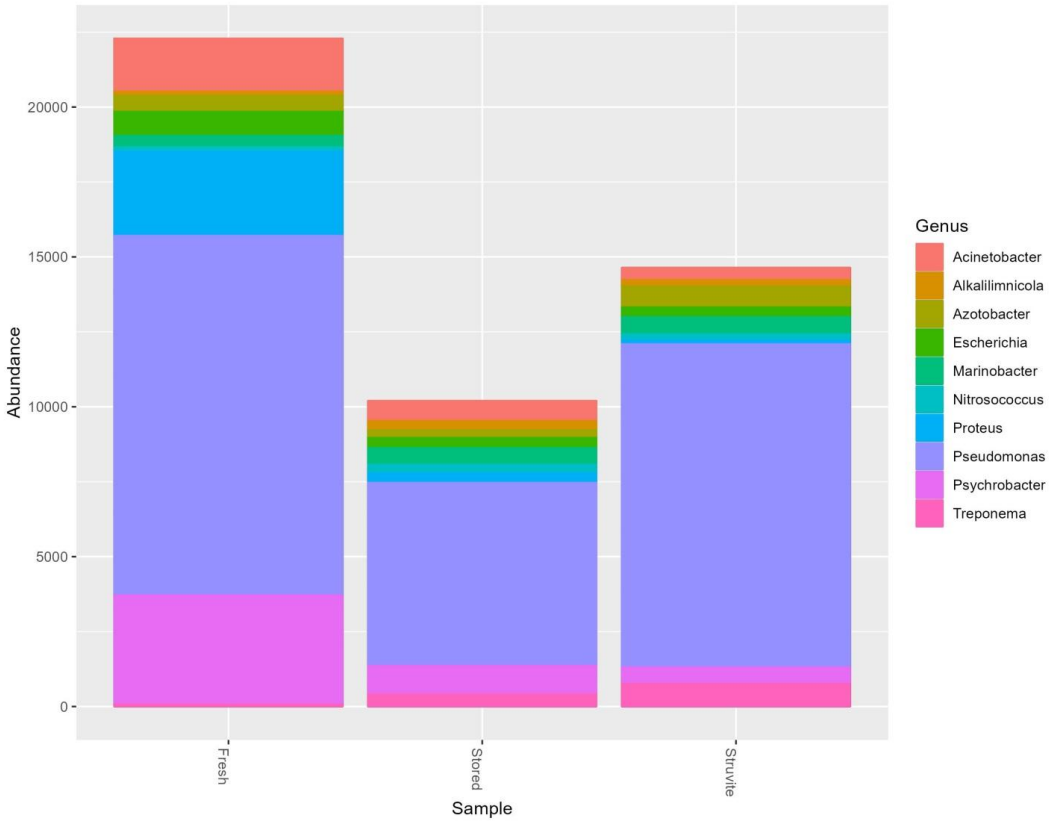


Figure 9: Bacterial genera of gamma-proteobacteria found in fresh, stored urine and struvite

4.3.3. Pathogenic bacteria identified from metagenome assembled genomes (MGS) through struvite production

Approximately forty-two bacterial pathogens have their genomes binned and identified using MGS. Just 19% (n=8) of the bacterial genomes were shared by the fresh, stored urine and struvite samples, whereas the pathogens' genomes were dispersed throughout the three samples (Fig. 10). Only 23% (n=10) of the bacterial genome was detected in fresh urine, whereas struvite and stored urine shared roughly 81% (n=34) of the genome. Members of the genera *Bacillus*, *Clostridium*,

and *Carnobacterium* comprised the phylum *Firmicutes*, which had the dominating bacterial genome in the fresh urine sample (n=10).

The phylum Firmicutes, which made about 33% of all the infectious, had a dominant and sustained genome in the urine that was stored. Similarly, with 33% of the entire pathogen genome, members of the phylum *Firmicutes* were similarly consistently prevalent in struvite. Struvite contained all of the bacterial genomes found in urine that had been stored (Fig. 10). In the urine that was stored, 23 percent of the bacterial genome were eliminated. However, 81% of the organisms survived in stored pee and the struvite that was formed, whereas 19% of the organisms persisted in both fresh and stored urine (Fig. 10).

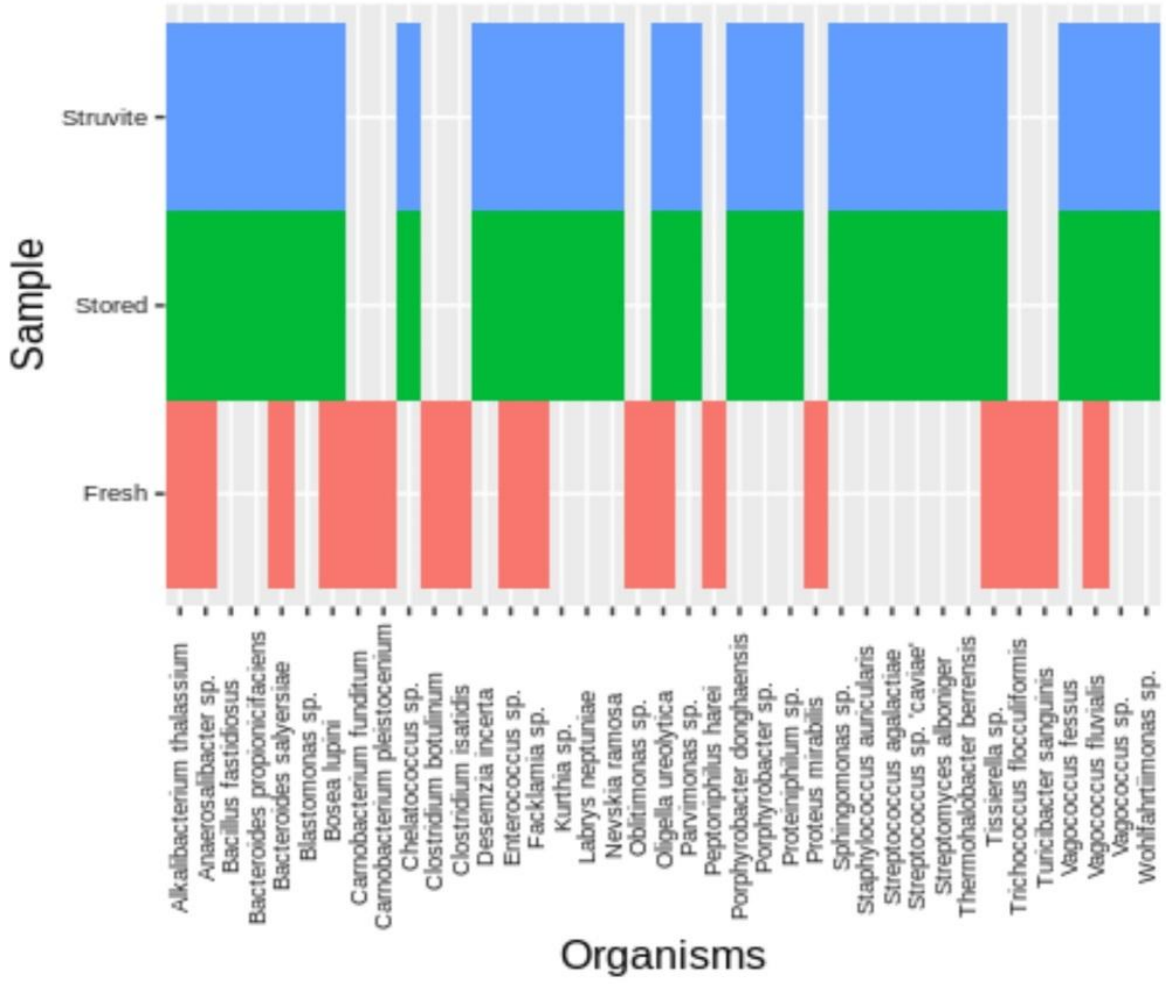


Figure 10: Heatmap illustrating metagenome-derived bacterial pathogens from struvite fresh and stored urine

4.3.4. RNA virus irradication through struvite production

The genome of RNA virus was observed to be degraded through the activity of struvite formation. No intact genome was able to be extracted from struvite. Eventhogh, we followed standard procedures to extract and sequence the genome, no meaningful result was produced. The genomes were observed to degrade when extracted. But we tried to produce sequence information from pieces of RNA as there is a protocol explained in the method for degraded RNA to treat and transfer to sequencing. However, we could not be able to produce any meaningful information.

4.4. Resistome and mobilome from struvite, fresh and stored urine

4.4.1. Phages identified in fresh, stored urine and struvite

The metagenome-derived genome sequence (MGS) in (Fig. 11) revealed several bacteriophages, and the phages in fresh urine differed somewhat from those in stored pee and struvite (Fig. 11). *Acinetobacter*, *Brevibacillus*, and *Morganella* phages were only detected in fresh urine; *Streptococcus*, *Staphylococcus*, *Bacillus*, and *Escherichia* phages were also present in stored urine and struvite-K. *Streptococcus* phage was the most prevalent phage in every sample, followed by *Bacillus* and *Escherichia* phages (Fig. 11).

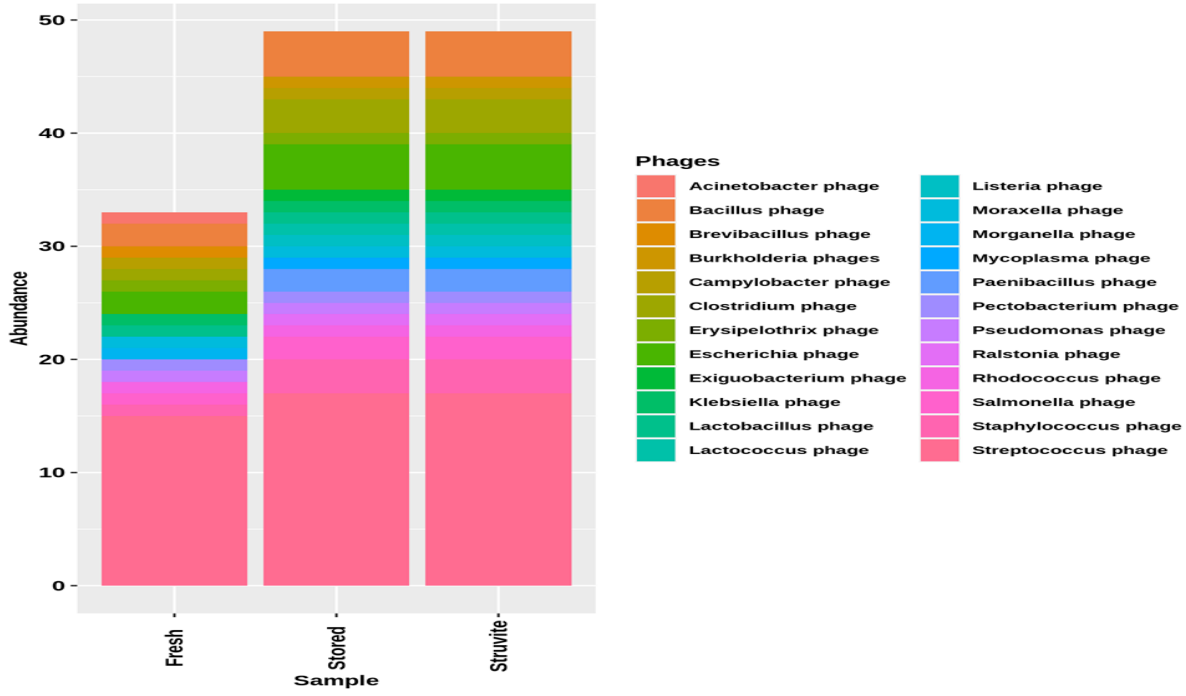


Figure 11: Phages identified in struvite, fresh and stored urine

4.4.2. Antibiotic resistance genes found from bacteria and phages

The fresh urine samples included seven antibiotic resistance genes that conferred resistance to seven antibiotic classes. With resistance to 11 antibiotic families, the number of ARGs found in stored urine rose to 23 (Fig. 12). In struvite samples, 18 ARGs that were resistant to eight antibiotic families were found (Fig. 12). Resistance to aminoglycosides (n = 10), carbapenem (n = 7), chloramphenicol and erythromycin (n = 6 each), and efflux pump (n = 5) ranked as the top five resistances across all sample types (Fig. 12). It was concluded that pathogenic organisms of the genus *Enterococcus*, *Clostridium*, and *Pseudomonas*, among others, were responsible for the presence of ARGs in fresh urine samples. Members of the genera *Escherichia*, *Staphylococcus*, *Proteus*, and *Acenitobacter* were found to carry the majority of the ARGs in the urine sample that was stored. The majority of the ARGs found in the struvite sample were assumed to be carried by members of the *Enterococcus*, *Aeromonas*, and *Acenitobacter* genera.

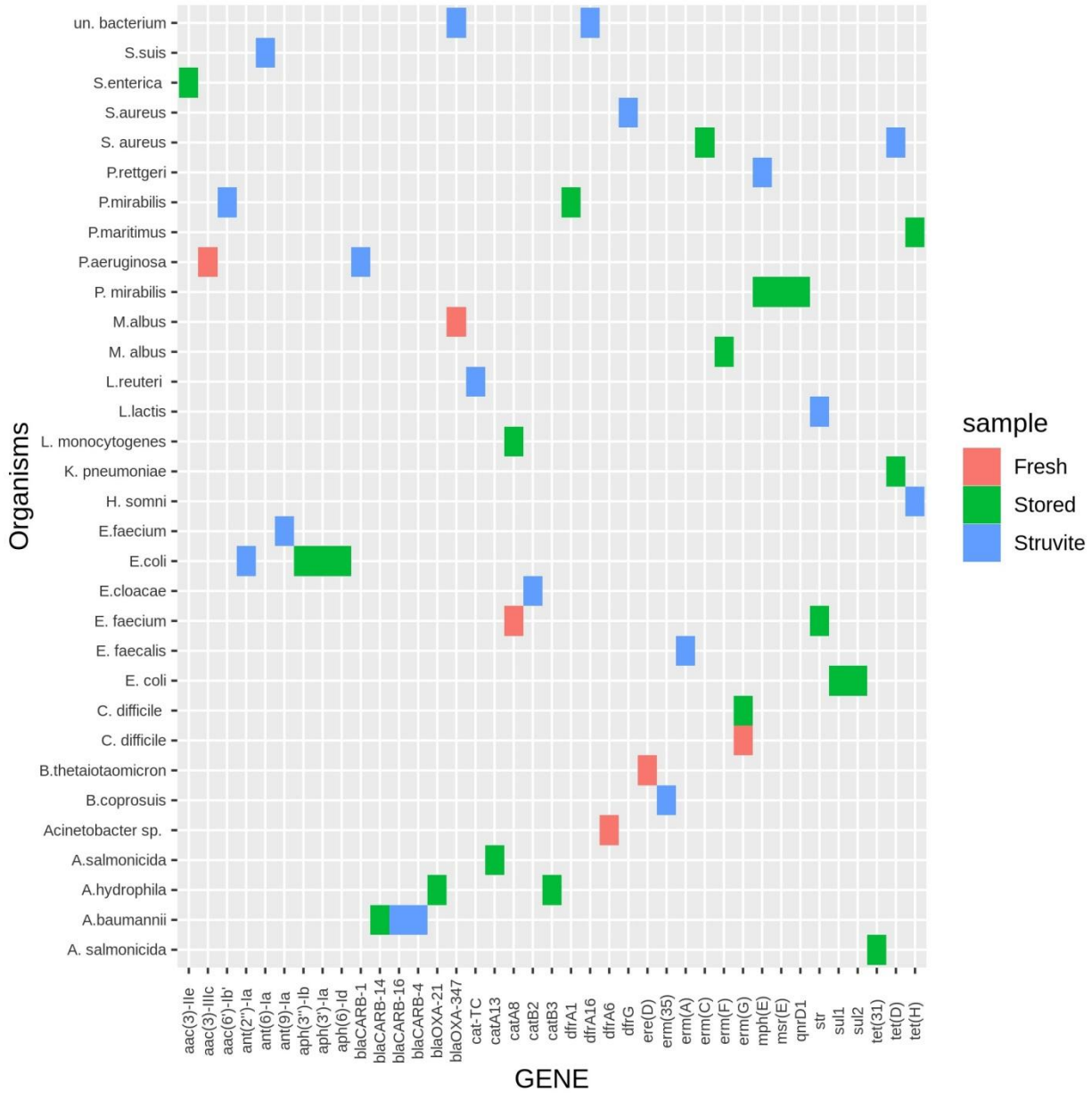


Figure 12: Antibiotic resistance identified in struvite, fresh and stored urine inferred by bacteria on NCBI

MGS produced phage genomes were analyzed for antibiotic resistance carraige. It was found out that resistances for aminoglycosides, chloramphenicol and carbapenem among the others (Fig. 13). The carbapeneme resistance genes were found out to undergo a production of two varients in the derived fertilizer from a single varient in fresh urine (Fig. 13).

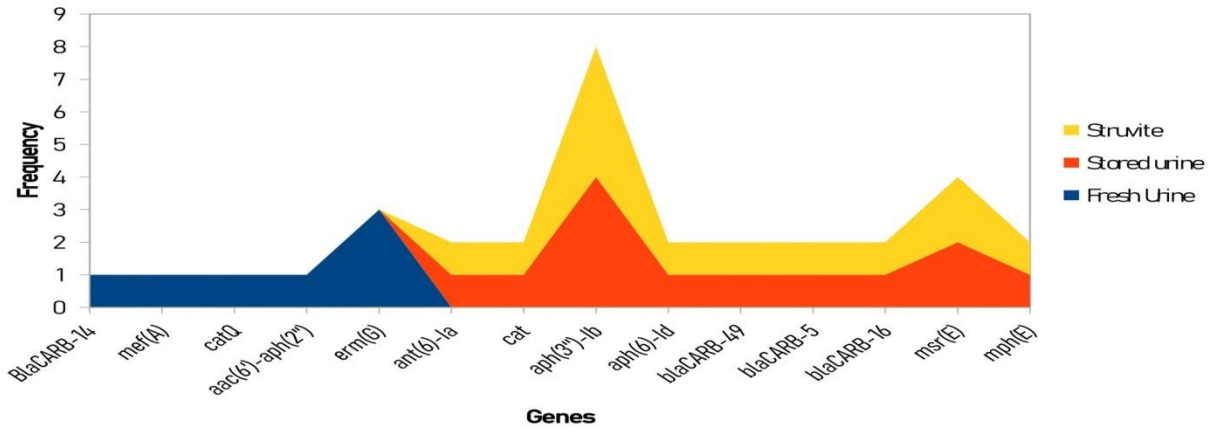


Figure 13: Antibiotic resistance gene identified on phage sequence derived from struvite, fresh and stored urine identified by ResFinder

4.4.3. Plasmids carrying ARGs

From the pathogenic organisms previously mentioned in (Fig. 10), many plasmids containing ARGs were found. Both fresh and stored urine and struvite included plasmids containing erythromycin, vancomycin, and multi-drug resistance genes (Fig. 14 and Table 3). In all three samples, the most prevalent resistance types were vancomycin and multi-drug carrying plasmids. Only fresh and stored urine samples contained carbapenemase-producing gene carrier plasmids. Struvite and stored urine shared several resistance genes, including those for methicillin-resistant type resistance, phenicols, aminoglycosides, and tetracycline. It was found that the struvite sample exhibited colistin resistance. As the detected plasmids were stored until the struvite synthesis process at 8 ºC, it was found that their copy number increased. However, struvite included plasmids containing colistin, which were absent from both fresh and stored urine.

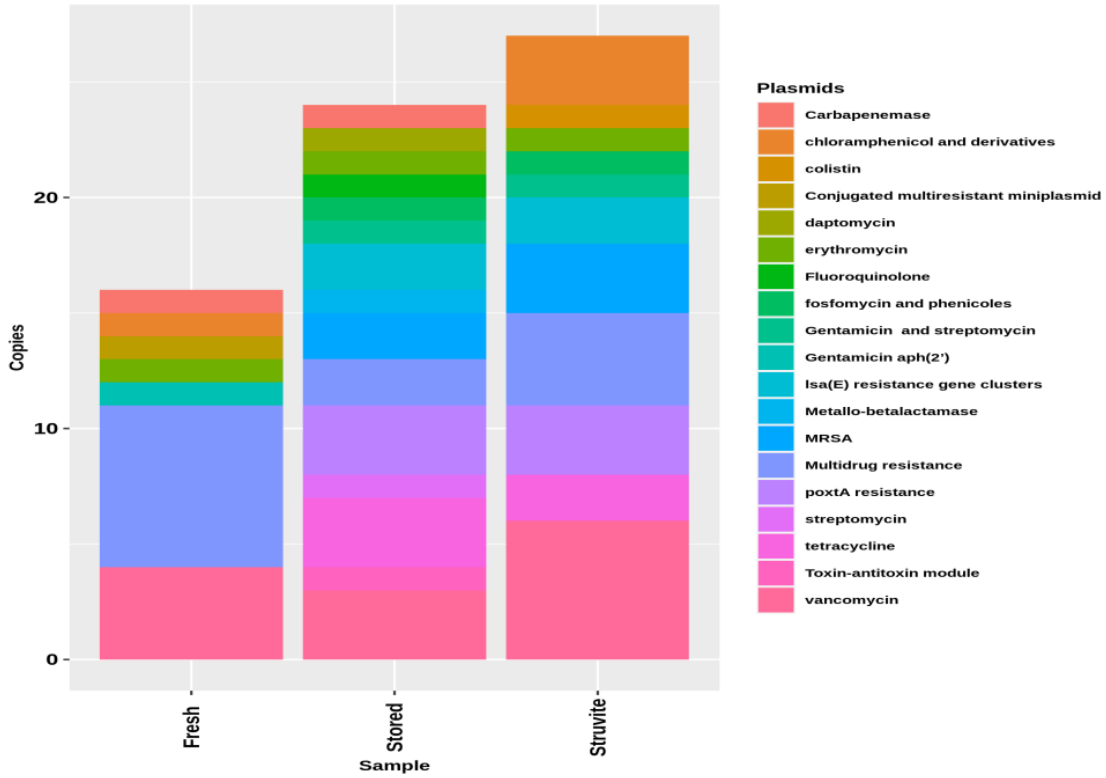


Figure 14: Plasmids predicted by FS machine learning and inferred on NCBI

The FS-plasmid machine learning tool predicted distinct plasmids in fresh, stored, and struvite urine (Table 3). After being inferred on NCBI, the plasmids were found to carry resistance. Numerous plasmids that carry antibiotic resistance were shared by the struvite, fresh, and stored urine.

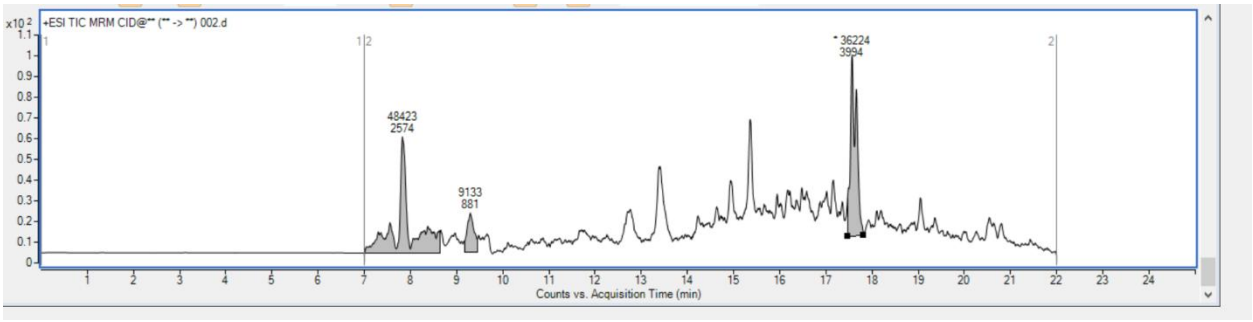
Table 3: Different ARG carrier plasmids derived from struvite, fresh and stored urine (F stands for fresh urine, S stands for stored urine and SU stands for struvite)

Sample	Plasmids predicted by FS machine learning & inferred from NCBI	Resistance inferred on NCBI
Struvite, stored	**plasmid p3^{S}, plasmid p3^{SU},**	Chloramphenicol
Struvite, stored	**Plasmid^{S}, plasmid pYN2-1^{S}, plasmid pNT1N31-93k^{S}, plasmid pJAT2091^{S}, plasmid pYH12068-2^{SU}, plasmid pYN2-1^{SU}, Plasmid^{SU},**	Tetracycline
Struvite, stored, fresh	**plasmid pVE80-1^{F}, plasmid pVE80-1^{F}, plasmid pA10290_P2^{F}, plasmid p41-1^{S}, pVEF4-like plasmid^{S}, plasmid pVE80-1^{S}, plasmid pHLSA^{SU}, plasmid pVE80-1^{SU}, plasmid pZA5-1_01^{SU}, plasmid pVE80-1^{SU}, plasmid pELF1^{SU}, plasmid p41-1^{SU},**	Vancomycin
Struvite, stored, fresh	**plasmid pE211-2^{F} , plasmid pT413A^{F} , plasmid pBS-02^{F}, plasmid pF104_1^{F},plasmid p1^{F}, plasmid pBS 02^{F}, plasmid delta pWDB100^{F}, plasmid pSTE1^{F}, plasmid pCQP3-9_1^{S}, plasmid pSTE1^{S}, plasmid unnamed2^{S}, plasmid pE505-1^{S}, plasmid pE211-2^{S}, plasmid pLI47-1^{SU}, plasmid unnamed2^{SU}, plasmid pE505-1^{SU}, plasmid pE211-2^{SU}, plasmid pCQP3-9_1^{SU}, plasmid pSTE1^{SU},ICE element^{SU},**	Multi-drug resistant
Struvite, stored, fresh	**plasmid p2^{F}, ICESsuYS146^{F}, plasmid pRHB16-C24_3^{F}, plasmid pRHB16-C24_3^{SU}**	Resistance claimed
Struvite, stored	**plasmid paadD^{S}, plasmid pKM0218^{S}, plasmid paadD^{SU}**	LA-MRSA
Struvite, stored	**plasmid p17-508_1^{S}, plasmid p17-508_1^{SU}**	linezolid
Struvite, stored, fresh	**transposon Tn6003^{F} , plasmid pLFE1^{S}, plasmid pLFE1^{SU}**	erythromycin
Struvite, stored, fresh	**plasmid TnpA^{F}, plasmid TnpA^{S}, plasmid TnpA^{SU}**	gentamicine

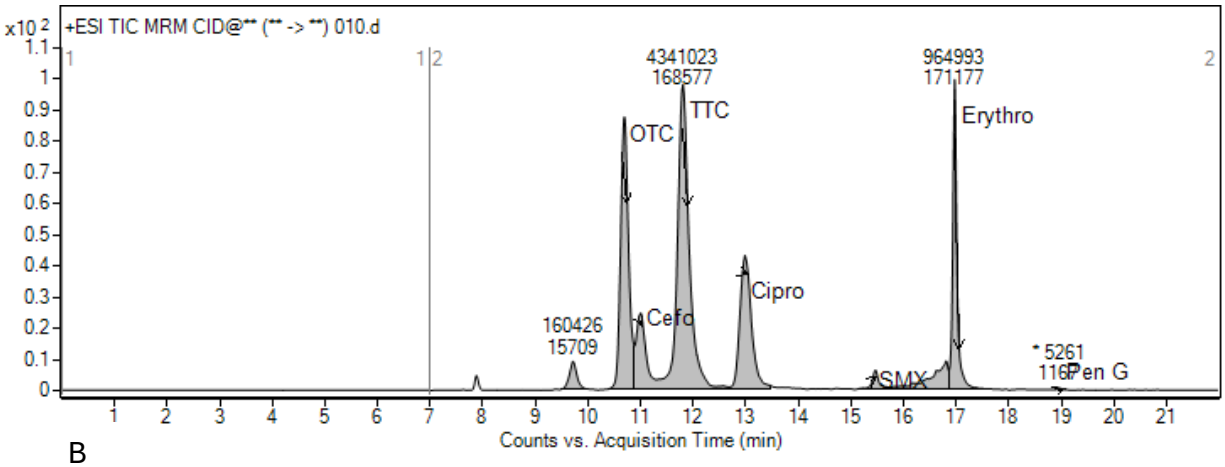
Struvite	**plasmid pCFS3292-1**[SU]	colistin
stored	**plasmid pPPM1000** [S],	mercury resistance
stored	**plasmid pN55972-1**[S],	Fluoroquinolone
stored	**pVEF3 plasmid**[S],	omega-epsilon-zeta toxin-antitoxin
stored	**plasmid p1**[S],	daptomycin
Stored, fresh	**plasmid pVIM_Pse-KSS435**[F], **plasmid pYPR31**[S], **plasmid pJNQH498-1**[S]	carbapenem
fresh	**plasmid p1525-a**[F],	Florfenicol

4.5. Micropollutants from struvite, fresh and stored urine traced by LC-MS/MS

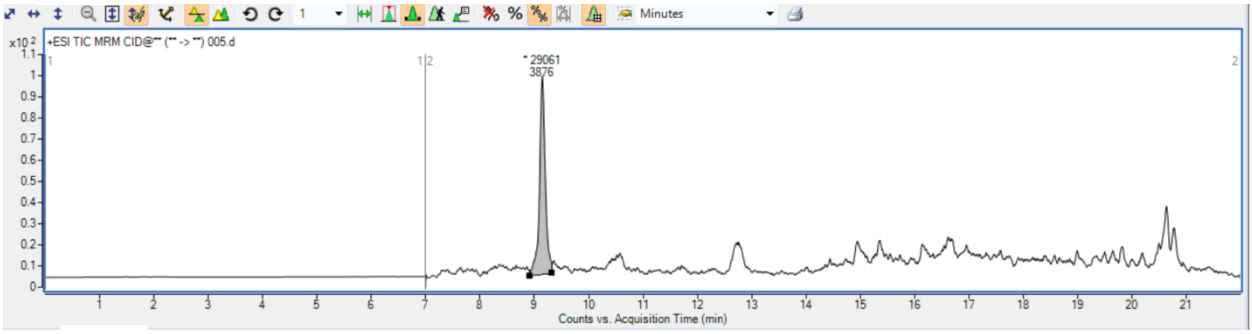
The standards of the assessed antimicrobials such as cefotaxime, ciprofloxacine, erythromycine oxytetracycline, penicillin G, tetracycline, sulfomethoxazole and doxycycline in this work were detected in urine matrix which its history was known. It was after spiked by the fore mentined antibiotics. (Fig.15A). According to their retention time they were displayed below in figure 15A. In comarison the analytes under search in fresh and stored urine sample are displayed in figure 15B & C. All the antibiotics under search were found in the samples except doxycycline and erythromycine according to their retention time and mass to charge ratio compatibility.



A



B



C

Figure 15 A, B, C: Total Ion Chromatogram (TIC) of Seven Antimicrobial Standards Mix Fortified in history known fresh urine and the chromatogram of six antibiotic types in fresh and stored urine respectively

4.5.1. UHPLC-MS/MS condition during analyte detection

UPLC-MS/MS technique is commonly for detection of multi antimicrobial residues in animal source food (Lee *et al*., 2018) from this we adopted for detection in urine matrix. From the LC-MS/MS optimization, the two strong precursor to product ion transitions per target analyte were selected to confirm positive findings for operation in multiple reaction monitoring (MRM) mode. Table 4 shows the selected transitions and MRM optimized MS/MS parameters of all target antimicrobial analytes in the study.

Table 4: Multiple Reaction Monitoring (MRM) Mass Acquisition Parameters and Retention Times of Each Antimicrobials Analyzed

Classes of Antimicrobials	Antimicrobials	RT (min)	Precursor ions $[M+H]^+$	Product ions [m/z]	Fragmentation (V)	CE (V)	CAV
Sulfonamides	Sulfamethoxazole (SMX)	15.47	254.1	156.0	96	22	7
				92.1	96	8	7
Cephalosporin	Cefotaxime (CEFO)	10.91	456.0	125	60	28	4
				166.9	60	70	4
Tetracyclines	Oxytetracycline (OTC)	10.67	461.1	426.2	120	16	7
				443.4	120	8	7
	Tetracycline (TTC)	11.77	445.2	427	120	16	7
				154.1	120	15	7
	Doxycycline (DXY)	16.95	445.1	428.1	120	15	7
				153.9	120	34	7
Quinolones	Ciprofloxacin (CIP)	12.882445	332.1	314.1	131	21	4
				231.0	131	41	4
				576.3	171	16	
Beta lactams	Penicillin G (PnG)	18.95	335.1	176.0	110	13	4
				160.1	110	5	4

RT, retention time; CE, collision energy; CAV, cell accelerator voltage

4.5.2. LCMS/MS method performance evaluation

Specificity

Specificity was tested by assessing the retention time of each standard antimicrobial peak deviated by less than ±15 seconds from its mean retention time (Table 4). This was compared for meeting the US-Environmental Protection Agency (EPA, 2007) standard of ±15 seconds. The result of the assay showed that no significant interfering peaks at the retention time windows for all of the target antimicrobials. The method shows adequate selectivity.

Linearity

Antimicrobial standards of eight types (cefotaxime, ciprofloxacin, sulfamethoxazole, erythromycin, doxycycline, oxy-tetracycline, tetracycline and penicillin G) were prepared by spiking certified reference antimicrobials at concentrations of 50-200 µg/L. Calibration curves were generated, and linearity coefficients (R^2) were found out to be in the range of (0.99-0.97) (Table 4 and Appendix 1) The calibration curves were with reasonable correlation of linearity over the range of the concentrations used in constructing the curves characterized by a high coefficient. The regression equation (y = ax + b) and the determination coefficient (r^2) were assessed by the least squares method. While, y is the response signal or peak, and x the concentration of standard solution in µg/L. Certainly, it is possible to say that this analytical method is linear for all targeted analytes in the selected concentration ranges (EPA, 2007 and EU, 2002).

Accuracy and precision

The accuracy or recovery of the analytical method was illustrated by calculating the mean recovery % ± % relative standard deviation RSD of triplicates of spiked concentrations, ranged from 50 - 200 ug/L. The mean recovery ranged from 71.46 % ± 12.62 % for sulfomethoxazol to 92.86 % ±7.26 % in tetracycline (Table 3). These values fall within the acceptable limit of 100 % ± 20 % recommended by the EPA standard (EPA, 2007 and EU, 2002). Except for erythromycine which its validation of methods failed. On the otherhand, precision was assessed using %RSD of triplicate recovery at concentrations of 50, 100 and 150 µg/L. The RSD ranged from 5.05 % in oxytetracycline to 17.66 % in Sulfomethoxazole (Table 4), meeting EPA standards (2007) of ≤20 % RSD.

4.5.3. Limits of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification was measured according to (Magnusson and Örnemark , 2014). The LOD and LOQ values of the antibiotics ranged between (1.47-0.91) µg/kg and (3.03-9.77) µg/kg respectively according to table 5.

Table 5: Validation of method-results of solid phase extraction UHPLC-MS/MS

Class	Antibiotics	Accuracy (%)	Precision Intra-day (%)	Linearity (R^2)	LOD (µg/kg)	LOQ (µg/kg)
Tetracyclines	Doxcycline	88.51± 8.15	9.21	0.9778	0.91	3.03
Tetracyclines	Oxytetracycline	90.33± 5.01	5.59	0.9942	0.91	3.03
Tetracyclines	Tetracycline	92.86± 7.26	7.82	0.9945	2.22	7.40
Beta-lactam	Penicillin G	84.48± 14.14	16.74	0.9895	2.93	9.77
Sulfonamides	Sulfomethoxazole	71.46± 12.62	17.66	0.9921	1.47	4.91
Quinolones	Ciprofloxacine	88.62± 11.16	12.59	0.9909	1.15	3.83
Cephalosporine	Cefotaxime	90.95± 5.37	5.90	0.9874	1.56	5.19

4.5.4. Analyte identification and quantification

For confirmatory identification, two mass transitions should be there, for a peak at the same RT. This should correspond to that of calibration solutions of the standards with a relative deviations of ± 2.5 % and an absolute deviation of 0.1 min in RT were acceptable (EPA, 2007 and EU, 2002). The ion ratio (qualifier /quantifier) must be within the acceptance criteria for ion ratio, typically not more than ± 25% (EPA, 2007 and EU, 2002). Positive identification was done only for those mass/ion transitions that simultaneously meet both the retention time and relative ion ratio criteria. Thus, accordingly, seven antimicrobials were detected in both fresh urine and stored urine samples (Table 6). Rather, there was no any detection of any of the seven antibiotics in struvite sample. All

the detected mass of the antibiotics in fresh and stored urine were between the value of LOD and LOQ.

Table 6: Detected and quantified antibiotics in struvite, fresh urine and stored urine

Antibitics	Fresh urine (µg/L)	Stored urine (µg/L)	Struvite (µg/Kg)
Doxycycline	< LOD	< LOD	< LOD
Oxytetracycline	8.47	8.49	< LOD
Tetracycline	9.41	9.39	< LOD
Sulfomethoxazole	25.92	25.42	< LOD
Penicillin G	18.15	13.63	< LOD
Ciprofloxacine	10.24	10.22	< LOD
Cefotaxime	1.79	1.79	< LOD

CHAPTER FIVE

5. DISCUSSION

5.1. X-ray diffraction analysis to estimate the existence of struvite

Struvite-K was found in our XRD study (Fig. 14) however, struvite-NH4 was already identified in our urine derived fertilizer by qualitative chemical analysis prior to this study as part of a masters thesis work (unpublished data). The finding of a different mixture of struvite families at pH 10 is possible according to former works (Lind *et al*., 2000 and Bouropoulos and Koutsoukos, 2000). Struvite-K presence in the fertilizer could be as a result of pH rise during our optimization process. This resulted the change of much of ammonium ion into ammonia gas (Huang and Shang, 2006). On the other hand, during the pilot scale stirring of the reaction mixture was by leaving container opened, thus this may cause for the escape of gaseous ammonia from the reactor, which led to formation of struvite-K in addition to the presence of struvite-NH4. Struvite-K is an analogous fertilizer to struvite-NH4 which has similar chemical and physical properties (Wilsenach *et al*., 2007, Graeser *et al*., 2008, Shih *et al*., 2016 and Mathew *et al*., 1979). Struvite-K formation in urine is likely higher when pH rises to 10 (Xu *et al*., 2011 and Wilsenach *et al*., 2007).

5.2. Microbial community structure and distribution of pathogens and non pathogens derived from struvite, fresh and stored urine

Microbial community structure and distribution was studied at different levels of taxa such as phyla class, family and genus. In our study at phyla level *Pseudomonadota*, *Bacillota* and *Actinomycetota* were the most dominant among the others in fresh urine (Fig. 3). Similarily, previous studies have revealed that the members of *Pseudomonadota*, *Bacillota* and *Actinomycetota* dominantely observed in fresh urine (Perez- Carrasco *et al*., 2021; Nelson *et al*., 2010; Zuber *et al*., 2023). On the other hand, members of *Bacillota*, *Pseudomonadota* and *Bacteroidota* were represented in struvite-NH4 in other study (Lahr *et al*., 2016) which also exihibited comparable results with the current work.

At class level, a decrease in microbial population was observed in stored urine samples to the representation of both *alphaproteobacteria* and *gammaproteobacteria* only (Fig. 4). This might be due to the harsh environmental conditions such as high pH value of stored urine (Starliper *et al.,*

2015), bactericidal effect of NH3 (Mendonça *et al*., 2021 and Avalos *et al*., 2020), and the low moisture and ventilation conditions of struvite (Qiu *et al.,* 2022) which generally limit the survival of the microbes. Besides, some of the groups of this class are claimed to be sensitive to external environments outside human host (pressbook.pub on the web). But others such as Bacilli were observed to sustain with a slight difference. This happened may be as a result of the ability of cells to enter into dormancy state (Wetzel and McBride, 2020). The same outcome was observed by Bischel *et al.,* 2015 the Bacilli to persist in stored urine. On the other hand, the others (*Clostridia, Tissierellia* and *Bacterodia*) were observed to increase in number through fresh urine to struvite (Fig. 4). This could be they survived by spore formation and persistence mechanism the harsh conditions in stored urine (La Rosa *et al*., 2022; Wetzel and McBride, 2020), later on they may started to change to vegetative cells in the moisture with less ammonia in decanted struvite solution. Since the decanted struvite was dried at 8 ºC in a refrigerator while container opened. The persistence of *Clostridia* and *Bacterodia* was also observed in stored urine by (Bishel *et al*., 2015 and Lahr *et al*., 2016) and in struvite (Lahr *et al*., 2016).

Quite a number of microorganisms were observed to resist storage treatment and struvite drying and seen increasing in number (Fig. 3 & 4). These phenomena could be is true as result of the intrinsic strength of some bacteria to switch their dividing vegetative cell in to a metabolically dormant state by switching off their vital cellular processes (La Rosa *et al*., 2022; Wetzel and McBride, 2020). In addition, spore forming bacteria can pass the moment of harsh environment by making spores in stored urine and in struvite-NH4 (Bishel *et al*., 2015; Lahr *et al*., 2016). Unlike our results most of the gram negatives were observed to be inhibited during storage (Bishel *et al*., 2015 and Lahr *et al*., 2016). This difference could be caused by the length of the storage which increases the exposure of the bacteria to higher amount of ammonia and alkaline pH (Goetsch *et al*., 2020; Lahr *et al*., 2016; Xu *et al*., 2022). Others many decreased during storage and struvite drying. In this case the higher pH and the large amount of ammonia may be the reasons to kill many cells (Bishel *et al*., 2015 and Lahr *et al*., 2016). According to the measurement of different alpha diversity analysis the fresh urine had more richer in individual species of bacteria than the stored urine and the struvite. This could be as a result of the nutritious and favorable physiological condition in fresh urine (Lahr, *et al*., 2016) The next in richness was struvite. The stored urine as a result of its harsh alkaline pH and increased ammonia concentration may kill unfit bacteria or inhibit them to unrecognizable amount at our sequencing depth (Lahr *et al*., 2016).

Some of the genera from the *gammaproteobacteria* were found out to decrease in stored urine (Fig. 8 & 9) may be as a result of harsh environment (Lahr *et al*., 2016) as a result of ammonia and alkaline pH. Some other were observed to increase in their number in struvite. This was observed by Lahr *et al*., 2016 too in struvite. This could be as a result of the favorable environment in the decanted struvite solution at decreased temperature some proliferated. They can achieve this after resisting the harsh environment in stored urine by cellular mechanisms such as dormancy, spore formation and other cellular fitnesses (La Rosa *et al*., 2022; Wetzel and McBride, 2020; Bishel., 2015; Lahr *et al* 2016). For example, *Pseudomonas aeruginosa* has cellular fitness under law moisture (Mund *et al*., 2017) and at cold temperatures even as low as 8^0C (Chauhan *et al*., 2023). On the other hand *Treponema pallidum* DNA could be isolated from human urine (Wang *et al*., 2022). Besides, the ability of their growth in struvite in our sample could be as a result of the fatty acids required by these species were found in human urine thus in decanted struvite solution (Salvatore, *et al*., 2013).

Besides, clinically important microbes were found to persist in struvite which was arisen from fresh urine (Fig. 8, 9 & 10). This was identified by binning according to the method section. Twenty three percent (23%) of the bacteria were removed from the stored urine. This could be as a result of the toxic effect of ammonia and subsequent genome degradation because of the elevated pH (Starliper *et al*., 2015). On the other hand, 19% of the organisms persisted in fresh and stored urine while 81 % survived in stored urine and the produced struvite (Fig. 9). This could be due to spore formation in some cases, or it could be because their membrane is fit, allowing the organisms to survive either actively or persistently (La Rosa, *et al*., 2022).

Most of the genomes of bacteria persisted through the process of struvite production. This could be as a result we sanitized urine minimally for about 20 days. On the other hand, a small portion of the bacterial genera were not found in stored urine though found in fresh urine and struvite. This could be because of the limitations of metagenome technique to reveal the microbial genera from small quantity of genomes of stored urine samples. Another small portion of the genera were detected only in struvite only. The genera could be originated from fresh urine though hardly detected in fresh urine and stored urine by the technique used. On the other hand the other portion of the genera found in stored and struvite only however the others were only found in stored urine.

Rather we have not found any RNA virus in struvite in our study. This problem with no any trace of viral RNA genome in our study in struvite could be as a result of formerly claimed reasons. One was also indicated in a Japanease paper (Bharucha *et al*., 2019) that RNA viruses are unstable in urine. They simply degrade as a result of genome instability in urine sample thus no traces of RNA genomes in urine. Thus, our struvite was made passing a harsh urine storage environment which could be deleterious for RNA viruses.

5.3. Resistome and mobilome from struvite, fresh and stored urine

Both mobilome and resistome were discovered in struvite and through struvite formation in our investigation (Figure 11-14 and Table 2). Streptococcus phages were shown to be the most prevalent in all three samples (struvite, fresh urine, and preserved urine) in the current investigation. Research on the urinary tract microbiome has also corroborated this indirect indication of the quantity of *Streptococcous* hosts in urine (Wojciuk *et al*., 2019; Żaczek *et al*., 2020). According to Lahr *et al*., (2016), *Streptococcus* was also one of the most prevalent bacteria in fresh urine samples. *Bacillus* phages are a sign that the urine and struvite samples include the species *Bacillus*. Members of the order *Bacillales* were discovered in fresh, one-month-stored pee, and struvite, despite the fact that the genus *Bacillus* was not separately reported in a comparable urine processing investigation (Lahr *et al*., 2016). Although Escherichia phages have been described in numerous clinical investigations, including (Peng *et al*., 2018 and Lukman *et al*., 2020).

Three phages—*Acinetobacter*, *Brevibacillus*, and *Morganella*—were not found in the urine and struvite that were stored for this investigation (Fig. 13). Their sensitivity to the pH rise and ammonia concentration of urine that has been kept may be the cause (Muniesa *et al*., 2013a; Muniesa *et al*., 2013b). However, *Streptococcous* phages were shown to be persistent in both dried struvite and preserved urine. This could be because *Streptococcous* bacteria are resistant to both conditions (Lahr *et al*., 2016).

It is well known that phages can transfer antibiotic resistance genes horizontally (Fig. 14) and that they can continue to resist in unfavorable extracellular conditions (Muniesa *et al*., 2013a; Muniesa *et al*., 2013b). Additionally, their ability to infect a variety of bacterial taxa helps them transmit resistant genes widely (Evans *et al*., 2010; Petrovski *et al*., 2011; Souza *et al*., 1972). Accordingly, the role of phages in the environment is considered significant (American Academy of

Microbiology, 2009). Research indicates that the presence of ARGs in viral particles extracted from polluted water sources (Balcazar, 2014; Colomer-Lluch *et al*., 2011a; Colomer-Lluch *et al*., 2011b; Colomer-Lluch *et al*., 2014 and Muniesa *et al*., 2004).

However, the stored urine and dried struvite retained the ARGs in the inferred organisms (Fig. 12 & 14). According to Vermeulen *et al*., (2007), this might be because the organisms that were found managed to survive despite the high pH, high ammonia content, and low water activity. It is concerning that ARGs, which are known as critical priority bacterial strains, were found in struvite carried by inferred *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli* (Havenga *et al*., 2019). *A. baumannii* hosted persistent ARGs in stored urine and struvite, including *MphE* (macrolide), which necessitate an intervention solution during the production process. These bacteria were found to survive following struvite drying and undertake ARG transfer through plasmids in certain cases, albeit this was not demonstrated in the current investigation (Fig. 11).

The ARGs found in phages were found to be associated with erythromycin, carbapenem, aminoglycoside, efflux pump, and chloramphenicol (Fig. 14). A change in the resistance gene variations from fresh to stored urine and struvite was noted while preserving the gene families (Fig. 14). Similar resistance gene variations were discovered in the struvite and stored urine samples. In struvite from fresh urine, the number of carbapenem resistance genes rose from one variant (*blaCARB-14*) to three additional variants (*blaCARB-49*, *blaCARB-5*, and *blaCARB-16*).

The ecosystem may be at risk from antibiotic resistance genes present on struvite. The genes responsible for ARG mobility in bacteria that were discovered on the struvite were located on phages and plasmids (Figs. 12 and 13). Applying such a pool of hazards to agriculture necessitates adequate urine treatment that takes resistomes and mobilomes into account. According to a study by Bischel *et al*., (2016), a significant decrease in microbial groups was observed when the generated struvite-NH4 was dried at a range of temperatures. Given the low sanitizing capacity of urine storage in this investigation, particular emphasis should be paid to the observed shift of resistance genes and intensity of phage-encoded ARGs. The following resistant genes withstood storage and struvite drying: *ant* (streptomycine), *cat* (chloramphenicol), *aph* (aminoglycoside), *bla* (beta-lactamease), *msr* (streptogamin, macrolide, and phosphnic acid), and *mph* (macrolide) (Fig. 12). In Addis Ababa, a prior survey found that amoxicillin, penicillin G, and ampicillin are among the most often administered antibiotics (Nebiyat and Adey, 2023: Supplementary material).

Techniques for lowering ARGs in struvite are crucial. Since daptomycin is the last treatment for *Staphylococcal* infections, finding a daptomycin-carrying plasmid in stored urine and struvite was another major worry (Bayer *et al*., 2013). Furthermore, the improvement of struvite drying processes is crucial for the identification of antibiotic resistances, including colistin (Gündoğdu *et al*., 2016). According to a study, sanitizing and storing struvite at high temperatures led to a considerable decrease in certain antibiotics and microorganisms (Bischel *et al*., 2016). However, given Ethiopia's lack of clean water and the fact that temperatures have risen globally, washing is not a viable option for Ethiopian-owned struvite facilities. Therefore, it is advised to use cold and ambient sanitizing techniques including UV treatment and storage at room temperature.

5.4. Method validation and investigating the fate of antibiotics in struvite, fresh and stored urine

About six antimicrobials from five classes were detected in fresh and stored urine. The antibitoics reasonably detected were oxytetracycline, tetracycline, penicillin G, sulfomethoxazole, ciprofloxacine and cefotaxime. The conditions for the technique validation in this work were satisfied by seven antibiotics. According to Table 5, they are oxytetracycline, tetracycline, cefotaxime, sulfomethoxazole, penicillin G, doxycyclin and ciprofloxacin. We obtained fairly consistent results using mobile phases A (water with 0.1% formic acid), B (ACN with 0.1% FA), and C (methanol with 0.1% formicacid) 0.1% (Table 1). In contrast to our method, the studies of Venkatesh *et al.,* 2013, Radovanovic *et al.,* 2022 and Patyra *et al.,* 2024 have been successful in assessing numerous antibiotics utilizing two mobile phases combining the claimed compounds in different amounts. The standards employed in this work to validate the method have a working concentration ranging from 50 to 150 µg/L. Comparable to other analytical work is this (Venkatesh et al., 2013). However, our work included a narrow range of concentrations (50 to 200) µg/L for the linearity assessment, which set it apart from previous studies. (Mullen *et al.,* 2017).

Using the UHPLC-MS/MS, separation and detection were accomplished. Since all of the analytes showed higher ionization efficiencies in positive mode than in negative mode, the MS was run under +ESI. The tetracycline class of drugs, cefotaxime, and ciprofloxacin all exhibited higher ionization efficiencies in positive mode when using our approach. (Mullen *et al*., 2017 and Radovanovic *et al*., 2022).

Precursor ions (m/z), qualitative ions (m/z), and quantitative ions (m/z) were used to monitor the antibiotics during the MS analysis (Table 3). Antibiotics were quantified using an external calibration method; Table 4 presents the results. To identify the analytes, method validation was monitored using specificity, linearity, accuracy, and precision; Table 5 shows the results. According to the EPA, 2007 and EU, 2002 guidelines, our separation and detection technique satisfied each of these requirements within an acceptable range. (EPA, 2007 and EU, 2002). Similar to our work, other investigations also included linearity, specificity, precision, and accuracy; they also validated methodologies and detected and quantified multiplexed antibiotic mix in various matrices. (Venkatesh *et al*., 2013 and Patyra *et al*., 2024).

Method validation was carried out with specificity, linearity, accuracy, and precision in order to identify the analytes; the results are displayed on Table 5. Our separation, quantification and detection method fell within an acceptable range for each of these criteria, as per the EPA, 2007 and EU, 2002 standards. The highest value of mean square for linearity in our work was 0.9945. On the contrary in other works the linearity is with mean square (R2) above 0.995 (Venkatesh *et al*., 2013, Radovanovic *et al*., 2022 and Patyra *et al*., 2024). The inter day, precision was 16.7 % in this work. This is unlike other works which is less than 7 % (Venkatesh *et al*., 2013, Radovanovic *et al*., 2022 and Patyra *et al*., 2024). The accuracy ranged in our work 88.51 %–90.95 %. Rather, in another works we compared is greater than 95%, (Radovanovic *et al*., 2022 and Patyra *et al*., 2024) and in other occasions closer to our results (Venkatesh *et al*., 2013). Just like our work, others detected and measured multiplexed antibiotic mix in different matrices (Radovanovic *et al*., 2022 and Patyra *et al*., 2024). Though, they referenced different validation standards and their procedure is different from ours, some of them in using internal standards.

Despite the fact that their process differs from ours in that some of them use internal standards, they made reference to several validation standards. Furthermore, the EPA and EU criteria were met for the detection of analytes based on their respective retention duration and the qualifier to quantifier ion ratio. These parameters were found to be within an acceptable range. (EPA, 2007 and EU, 2002) In contrast to our approach, in another work by (Mullen *et al*., 2017), they tracked antibiotics by detecting the ratio of quantifier to qualifier ion area for the analytes vs standard. Our work's measured analytes amount exceeded the limit of detection (LOD), demonstrating the

accuracy of the findings. (EPA, 2007 and EU, 2002) Compared to other research, we discovered that our LOD was greater. (Mullen *et al*., 2017, Venkatesh *et al*., 2013, Miller *et al*., 2016)

The detection of different antibitics in fresh urine and stored urine is not a wonder since many antibitics degrade at acidic pH (Kurjogi *et al*., 2019, Loftin *et al*., 2008 and Dehghani *et al*., 2013). However, at room temprature in fresh urine a shift of pH occurs very rapidly to neutral and alkaline pH (Lahr *et al*., 2015). As a result, stability of antibiotics increaseses (Kurjogi *et al*., 2019 and Dehghani *et al*., 2013) through increament of pH from 5 to 8.9. In case of fresh and stored urine which pH ranges from 5 to 8.9 in stored urine according to our sample can be a comfortable place for most antibiotics with minimal degradation. For example, Penicllin G, tetracycline, erythromycin, doxycycline and oxytetracycline remains active with minimal degradation at basic pH range around 8-8.9 (Kurjogi *et al*., 2019, Dehghani *et al*., 2013, Xuan *et al*., 2009 and Toala *et al*., 1970).

On the contrary, at higher pH where struvite was prepared (pH=10), different antibiotics are unstable if exposed for long time (Xuan *et al*., 2009) thus, degraded. As a result, in this work no residual antibiotics was detected in struvite sample. According to (Ronteltap *et al*., 2007) who claimed about washing of struvite cleans it from pharmaceuticals sounds good. However, for water scarce areas like Ethiopia preparing struvite at alkaline pH is profitable to get pharmaceutical free product according to the results in this work.

On the other hand, for the reason the antibiotics from fresh and stored urine was not detected in struvite could be as a result of microbial degradation while in struvite solution till dehydration. The fresh urine was put into -20^{0}C with an hour of collection. Within this moment may be no or small degradation occurred on the antibiotics. However, during urine storage, the temperature, constituents of the matrix, metabolic activity of microbes and type of dominant species vary from the struvite. These factors are found to affect bio-degradation of antibiotics in all the samples of struvite, fresh and stored urine therefore antibiotics remained intact in stored urine but not in struvite-K (Yang *et al*., 2021). In opposite, in the decanted struvite where ammonia concentration dropped and there was moisture and there were nutrients, microbes may manage to grow and degrade antibiotics derived from stored urine (Nebiyat and Adey, 2023). Especially the antibiotics sulfomethoxazole was observed to be easily degradable in the dominance of *Acinetobacter* and *Pseudomonas* species (Yang, *et al.,* 2016). In struvite *Pseudomonas* species were the dominant

species which agrees with the idea of the presence of these bacteria may favor degradation as a result the antibiotics was not detected in struvite in our work. On the other hand, the reason for the absence of ciprofloxacin in struvite sample could be a biological degradation (Amorim *et al*., 2014, Liao *et al*., 2016, Alexandrino *et al*., 2017, Maia *et al*., 2014 and Pan *et al*., 2018). As, this was observed on different samples in different works ciprofloxacin to be degraded by microbes (Amorim *et al*., 2014, Liao *et al*., 2016, Alexandrino *et al*., 2017, Maia *et al*., 2014 and Pan *et al*., 2018). According to the aforementioned works Bacteriodete and Proteobacteria species were found to degrade ciprofloxacin. These species were abundant in struvite which implies the degradation could be because of these. The same fate was observed for erythromycin in struvite it was also as a result of biodegradation as this occurred some-where in a different work and (Zhang *et al*., 2017). In conclusion, the synergestic effect of pH and microbial degradation may cleanse the micropollutants from struvite as it was referenced on by the above claimed former works. As the basic struvite solution around 10 with decreased amount of ammonia as a result of volatilization during drying may be was a comfortable place for growth. Evidently, some microbes were observed to grew in this solution (Nebiyat and Adey 2021).

CHAPTER SIX

6. CONCLUSION AND RECOMMENDATION

Mobilomes, pathogens and resistome were found in struvite thus either on struvite drying, storage conditions of urine, chemical and physical methods such as UV or biological treatments (engineered organisms) should be taken in to consideration to free struvite from biological contaminants. Most of all the results in our work shows that the human derived fertilizer can be a source of biological hazards to the soil environment if untreated properly. This may join the food chain of humans and animals. On the other hand, micro-pollutants were not found in struvite this may entails that the product can be safe from micropollutants at least in this work that may be was affected by pH and microbial degradation. Therefore, I recommend the study of the right reasons of the free state of struvite from antibiotics should be studied so that one can use it in future applications.

7. REFERENCES

Abe, S. (1995). Phosphate removal from dewatering filtrate by MAP process at Seibu treatment plant in Fukuoka City. Sewage Works in Japan. pp. 59–64.

Ackerman, A. L., & Chai, T. C. (2019). The Bladder is Not Sterile: an Update on the Urinary Microbiome. *Current bladder dysfunction reports*, 14(4), 331–341.

Adewole, A.T., Oyekale, A. S., Adeoluwa, A., & Cofie, O. (2013). Farmers' perception on the use of urine for growing vegetables in Ibadan. *Journal of Human Ecology, 41*(1), 19-23.

Agency for Toxic Substances and Disease Registry, ATSDR (2004). *Toxicological Profiles for Ammonia. US Department of Health and Human Services, Public Health Services, Atlanta*. Retrieved June 17, 2017 from http://www.atsdr.cdc.gov/toxpro2.html

Aguilera, E., Lassaletta, L., Sanz-Cobena, A., Garniere, J., & Vallejo, A. (2013). The potential of organic fertilizers and water management to reduce N_2O emissions in Mediterranean climate cropping systems. *Agriculture, Ecosystems & Environment*, *164*, 32–52.

Ahmed, W., Hamilton, K. A., Vieritz, A., Powell, D., Goonetilleke, A., Hamilton, M. T., & Gardner, T. (2017). Microbial risk from source-separated urine used as liquid fertilizer in sub-tropical Australia. *Microbial Risk Analysis, 5*, 53–64.

Alexandrino, D. A., Mucha, A. P., Almeida, C. M. R., Gao, W., Jia, Z., & Carvalho, M. F. (2017). Biodegradation of the veterinary antibiotics enrofloxacin and ceftiofur and associated microbial community dynamics. *Science of the Total Environment*, *581*, 359-368.

Ali, J., Rafiq, Q. A., & Ratcliffe, E. (2018). Antimicrobial resistance mechanisms and potential synthetic treatments. *Future science OA*, *4*(4), FSO290.

Al-Odaini, N.A., Zakaria, M.P., Yaziz, M.I., & Surif, S. (2010). Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *Journal of chromatography A, 1217,* 6791–6806.

Aminov, R.I. (2011). Horizontal gene exchange in environmental microbiota. *Fronteirs in Microbiolog, 2,* 1–9.

American Academy of Microbiology (2009). Antibiotic Resistance: An Ecological Perspective on an Old Problem. Washington, DC.

Amorim, C. L., Moreira, I. S., Maia, A. S., Tiritan, M. E., & Castro, P. M. (2014). Biodegradation of ofloxacin, norfloxacin, and ciprofloxacin as single and mixed substrates by Labrys portucalensis F11. *Applied microbiology and biotechnology*, *98*, 3181-3190.

Andersson, E. (2015). Turning waste into value: using human urine to enrich soils for sustainable food production in Uganda. *Journal of Cleaner Production, 96,* 290-298.

Andreu, A. (2005). Pathogenesis of urinary tract infections. *Enfermedades Infecciosas y Microbiología Clínica, 23*(4), 15–21.

Aniko Zseni (2015) Human Excreta as an Important Factor of Sustainable Water Management and Agriculture4th International Conference on Informatics, Environment, Energy and Applications Volume 82 of IPCBEE *DOI: 10.7763/IPCBEE. 2015.V82.22.*

Ankley, G. T., & Johnson R. D. (2004). Small fish models for identifying and assessing the effects of endocrine–disrupting chemicals. *ILAR Journal, 45,* 469–483.

Antonini, S., Paris, S., Eichert, T., & Clemens, J. (2011). Nitrogen and phosphorus recovery from human urine by struvite precipitation and air stripping in Vietnam. *CLEAN–Soil, Air, Water*, *39*(12), 1099-1104.

Avalos, M., Garbeva, P., Raaijmakers, J. M., & van Wezel, G. P. (2020). Production of ammonia as a low-cost and long-distance antibiotic strategy by Streptomyces species. *The ISME Journal 14,* 569–583.

Ayukekbong, J. A., Ntemgwa, M., & Atabe, A. N. (2017). The threat of antimicrobial resistance in developing countries: Causes and control strategies. *Antimicrobial Resistance & Infection Control, 6,* 47.

Baharoglu, Z., & Mazel, D. (2014).SOS, the formidable strategy of bacteria against aggressions. *FEMS microbiology reviews, 38,* 1126–1145.

Baker-Austin, C.,Wright, M. S., Stepanauskas, R., & McArthur, J. V. (2006).Co- selection of antibiotic and metal resistance. *Trends in Microbiology, 14,* 176–182.

Balcazar, J. L. (2014). Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS pathogens*, *10*(7), e1004219.

Banks, E., Chianelli, R., & Korenstein, R. (1975). Crystal Chemistry of Struvite Analogs of the Type MgMPO 4 · 6H 2 O (M + = K + , Rb + , Cs+, Ti + , NH4 +). *Inorganic Chemistry, 14*, 1634–1639.

Banks, D. J., Porcella, S. F., Barbian, K. D., Martin, J. M., & Musser, J. M. (2003). Structure and distribution of an unusual chimeric genetic element encoding macrolide resistance in phylogenetically diverse clones of group A Streptococcus. *The Journal of infectious diseases,* 188, 1898–1908.

Barlow, M., & Hall B.G. (2002). Phylogenetic analysis shows that the OXA b-lactamase genes have been on plasmids for millions of years. *Journal of Molecular Evolution, 55,* 314–321.

Baron, S., & Davis, C. P. (1996). Normal flora. *Medical Microbiology, 4th ed.; Baron, S., Ed.; University of Texas Medical Branch: Galveston, TX, USA*.

Baroud, Á., Dandache, I., Araj, G. F., Wakim, R., Kanj, S., Kanafani, Z., ... & Matar, G. M. (2013). Underlying mechanisms of carbapenem resistance in extended-spectrum β-lactamase-producing Klebsiella pneumoniae and Escherichia coli isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases. *International journal of antimicrobial agents*, *41*(1), 75-79.

Batchelder, A. R. (1981). Chlortetracycline and oxytetracycline effects on plant growth and development in liquid cultures. *Journal of Environmental Quality, 10*(4), 515-518.

Batchclder A. R. (1982) Chlortelracyline and oxytetracykline effects on plant growth and development in soil systems. *Journal of Environmental Quality, 11*(4), 675-678.

Battistoni, P., Pavan, P., Prisciandaro, M., & Cecchi, F. (2000). Struvite crystallization: a feasilble and reliable way to fix phousphourous in anaerobic supernatants. *Water Research, 34*(11), 3033-3041.

Båth, B. (2003). Field trials using human urine as fertilizer to leeks (In Swedish). *Manuscript, Department of Ecology and Plant Production Science*, Swedish University of Agricultural Sciences. Uppsala, Sweden.

Bayer, A.S., Schneider, T. and Sahl, H-G. (2013). Mechanisms of daptomycin resistance in Staphylococcus aureus: role of the cell membrane and cell wall. Ann N Y Acad Sci. 2012 Dec 5;1277(1):139-158.

Beaber, J. W., Hochhut, B., & Waldor, M. K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature, 427*, 72–74.

Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J. A., Doumith, M., Hornsey, M., & Woodford, N. (2011). Phosphoethanolamine modification of lipid A in colistin-resistant variants of Acinetobacter baumannii mediated by the pmrAB two-component regulatory system. *Antimicrobial agents and chemotherapy*, *55*(7), 3370-3379.

Beler Baykal B., Allar A., & Bozkir E. (2011). A preliminary survey of the public acceptance of the use of human urine as fertilizer in paper presented at the 3rd international congress Waste water in small communities towards the water frame work directive and the development goals; Istanbul, Turkey.

Bharucha, T., Sengvilaipaseuth, O., Seephonelee, M., Vongsouvath, M., Vongsouvath, M., Rattanavong, S., ... & Dubot-Pérès, A. (2019, March). Viral RNA degradation makes urine a challenging specimen for detection of Japanese encephalitis virus in patients with suspected CNS infection. In *Open forum infectious diseases* (Vol. 6, No. 3, p. ofz048). US: Oxford University Press.

Bhuiyan, M. I. H., Mavinic, D. S., & Koch, F. A. (2008). Thermal decomposition of struvite and its phase transition. *Chemosphere, 70,* 1347-1356.

Billard-Pomares, T., Fouteau, S., Jacquet, M. E., Roche, D., Barbe, V., Castellanos, M., ... & Branger, C. (2014). Characterization of a P1-like bacteriophage carrying an SHV-2 extended-spectrum β-lactamase from an Escherichia coli strain. *Antimicrobial agents and chemotherapy*, *58*(11), 6550-6557.

Bischel, H.N., Schindelholz, S., Schoger, M., Decrey, L., Buckley, C.A., Udert, K.M. and Kohn, T. (2016). Bacteria Inactivation during the Drying of Struvite Fertilizers Produced from Stored Urine. Environmental Science and Technology 50(23) DOI:10.1021/acs.est.6b03555

Bischel, H. N., Duygan, O. D. B, Strande, L., McArdell, S. C., Udert, M. K., & Kohn, T. (2015). Pathogens and pharmaceuticals in source-separated urine in eThekwini, South Africa. *Water Research, 85,* 57-65.

Bischel, H. N., Özel, B. D., Strande, L., McArdell, C. S., Udert, K. M., & Kohn T. (2015). Pathogens and pharmaceuticals in source-separated urine in eThekwini, South Africa. *Water Research, 85,* 57–65.

Bischel, N. H., Schindelholz, S., Schogger, M., Decrey, L., Buckley, A. C., Udert, M. K., & Kohn, T. (2016). Bacteria inactivation during the drying of struvite fertilizers produced from stored urine. *Environmental Science and Technology,* 50(23), 13013-13023.

Black, Z., Balta, I., Black, L., Naughton, P. J., Dooley, J. S. G., & Corcionivoschi, N. (2021). The Fate of Foodborne Pathogens in Manure Treated Soil. *Frontiers in Microbiology,* 12, 781357.

Blahová, J., Hupková, M., Babálová, M., Krcméry, V., & Schäfer, V. (1993). Transduction of resistance to Imipenem, Aztreonam and Ceftazidime in nosocomial strains of Pseudomonas aeruginosa by wild-type phages. *Acta virologica, 37*, 429–436.

Blanco, L. E., Aragon, A., Lundberg, I., & Lidén, C. (2005). Determinants of dermal exposure among Nicaraguan subsistence farmers during pesticide applications with backpack sprayers. *Annals of Occupational Hygiene, 49,* 17-24.

Blanco, P., Hernando-Amado, S., Reales-Calderon, J. A., Corona, F., Lira, F., Alcalde-Rico, M., ... & Martinez, J. L. (2016). Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. *Microorganisms*, *4*(1), 14.

Blume, S., & Winker, M. (2011). Three years of operation of the urine diversion system at GTZ headquarters in Germany: user opinions and maintenance challenges. Water Science and Technology, *64*(3), 579-586.

Bofill-Mas, S., Pina, S., & Girones, R. (2000). Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. *Applied Environmental Microbiology, 66*(1), 238–245.

Bouatra, S., Aziat, F., Mandal, R., Guo, A. C., Wilson, M. R., Knox, C., ... & Wishart, D. S. (2013). The human urine metabolome. *PloS one*, *8*(9), e73076.

Bouropoulos, N. C., & Koutsoukos, P. G. (2000). Spontaneous precipitation of struvite from aqueous solutions. *Journal of Crystal Growth, 213,* 381–388.

Boxall, A. B. A., Johnson, P., Smith, E. J., Sinclair, C. J., Stutt, E., & Levy, L. S. (2006). Uptake of veterinary medicines from soils into plants. *Journal of Agriculture and Food Chemistry, 54,* 2288–2297.

Burke, E.A.J. and Ferraris, G. (2004). New Minerals and Nomenclature Modifications Approved In 2003 By the Commission on New Minerals and Mineral Names, International Mineralogical Association. *The Canadian Mineralogist*, 42:905-913.

Brabban, A. D., Hite, E., & Callaway, T. R. (2005). Evolution of food borne pathogens via temperate bacteriophage-mediated gene transfer. *Food borne Pathogens & Disease*, *2*(4), 287-303.

Brambilla, G., Civitareale, C., & Migliore, L. (1994). Experimental toxicity and analysis of bacitracin, flumequine and sulphadimethoxine in terrestrial and aquatic organisms as a predictive model for ecosystems damage. *QUIMICA ANALITICA-BELLATERRA-*, *13*, S114-S114.

Braun, A. M., & Thomas P. (2003). Androgens inhibit estradiol-17beta synthesis in Atlantic croaker (*Micropogonias undulatus*) ovaries by a nongenomic mechanism initiated at the cell surface. *Biology of Reproduction, 69*(5), 1642-1650.

Burke, E.A.J. and Ferraris, G. (2004). New Minerals and Nomenclature Modifications Approved In 2003 By the Commission on New Minerals and Mineral Names, International Mineralogical Association. The Canadian Mineralogist, 42:905-913.

Burkhardt-Holm, P., Peter, A., & Segner, H. (2002). Decline of fish catch in Switzerland-project Fishnet: a balance between analysis and synthesis. *Aquatic Sciences, 64,* 36-54.

Bushman, F., (2002). Lateral DNA Transfer: Mechanisms and Consequences. Cold Spring Harbor Laboratory Press, New York.

Cao, X., Hao, G., Li, Y., Wang, M., & Wang, J. (2022). On male urination and related environmental disease transmission in restrooms: From the perspectives of fluid dynamics. *Sustainable Cities and Society, 80*, 103753.

Cargill, J. S., & Upton, M. (2009). Low concentrations of vancomycin stimulate biofilm formation in some clinical isolates of *Staphylococcus epidermidis*. *Journal of Clinical Pathology, 62,* 1112–1116.

Carne, G., Leconte, S., Sirot, V., Breysse, N., Badot, P. M., Bispo, A., & Crépet, A. (2021). Mass balance approach to assess the impact of cadmium decrease in mineral phosphate fertilizers on health risk: The case-study of French agricultural soils. *Science of the Total Environment*, 760, 143374.

Chaix-Bar, M. (2014). Opportunities and constraints of using urine as a fertiliser in Silasse, Ethiopia. BSc Thesis. University of York. Environment and Geography Department. Triple Green Project, Stockholm Environment Institute.

Chandran, A. Pradhan, S. K., & Heinonen-Tanski, H. (2009). Survival of enteric bacteria and coliphage MS2 in pure human urine. *Journal of Applied Microbiology, 107,* 1651-1657.

Chatzitakis, A., Berberidou, C., Paspaltsis, I., Kyriakou, G., Sklaviadis, T., & Poulios, I. (2008). Photocatalytic degradation and drug activity reduction of chloramphenicol. *Water Research, 42,* 386–94.

Chauhan, C. K., Vyas, P. M., & Joshi, M. J. (2011). Growth and characterization of Struvite-K crystals. *Crystal Research and Technology, 46*, 187–194.

Chauhan, M., Kimothi, A., Sharma, A., & Pandey, A. (2023). Cold adapted Pseudomonas: ecology to biotechnology. *Frontiers in microbiology*, *14*, 1218708.

Chen, Q., An, X., Zhu, Y. Su, J., Gillings, M. R., Ye, Z., & Cui, L. (2017). Application of struvite alters the antibiotic resistome in soil, rhizosphere and phyllosphere. *Environmental Science & Technology, 51(*14), 8149–8157.

Chen, J., Quiles-Puchalt, N., Chiang, Y. N., Bacigalupe, R., Fillol-Salom, A., Chee, M. S. J., ... & Penadés, J. R. (2018). Genome hypermobility by lateral transduction. *Science*, *362*(6411), 207-212.

Chiaka, C. (2009). Management of EcoSan System in Urban Areas: "EcoSan_UE Project Case Study'' in four sectors of Ouagadougou, *BF*. Presentation to the West Africa Regional Sanitation and Hygiene Symposium, Accra, Ghana, 10-12 November 2009.

Chirmuley, D. G. (1994). Struvite precipitation in WWPTS: causes and solutions. *WATER-MELBOURNE THEN ARTARMON-*, *21*, 21-21.

Chu, H., Hosen, Y., & Yagi, K. (2007). NO, N_2O, CH_4 and CO_2 fluxes in winter barley field of Japanese Andisol as affected by N fertilizer management. *Soil Biology & Biochemistry, 39,* 330–339.

Clean water services, rock creek advances waste water treatment facility, Hillsboro, Oregon, USA.Cofie, O., Olubenga, A. & Amoah, P. (2010). Introducing urine as an alternative fertilizer source for urban agriculture: Case studies from Nigeria and Ghana. *Urban Agriculture Magazine, 23*(2), 49-50.

Cofie, O., Olubenga, A. and Amoah, P. (2010). Introducing urine as an alternative fertiliser source for urban agriculture: Case studies from Nigeria and Ghana. *Urban Agri. Mag*. 23 (2):49-50.

Colomer-Lluch, M., Imamovic, L., Jofre, J., & Muniesa, M. (2011a). Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. *Antimicrobial Agents & Chemotherapy, 55*, 4908–4911.

Colomer-Lluch, M., Jofre, J., & Muniesa, M. (2011b). Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLOS ONE, 6(3), e17549.*

Colomer-Lluch, M., Jofre, J., & Muniesa, M. (2014). Quinolone resistance genes (qnrA and qnrS) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes. *Journal of Antimicrobial Chemotherapy, 69*, 1265–1274.

Combalbert, S., & Hernandez-Raquet, G. (2010). Occurence, fate nad biodegradation of estrogens in sewage and manure. *Applied Microbiology and Biotechnology, 86*(6), 1671-1692.

Connor, T. H., Stoeckel, M. Evrard, J. and Legator, M. S. (1977). The contribution of metronidazole and two metabolites to the mutagenic activity detected in urine of treated humans and mice. *Cancer Res.* 37, 629-633.

Cooper, R.C. and Olivieri, A.W. 1998. Infectious disease concerns in wastewater reuse. In: Asano, T. (ed.) Wastewater reclamation and reuse. Technomic Publishing Company, Inc., Lancaster, PA, USA. p. 489-520.

Cordell, D., & White, S. (2006). Peak phosphorus: Clarifying the key issues of vigorous debate about long term phosphorus security. *Sustainability, 3*: 2027-2049.

Cordell, D., Drangert, J. O., & White, S. (2009). The story of phosphorous: global food security and food for thought. Global environmental change: *Human and policy dimensions, 19*(2), 292-305.

Cramer, W. N., Burge, W. D., & Kawata, K. (1983). Kinetics of virus inactivation by ammonia. *Applied and Environmental Microbiology, 45,* 760-765.

Daher, E. D. F., Silva, Junior, G. B. D. Damasceno, R. T., Santosd, G. M., Corsino, G. A., Silva, S. L. D., & Gutierrez-Adrianzen O. A. (2007). End-stage renal disease due to delayed diagnosis of renal tuberculosis: a fatal case report. *Brazilian Journal of Infectious diseases, 11*(1), 169-171.

Dagerskog, L. (2010). Opening minds and closing loops: productive sanitation initiatives in Burkina Faso and Niger.

Dai, J., Tang, W. T. Zheng, Y. S. Mackey, H. R. Chui, H. K., van Loosdrecht, M. C. M., & Chen, G. H. (2014). An exploratory study on sea water-catalysed urine phosphorus recovery. *Water Research, 66*(0):75-84.

Darlymple, O. K. Yeh, D. H., & Trotz, M. A. (2007). Removing pharmaceuticals and endocrine disrupting compounds from wastewater by photocatalysis. *Journal of Chemical Technology & Biotechnology, 82,* 121–34.

Davidson, A. R. (2018). A common trick for transferring bacterial DNA. *Science*, *362*(6411), 152-153.

de Boer, M. A., Hammerton, M., & Slootweg, J. C. (2018). Uptake of pharmaceuticals by sorbent-amended struvite fertilisers recovered from human urine and their bioaccumulation in tomato fruit.*Water Research, 133,*19-26.

Decrey, L., Udert, K. M., Tilley, E., Pecson, B. M., & Kohn, T. (2011). Fate of the pathogen indicators phage ΦX174 and *Ascaris suum* eggs during the production of struvite fertilizer from source-separated urine. *Water Research, 45,* 4960 – 4972.

Decrey, L., Kazama, S., & Kohn, T. (2016). Ammonia as an in situ sanitizer: influence of virus genome type on inactivation. *Applied and environmental microbiology, 82*(16), 4909-4920.

Decrey, L. Kazama, S., Udert, K. M., & Kohn, T. (2015). Ammonia as in situ sanitizer: Inactivation kinetics and mechanisms of the ssRNA virus MS2 by NH3. *Environmental Science & Technology, 49*(2), 1060-1067.

De Gisi, S., Petta, L., & Wendland, C. (2014). History and Technology of Terra Preta Sanitation. *Sustainability.* 6, 1328-1345.

Dehghani, M., Ahmadi, M., & Nasseri, S. (2013). Photodegradation of the antibiotic penicillin G in the aqueous solution using UV-A radiation. *Iranian journal of health sciences*, *1*(3), 43–50.

Deinert, L., Ikoyi, I., & Schmalenberger, A. (2020). Assessment of Recycling-derived Fertilizers as an Alternative to Mineral Fertilizers–Effects on the Soil Microbiome.

Dever, L. A., & Dermody T. S. (1991). Mechanisms of bacteria resistance to antibiotics. *Archives of Internal. Medicine, 151*, 886–895.

Devier, M. H., Labadie, P., Togola, A., & Budzinski, H. (2010). Simple methodology coupling microwave-assisted extraction to SPE/GC/MS for the analysis of natural steroids in biological tissues: application to the monitoring of endogenous steroids in marine mussels *Mytilus* sp. *Analytica Chimica Acta, 657,* 28-35.

Diasi, M., Singh, R., Mahapatra, A.D., Renuka, L., Patel, H., Ganatra, H. and Datta, B. (2024). Ammonium release in synthetic and human urine by a urease immobilized nanoconstruct. *RSC Adv.* 14:6972

Díaz, V., Leyva-Díaz, J. C., Almécija, M. C., Poyatos, J. M., del Mar Muñío, M., & Martín-Pascual, J. (2022). Microalgae bioreactor for nutrient removal and resource recovery from wastewater in the paradigm of circular economy. *Bioresource Technology*, *363*, 127968.

Dojmi di Delupis, G., Macrì, A., Civitareale, C., & Migliore, L. (1992). Antibiotics of zootechnical use: effects of acute high and low dose contamination on Daphnia magna Strauss. *Aquatic Toxicology, 22,* 53–60.

Dolliver, H. A., Kumar, K., & Gupta, S. C. (2007). Sulfamethazine uptake by plants from manure-amended soil. *Journal of Environmental Quality, 36,* 1224–1230.

Dolejska, M., Villa, L., Poirel, L., Nordmann, P., & Carattoli, A. (2013). Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase

and a resistance nodulation cell division/multidrug efflux pump. *Journal of Antimicrobial Chemotherapy, 68*, 34–39.

Drangert, J. O. (1998). Fighting the urine blindness to provide more sanitation options. *WATER SA-PRETORIA-*, *24*, 157-164.

Du, X. H., Song, Y. M., Cai, J. A., & Zhu,Y. G. (2024). Utilization of human excreta in Premodern China, its theoretical interpretations and ecological significance. Soil *Ecology Letters,* 6, 240226.

Dunseth, M. G., Salutsky, M. L., Ries, K. M., & Shapiro, J. J. (1970). Ultimate disposal of phosphate from waste water by recovery as fertilizer.

Dunne, W. M. J. (1990). Effects of subinhibitory concentrations of vancomycin or cefamandole on biofilm production by coagulase-negative staphylococci. *Antimicrobial Agents Chemotherapy, 34*, 390–393.

Dussault, E. B., Balakrishnan, V. K., Borgmann, U., Solomon, K. R., & Sibley, P. K. (2009). Bioaccumulation of the synthetic hormone 17 alpha-ethinylestradiol in the benthic invertebrates Chironomus tentans and Hyalella azteca. *Ecotoxicology and Environmental Safety,* 72, 1635-1641.

El Diwani, G., El Rafie, S., El Ibiari, N. N. & El-Aila, H. I. (2007). Recovery of Ammonia Nitrogen from Industrial Wastewater Treatment as Struvite Slow Releasing Fertilizer. *Desalination, 214*, 200–214.

Eliopoulos, G. M., Meka, V. G., & Gold, H. S. (2004). Antimicrobial resistance to linezolid. Clinical Infectious Diseases, 39(7), 1010-1015.

Ek, M. Bergstrom, R., Bjurhem, J. E. Bjorlenius, B., & Hellstrom, D. (2006). Concentration of nutrients from urine and reject water from anaerobically digested sludge. *Water Science & Technology, 54*(11-12):437-444.

Elvers, K.T., & Wright S. J. L. (1995) Antibacterial Activity of the Anti-inflammatory Compound Ibuprofen. *Letters in Applied Microbiology, 20,* 82-84.

Emerson, K., Russo, R., Lund, R., & Thurston, R. (1975). Aqueous ammonia equilibrium calculations: effects of pH and temprature. *Journal of Fisheris Research Board of Canada, 32*, 2379-2383.

Encyclopaedia Britannica, browsed on December 5 at 8:00 pm EAT

EPA, (2007). EPA Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS. U.S. Environmental Protection Agency. 2007; 1-34. https://www.epa.gov/sites/default/files/2015-10/documents/method_1694_2007.pdf

Epel, D., & Smital, T. (2001). Multidrug-multixenobiotic transporters and their significance with respect to environmental levels of pharmaceuticals and personal care products.

Escher, I. B., Pronk, W., Suter, M. J. F., & Maurer, M. (2006). Monitoring the Removal Efficiency of Pharmaceuticals and Hormones in Different Treatment Processes of Source-Separated Urine with Bioassays. *Environmental Science & Technology, 40,* 5095-5101.

Esrey, S. A., Gough, J., Rapaport, D., Sawyer, R., Simpson-Hebert, M., Vargas, J., & Winblad, U. (1998). *Ecological sanitation. Swedish International Development Cooperation Agency, Stockholm*.

Espinosa-Aquirre, J. J., dc la Torte, R. A., Lares-Asseff, I., Rubio, J., Dorado, V., Wong, M., & Hermindez, J. M. (1996). Bacterial mutagens in the urine of patients under tinidazole treatment. *Mutation Research, 359,*133-140.

Etter, B., Tilley, E. Khadka, R., & Udert, K. M. (2011). Low cost struvite production using source separated urine in Nepal. *Water Research, 45*(2), 852-862.

EU, (2002). 30 96/23/Ec Commission Decision, 96/23/Ec Commission Decision, 96/23/EC COMMISSION DECISION of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results (Notified under Document Number C (2002) 3044) (Text with EEA Relevance) (2002/657/EC), p. 29, 96/23/Ec Commission Decision, 2002, https://op.europa.eu/en/publication-detail/-/publication/047cb006-40eb-43db-872c-075640fd6668/language-en/format-PDF/source

Evans, T. J., Crow, M. A., Williamson, N. R., Orme, W., Thomson, N. R., Komitopoulou, E., & Salmond, G. P. C. (2010). Characterization of a broad-host-range flagellum-dependent phage that mediates high-efficiency generalized transduction in, and between, Serratia and Pantoea. *Microbiology*, *156*(1), 240-247.

Fane, S. (2004). Life cycle microbial risk analysis of sustainable sanitation alterna- tives. In: Ecosan- Closing the Loop. Proceedings of the 2nd International Symposium on

Ecological Sanitation. Incorporating the 1st IWA Specialist Group Conference on Sustainable Sanitation. GTZ. Germany, pp. 389–396.

Feachem, R.G., Bradley, D.J., Garelick, H. and Mara, D.D. (1983). Sanitation and Disease-Health aspects of excreta and wastewater management. John Wiley and Sons, Chichester, UK.

Fenlon, K. A., Johnson, A. C., Tyler, C. R., & Hill, E. M. (2010). Gas-liquid chromatography tandem mass spectrometry methodology for the quantitation of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents and from wild populations. *Journal of Chromatography A , 1217,* 112-118.

Fenske, M., Maack, G., Schäfers, C., & Segner, H. (2005). An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *danio rerio. Environmental Toxicology and Chemistry, 24*(5), 1088–1098.

Ferrer, M. D, Rodriguez, J. C., Alvarez, L., Artacho, A., Royo, G., & Mira, A. (2016). Effect of antibiotics on biofilm inhibition and induction measured by real-time cell analysis. *Journal of applied Microbiology, 122,* 640—650.

Fertuck, K. C., Eckel, J. E., Gennings, C., & Zacharewski, T. R. (2003). Identification of temporal patterns of gene expression in the uteri of immature, ovariectomized mice following exposure to ethynylestradiol. *Physiological genomics, 15,*127–141.

Fewless, K. L., Sharvelle, S., & Roesner, L. A. (2011). *Source separation and treatment of anthropogenic urine*. Alexandria, VA: Water Environment Research Foundation.

Filby, A. L., & Tyler, C. R. (2005). Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biology of Reproduction, 73,* 648–662.

Floyd, J. L., Smith, K. P., Kumar, S. H., Floyd, J. T., & Varela, M. F. (2010). LmrS is a multidrug efflux pump of the major facilitator superfamily from Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 54*, 5406–5412.

Foran, C. M., Weston, J., Slattery, M., Brooks, B. W., & Huggett, D. B. (2004). Reproductive assessment of Japanese medaka (*Oryzias latipes*) following a four week fluoxetine (SSRI) exposure. *Archives of environmental contamination and toxicology, 46,* 511–517.

Foran, M., Petersen, B. N., & Benson, W. H. (2002). Transgenerational and developmental exposure of Japanese medaka (*Oryzias latipes*) to ethinylestradiol results in endocrine and reproductive differences in the response to ethinylestradiol as adults. *Toxicological Sciences, 68,* 389–402.

Forslund, K., Sunagawa, S., Kultima, J. R., Mende, D. R., Arumugam, M., Typas, A., & Bork, P. (2013). Country specific antibiotic use practices impact the human gut resistome. *Genome Research, 23,* 1163–1169.

Fricke, W. F., Maddox, C., Song, Y., & Bromberg, J. S. Human Microbiota Characterization in the Course of Renal Transplantation. *American Journal of Transplant*, *14*, 416–427.

Frode-Kristensen, S. (1966). By the well at the turn of the century.(Vid brunnen-En kulturbild från sekelskiftet). Collected works from the archives of life of the people in Lund no. 8, Lund, Sweden.

Frost, R. L., Weier, M. L., & Erickson, K. L. (2004). Thermal decompositon of struvite - implications for the decomposition of kidney stones. *Journal of Thermal Analysis and Calorimetry, 76,* 1025-1033.

Fry, L. M., Mihelcic, J. R., & Watkins, D. W. (2008). Water and non-water-related challenges of achieving global sanitation coverage. *Environmental Science & Technology, 42*(4), 4298–4304.

Ganrot, Z. Dave, G., & Nilsson, E. (2007). Recovery of N and P from human urine by freezing, struvite precipitation and adsorption to zeolite and active carbon. *Bioresource Technology, 98*(16), 3112-3121.

Gardner, L. J., Walling, S. A., Lawson, S. M., Sun, S., Bernal, S. A., Corkhill, C. L., Provis, J. L., Apperley, D. C., Iuga, D., Hanna, J. V., & Hyatt, N. C. (2020). Characterization of and Structural Insight into Struvite-K, $MgKPO_4 \cdot 6H_2O$, an Analogue of Struvite. *Inorganic Chemistry 60*(1), 195–205.

Gaterell, M. R., Gay, R., Wilson, R., Gochin, R. J., & Lester, J. N. (2011). An Economic and Environmental Evaluation of the Opportunities for Substituting Phosphorus Recovered from Wastewater Treatment Works in Existing UK Fertiliser Markets. *Environmental Technology, 21*, 1067–1084.

Geissler, M., Isabel, S., Voisin, B., Fauvel, C., Boissinot, M., Bergeron, M. G., & Veres, T. (2012). Modular Ultrasonic Lysis System for Rapid Nucleic Acid Extraction and Sample Transfer of Bacillus Spores. *Journal of Bioterrorism & Biodefense, 03*, 119.

Gerba, C. P. (1996). Pathogens in the environment. *Pollution Science, edited by: Pepper, IL, Gerba, CP, and Brusseau, ML, Academic Press, New York*, 279-299.

Germer, J. Ö. R. N., & Sauerborn, J. O. A. C. H. I. M. (2006). Exploring the Potential for Recycling Nutrients from Waste Water to Enhance Agricultural Productivity-the Example of Valley View University in Accra, Ghana. Presentation at the Tropentag 2006-International Research on Food Security. *Natural Resource Management and Rural Development. University of Bonn, October*, 11-13.

Giger, W., Alder, A. C., Golet, E. M., Kohler, H. P. E., McArdell, C. S., Molnar, E., Siegrist, H., & Suter M. J. F. (2003a). Occurrence and fate of antibiotics as trace contaminants in wastewaters, sewage sludges, and surface waters. *Chimia, 57,* 485–491.

Girardi, C., Greve, J., Lamshöft, M., Fetzer, I., Miltner, A., Schäffer, A., & Kästner M. (2011). Biodegradation of ciprofloxacin in water and soil and its effects on the microbial communities. *Journal of Hazardous Materials, 198,* 22 – 30.

Golet, E. M., Strehler, A., Alder, A. C., & Giger, W. (2002). Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Analytical Chemistry, 74,* 5455–5462.

Graeser, S., Postl, W., Bojar, H. P. B., Armbruster, T., Raber, T., Ettinger, K. & Walter, F. (2008). Struvite-(K), $KMgPO_4$ $6H_2O$, the Potassium Equivalent of Struvite a New Mineral. *European Journal of Mineralogy, 20*, 629–633.

Grange, J. M., & Yates, M. D. (1992). Survey of *Mycobacteria* isolated from urine and the genitourinary tract in south-east England from 1980 to 1989. *British journal of urology, 69*(6), 640–646.

Grau, M. G., Rhoton, S. L., Brouckaert, C. J., & Buckley, C. A. (2015). Evaluation of an automated struvite reactor to recover phosphorus from source-separated urine collected at urine diversion toilets in eThekwini. *Water SA*, *41*(3), 383-389.

Grote, M., Schwake-Anduschus, C., Michel, R., Stevens, H., Heyser, W., Langenkämper, G., Betsche, T., & Freitag, M. (2007). Incorporation of veterinary antibiotics into crops from manured soil. *Landbauforschung Volkenrode, 57,* 25–32.

Gaterell, M. R., Gay, R., Wilson, R., Gochin, R. J., & Lester, J. N. (2000). An economic and environmental evaluation of the opportunities for substituting phosphourous recovered from waste water treatment works in existing UK fertilizer markets. *Environmental Techonology, 21,* 1067-1084.

Goetsch, H. E., Love, N. G., & Wigginton K. R. (2020). Fate of Extracellular DNA in the Production of Fertilizers from Source-Separated Urine. *Environmental Science & Technology, 54*, 1808−1815.

González-Ponce, R., López-de-Sá, E. G., & Plaza, C. (2009). Lettuce Response to Phosphorus Fertilization with Struvite Recovered from Municipal Wastewater. *HORTSCIENCE, 44*(2), 426–430.

Green, S. K., Schroth, M. N., Cho, J. J., Kominos, S. D., & Vitanza-Jack, V. B. (1974). Agricultural plants and soil as a reservoir for Pseudomonas aeruginosa. *Applied microbiology*, *28*(6), 987-991.

Guadarrama, R., Pichardo, N., & Morales-Oliver, E. (2002, November). Urine and compost efficiency applied to lettuce cultivation under greenhouse conditions, Intemixco, Morelos, Mexico. In *Proceedings of the First International Conference on Ecological Sanitation*.

Guney, K., Weidelener, A., & Krampe, J. (2008). Phosphorus recovery from digested sewage sludge as MAP by the help of metal ion separation. *Water Research, 42,* 4692–4698.

Gündoğdu, A., & Nalbantoğlu, Ö. U. (2016). Humans as a Source of Colistin Resistance: In Silico Analysis of Public Metagenomes for the *mcr*-1 Gene in the Gut Microbiome. *Journal of Clinical Practice & Research,* 3*8*(2), 59-61.

Gyllenhammar, I., Holm, L., Eklund, R., & Berg, C. (2009). Reproductive toxicity in Xenopus tropicalis after developmental exposure to environmental concentrations of ethynylestradiol. *Aquatic Toxicology, 91,* 171–178.

Hall, B. G., & Barlow, M. (2004). Evolution of the serineβ-lactamases: past, present and future. *Drug Resistance Updates, 7,* 111–123.

Halling-Sorensen B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lutzhoft, H.C., Jorgensen, S.E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment. A review. *Chemosphere,* 36(2):357-393.

Han, M., & Kim, M. (2014). Revisiting the technical and social aspects of wastewater management in ancient Korea. *Evolution of Sanitation and Wastewater Technologies Through the Centuries. IWA Publishing, London*, 301-312.

Haneaus, A., Hellström, D., & Johanssonn, E. (1996). Conversion of urea during storage of human urine. *Vatten, 52,* 263-270.

Hao, X. D., Wang, C. C., Lan, L., & Von Loosdrecht, M. C. M. (2008). Struvite formation analytical methods and effects of pH and Ca^{2+}. *Water Science & Technology, 58,* 1687–1692.

Hao, X., Wang, C., Van Loosdrecht, M. C. M., & Hu, Y. (2013). Looking beyond struvite for P-recovery. *Environmental Science & Technology, 47,* 4965-4966.

Hansen, P. D., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M., & Blaise C. (1998). Vitellogenin - a biomarker for endocrine disruptors. *Trac-Trends in Analytical Chemistry, 17,* 448-451.

Harrass, M.C., Kindig, A.C. and Taub, F.B., 1985. Responses of blue-green and green algae to streptomycin in unialgal and paired culture. *Aquatic Toxicology*, 6(1):1-11.

Havenga, B., Ndlovu, T., Clements, T., Reyneke, B., Waso, M., & Khan, W. (2019). Exploring the antimicrobial resistance profiles of WHO critical priority list bacterial strains. *BMC microbiology*, *19*, 1-16.

Heinonen-Tanski, H., & van Wijik-Sijbesma, C. (2005). Human excreta for plant production. *Bioresource Technology, 96,* 403-411.

Heinonen-Tanski, H. H., Pradhan, S. K., & Karinen, P. (2010). Sustainable sanitation -A cost effective tool to improve plant yields and the environment. *Sustainability, 2,* 341-353.

Hellström, D., Johansson, E. and Kerstin Grennberg, K. (1999). Storage of human urine: acidification as a method to inhibit decomposition of urea. Ecological Engineering, 12:253-269.

Hektoen, H. Berge, J. A., Hormazabal, V., & Yndeslad, M. (1995) Persistence of Antibacterial Agents in Marine Sediments. *Aquaculture, 133,* 175-184.

Heth, C. L. (2015). The skin they were in: leather and tanning in antiquity. In *Chemical technology in antiquity* (pp. 181-196). American Chemical Society.

Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A., & Miller, S. I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature*, *436*(7054), 1171-1175.

Hidayat, E., & Harada, H. (2021). Simultaneously recovery of phosphorus and potassium using bubble column reactor as struvite-K and implementation on crop growth. In *Crystallization and Applications*. IntechOpen.

Höglund, C., Stenström, T. A., Jönsson, H., & Sundin A. (1998). Evaluation of faecal contamination and microbial die-off in urine separating sewage systems. *Water Science & Technology,* 38(6), 17-25.

Höglund, C., & Stenström T. A. (1999). Survival of Cryptosporidium parvum oocysts in source separated urine used in agriculture. *Waste Management & Research, 45,* 740-746.

Höglund, C., Steinström, T. A., & Ashbolt, N. (2002). Microbial risk assessment of source separated urine used in agriculture. *Waste Management & Research, 20,* 150-161.

https://op.europa.eu/en/publication-detail/-/publication/047cb006-40eb-43db-872c-075640fd6668/language-en/format-PDF/source30 96/23/Ec Commission Decision, 96/23/Ec Commission Decision, 96/23/EC COMMISSION DECISION of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results (Notified under Document Number C(2002) 3044) (Text with EEA Relevance) (2002/657/EC), p. 29, 96/23/Ec Commission Decision, 2002

Huang, L., Wu, C., Gao, H., Xu, C., Dai, M., Huang, L., Hao, H., Wang, X., & Cheng, G. (2022). Bacterial Multidrug Efflux Pumps at the Frontline of Antimicrobial Resistance: An Overview. *Antibiotics*, **11**, 520.

Huang, Ju-C., & Chii Shang, C. (2006). Air Stripping: In Advanced Physicochemical Treatment Processes, PP 47-79 (Lawrence, K.W., Yung-Tse, H. and K.S. Nazih, K.S., Eds.) Humana Press, Totowa, New Jersey.

Hu, R. M., Liao, S. T., Huang, C. C., Huang, Y. W., & Yang, T. C. (2012). An inducible fusaric acid tripartite efflux pump contributes to the fusaric acid resistance in Stenotrophomonas maltophilia. *PLOS ONE, 7*, e51053.

Hu, Y., Yang, X., Qin, J., Lu, N., Cheng, G., Wu, N., Pan, Y., Li, J., Zhu, L., Wang, X., Meng, Z., Zhao, F., Liu, D., Ma, J., Qin, N., Xiang, C., Xiao, Y., Li, L., Yang, H., Wang, J., Yang, R., Gao, F. G. Wang, J., & Zhu, B. (2013). Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nature Communication, 4,* 2151.

Hyder, S. L., & Streitfeld, M. M., (1978). Transfer of erythromycin resistance from clinically isolated lysogenic strains of Streptococcus pyogenes via their endogenous phage. *Journal of Infectious Diseases, 138*, 281–286.

Jackson, B. (2005). A review of EcoSan experience in Eastern and Southern Africa. *Sanitation and hygiene series. WSP-Africa, Nairobi*.

Jenkins, M. B. Bowman, D. D., & Ghiorse, W. C (1998). Inactivation of Cryptosporidium parvum oocyst by Ammonia. *Applied and environmental microbiology, 64*(2), 784-788.

Jernberg, C., Löfmark, S., Edlund, C., & Jansson J. K. (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *The ISME Journal, 1,* 56–66.

Jiménez-Arias, D., Sierra, S. M., García-Machado, F. J., García-García, A. L., Borges, A. A., & Luis, J. C. (2022). Exploring the agricultural reutilisation of desalination reject brine from reverse osmosis technology. *Desalination*, *529*, 115644.

Jjemba, P. K. (2006). Excretion and ecotoxicity of pharmaceutical and personal care product in the environment. *Ecotoxicology and Environmental Safety, 63,* 113-130.

Jones, O. A. H., Green, P. G., Voulvoulis, N., & Lester, J. N. (2007). Questioning the Excessive Use of Advanced Treatment to Remove Organic Micropollutants from Wastewater. *Environmental Science & Technology, 41,* 5085-5089.

Jönsson, H., Stintzing, A. R., Vinnerås, B., & Salomon, E. (2004). *Guidelines on the use of urine and faeces in crop production*. EcoSanRes Programme.

Jönsson, H., & Vinneras, B. (2007). Experiences and suggestion for collection systems for source separated urine faeces. *Water Science & Technology, 56*(5): 71-76.

Jönsson, H., Vinnerås, B., Höglund, C., Stenström, T.A., Dalhammar, G. & Kirchmann, H. (2000). Källsorterad humanurin i kretslopp (Recycling source separated human urine). In Swedish, English summary. VA-FORSK Report 2000•1. VA-FORSK/VAV. Stockholm, Sweden.

Jönsson, H., Vinnerås, B., Höglund, C. & Stenström, T.-A. (1999). Source separation of urin (Teilstromerfassung von Urin). Wasser & Boden 51(11):21-25.

Jönsson, H., Stenström, T.A., Svensson, J. & Sundin, A. (1997). Source separated urine - Nutrient and heavy metal content, water saving and faecal contamination. *Water Science and Technology*, 35(9):145-152.

Johansson, M., Jönsson, H., Höglund, C., Richert Stintzing, A., & Rodhe, L. (2001). Urine separation–closing the nutrient cycle. *Final report on the R&D project Source-Separated Human Urine–a future source of fertiliser for agriculture in the Stockholm region*.

Johnson, A. P., & Woodford, N. (2013). Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. *Journal of medical microbiology, 62*, 499–513.

Kabdaşl, I., Kuşçuoğlu, S., Tünay, O., & Siciliano, A. (2022). Assessment of K-Struvite Precipitation as a Means of Nutrient Recovery from Source Separated Human Urine. *Sustainability, 14*, 1082.

Kabdaşl, I., Sertaç, B., & Tünay, O. (2019). Nutrient removal from human urine by chemical precipitation. *FEB-FRESENIUS ENVIRONMENTAL BULLETIN, 28*, 852–856.

Kabdaşl, I., Tünay, O., & Özcan, P. (2009). Application of Struvite Precipitation Coupled with Biological Treatment to Slaughterhouse Wastewaters. *Environmental technology, 30*, 1095–1101.

Kabdasli, I., Tüney, O., Islek, C. Erdinc, E., Huskalar, S., & Tatli, M. B. (2006). Nitrogen recovery by urea hydrolysis and struvite precipitation from anthropogenic urine. *Water Science & Technology, 53*(12), 305-312.

Kapikian, A. Z. (1996). *Overview of viral gastroenteritis* (pp. 7-19). Springer Vienna.

Kaplan, J. B., Jabbouri, S., & Sadovskaya, I. (2011). Extracellular DNA-dependent biofilm formation by Staphylococcus epidermidis RP62A in response to sub-minimal inhibitory concentrations of antibiotics. *Research in Microbiology, 162*(5), 535-541.

Katayama, Y., Ito, T., & Hiramatsu, K. (2000). A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 44*, 1549–1555.

Kayode-Afolayan, S.D., Ahuekwe, E.F. & Nwinyi, O.C. (2022). Impacts of pharmaceutical effluents on aquatic ecosystems. Scientific African, 17, e01288. doi.org/10.1016/j.sciaf.2022.e01288

Khan, F.A. and Ansari, A.A. (2005). Eutrophication: an ecological vision. The Bot. Rev. 71:449-482.

Kenner, B. A. (1978). Fecal streptococcal indicators.

Khan, U., & Nicell, J. (2010). Assessing the risk of exogenously consumed pharmaceuticals in land-applied human urine.*Water Science & Technology*, 62(6): 1335-1345.

Kim, C., Mwangi, M., Chung, M., Milheirço, C., de Lencastre, H., & Tomasz, A. (2013). The mechanism of heterogeneous beta-lactam resistance in MRSA: key role of the stringent stress response. *PLOS One, 8(12), e82814.*

King, F.H., (1911). Farmers of 40 Centuries; or, Permanent Agriculture in China, Korea, and Japan. Published by Mrs. King, F.H. Madi-son, Wis.

Kirchmann, H., & Pettersson, S. (1995) Human urine chemical composition and fertilizer efficiency. *Fertilizer Research, 40,* 149-154.

Kim, Y., Choi, K., Jung, J., Park, S., Kim, P. G., & Park, J. (2007). Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. *Environment International, 33,* 370–375.

Kitano, M., Inoue, Y., Yamazaki, Y., Hayashi, F., Kanbara, S., Matsuishi, S., Yokoyama, T., Kim, S., Hara, M., & Hosono, H. (2012). Ammonia synthesis using a stable electride as an electron donor and reversible hydrogen store. *Nature Chemistry, 4,* 934-940.

Kleinheinz, K. A., Joensen, K. G., & Larsen, M. V. (2014). Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and E. coli virulence genes in bacteriophage and prophage nucleotide sequences. *Bacteriophage, 4*(2), e27943.

Knutsson, M. & Kidd-Ljunggren, K. (2000). Urine from chronic hepatitis B virus carriers: Implications for infectivity. *Journal of Medical Virology, 60*(1), 17-20.

Kohanski, M. A., De Pristo, M. A., & Collins, J. J. (2010). Sub lethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Molecular Cell, 37,* 311-320.

Kolpin, D., Furlong, E., Meyer, M., Thurman, E., Zaugg, S., Barber, L., & Buxton H. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999 - 2000: *A national reconnaissance. Environmental Science & Technology, 36*(6), 1202-1211.

Kraft, L. (2010). EU-SIDA GTZ EcoSan Promotion Project. http://www2.gtz.de/Dokumente/oe44/ecosan/en-eu-sida-gtz-ecosanpromotion project-final-report-2010.pdf accessed June 2010.

Krol, J. E., Nguyen, H. D., Rogers, L. M. , Beyenal, H., & Krone, S. M. (2011). Top, Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. *Applied and Environmental Microbiology, 77,* 5079–5088.

Kumar, K., Gupta, S., Baidoo, S., Chander, Y., & Rosen, C. (2005). Antibiotic Uptake by Plants from Soil Fertilized with Animal Manure. *Journal of Environmental Quality, 34*(6), 2082-2085.

Kumar, N., Radhakrishnan, A., Wright, C. C., Chou, T. H., Lei, H. T., Bolla, J. R., ... & Yu, E. W. (2014). Crystal structure of the transcriptional regulator Rv1219c of Mycobacterium tuberculosis. *Protein Science*, *23*(4), 423-432.

Kurjogi, M., Issa, Mohammad, Y. H., Alghamdi, S., Abdelrahman, M., Satapute, P., & Jogaiah, S. (2019) Detection and determination of stability of the antibiotic residues in cow's milk. PLOS ONE 14(10), e0223475.

Kümmerer, K. (2009). The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. *Journal of Environmental Management, 90,* 2354-2366.

Kümmerer, K. (2009). Antibiotics in the aquatic environment – A review – *Part I Chemosphere*, *75,* 417–434.

Kümmerer, K., & Henninger, A. (2003). Promoting resistance by the emission of antibiotics from hospitals and households into effluents. *Clinical Microbiolgy Infection, 9,* 1203–1214.

Kuşcuoğlu, S. (2008). Determination of K-struvite Application Bases. Master's Thesis, İstanbul Technical University, İstanbul, Turkey.

Lahr, H. R., Goetsch, E. H., Haig, J. S., Noe-Hays, A., Love, G. N., Aga, S. D., Bott, B. C., Foxman, B., Jimenez, J., Luo, T., Nace, K, Ramadugu, K., & Wigginton, R. K. (2016). Urine bacterial community convergence through fertilizer production: Storage, pasteurization and struvite precipitation. *Environmental Science & Technology, 50,* 11619-11626.

La Rosa, R., Johansen, H. K., & Molin, S. (2022). Persistent Bacterial Infections, Antibiotic Treatment Failure, and Microbial Adaptive Evolution. *Antibiotics*, 11, 419.

Lambert, P. A. (2005). Bacterial resistance to antibiotics: Modified target sites. *Advanced drug delivery reviews, 57,* 1471–1485.

Lamichhane, K. & Babcock, R. (2012). An economic appraisal of using source separation of human urine to contain and treat endocrine disruptors in USA. *Journal of Environmental Monitoring, 14*(10), 2557-2565.

Landry, K. A., & Boyer, T. H. (2013). Diclofenac removal in urine using strong base anion exchange polymer resins. *Water Research, 47*(17), 6432-6444.

Länge, R. and Dietrich, D. (2002). Environmental risk assessment of pharmaceutical drug substances conceptual considerations. Toxicol Lett. 131(1-2):97-104. doi: 10.1016/s0378-4274(02)00071-1.

Larsen, T. A., & Gujer, W. (1996). Separate management of anthropogenic solutions (human urine). *Water Science & Technology, 34*(3-4), 87-94.

Larsen, B., & Monif, G. R. G. (2001). Understanding the bacterial flora of the female genital tract. *Clinical Infectious Diseases, 32*(4), 69-77.

Larsen, A. T., Peter, I., Alder, A., Eggen, R., Maurer, M., & Muncke, J. (2001). Re-engineering the toilet for sustainable waste water management. *Environmental Science & Technology, 35,* 192-197.

Larsen, A. T., Lienert, J., Joss, A., & Siegrist, H. (2004). How to avoid pharmaceuticals in the aquatic environment. *Journal of Biotechnology, 113,* 295-304.

Lee, H.C, Chen, C.M, Wei, J.T, & Chiu, H.Y. (2018). Analysis of veterinary drug residue monitoring results for commercial livestock products in Taiwan between 2011 and 2015. Journal of Food and Drug Analysis, DOI: https://doi.org/10.1016/j.jfda.2017.06.008

Lee, S. H., Kumar, R., & Jeon, B. H. (2016). Struvite Precipitation under Changing Ionic Conditions in Synthetic Wastewater: Experiment and Modeling. *Journal of colloid and interface science, 474*, 93–102.

Lee, S. I., Weon, S. Y., Lee, C. W., & Koopman, B. (2003). Removal of nitrogen and phosphate from wastewater by addition of bittern. *Chemosphere, 51,* 265–271.

Lee, J. E., Rahman, M. M., & Ra, C. S. (2009). Dose effects of Mg and PO_4 sources on the composting of swine manure. *Journal of Hazardous Materials, 169,* 801–807.

Lee, W.Y. and Arnold, C.R. (1983). Chronic toxicity of ocean-dumped pharmaceutical wastes to the marine amphipod Amphithoe valida. *Marine Pollution Bulletin* 14(4):150-153.

Le Corre, K. S., Jones, E. V., Hobbs, P., & Parsons, S. A. (2005). Impact of calcium on struvite crystal size, shape and purity. *Journal of Crystal Growth, 283,* 514–522.

Le Corre, K. S., Jones, E. V., Hobbs, P., & Parsons, S. A. (2009). Phosphorus recovery from wastewater by struvite crystallization: a review. *Crit. Rev. Environmental Science & Technology, 39,* 433–477.

Leisner, J. J., Cohn, T. M., Catalan-Moreno, A., Nielsen, B. J., Westh, H., Penadés, J. R., & Ingmer, H. (2016). Bacterial viruses enable their host to acquire antibiotic resistance genes from neighbouring cells. *Nature communications, 7,* 1-8.

Levy, S. B. (2002). The antibiotic paradox: how the misuse of antibiotics destroys their curative powers, 2nd ed. Cambridge, MA, USA: Da Capo Press, Perseus Publishing.

Lewis, J. R., & Winkler, M. (1991). Sludge parasites and other pathogens. Ellis Horwood Limited, Chichester, UK.

Liang, X. Q., Chen, Y. X., Li, H., Tian, G. M., Ni, W. Z., He, M. M., & Zhang, Z. J. (2007). Modeling transport and fate of nitrogen from urea applied to a near-trench paddy field. *Environmental Pollution, 50,* 313–320.

Liao, X., Li, B., Zou, R., Dai, Y., Xie, S., & Yuan, B. (2016). Biodegradation of antibiotic ciprofloxacin: pathways, influential factors, and bacterial community structure. *Environmental Science and Pollution Research*, *23*, 7911-7918.

Lienert, J., Güdel, K., & Escher, B. (2007). Screening Method for Ecotoxicological Hazard Assessment of 42 Pharmaceuticals Considering Human Metabolism and Excretory Routes. *Environmental Science & Technology*, *41*(*12*): 4471–4478.

Linares, J. F., Gustafsson, I., Baquero, F., & Martinez, J. L. (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proceedings of the National Academy of Sciences, 103,* 19484–19489.

Lind, B., Ban, Z., & Bydén, S. (2000). Nutrient recovery from human urine by struvite crystallization with ammonia adsorption on zeolite and wollastonite. *Bioresource technonology*, 73, 2, 169-174

Liu, J., Chen, D., Peters, B. M., Li, L., Li, B., Xu, Z., & Shirliff, M. E. (2016). Staphylococcal chromosomal cassettes mec (SCCmec): A mobile genetic element in methicillin-resistant Staphylococcus aureus. *Microbial pathogenesis*, 101, 56-67.

Liu, Y., Kumar, S., Kwag,JH. and Ra, C. (2013). Magnesium ammonium phosphate formation, recovery and its application as valuable resources: A review. *J. Chem. Technol. Biotechnol.,* 88, pp. 181-189.

Liu, Y. H., Rahman, M. M., Kwag, J. H., Kim, J. H., & Ra, C. S. (2011). Eco-Friendly Production of Maize Using Struvite Recovered from Swine Wastewater as a Sustainable Fertilizer Source. *Asian-Australasian Journal of Animal Sciences, 24*, 1699–1705.

Liu, Z. G., Zhao, Q. L. Wang, K. Lee, D. J., Qiu, W., & Wang, J. F. (2008a). Urea hydrolysis and recovery of nitrogen and phosphrous as MAP from stale human urine. *Journal of Environmental Sciences, 20*(8), 1018-1024.

Liu, Z. G., Zhao, Q. L., Wang, K., Qiu, W., & Wang, J. F. (2008b). Comparision between complete and partial recovery of N and P from stale human urine with MAP crystallization. *Journal of Environmental Engineering and Science, 7*(3), 223-228.

Loftin, K. A., Adams, C. D., Meyer, M. T., & Surampalli, R. (2008). Effects of ionic strength, temperature, and pH on degra-dation of selected antibiotics. *Journal of environmental quality,* 37, 378–386.

Lothenbach, B., Xu, B., & Winnefeld, F. (2019). Thermodynamic Data for Magnesium (Potassium) Phosphates. *Applied Geochemistry, 111*, 104450.

Lukman, C., Yonathan, C., Magdalena, S. & D. E. Waturangi, D.E. (2020). Isolation and characterization of pathogenic Escherichia coli bacteriophages from chicken and beef offal. *BMC Research*, 13:8 https://doi.org/10.1186/s13104-019-4859-y

Lynch III, J. P., Clark, N. M., & Zhanel, G. G. (2013). Evolution of antimicrobial resistance among Enterobacteriaceae (focus on extended spectrum β-lactamases and carbapenemases). *Expert opinion on pharmacotherapy*, *14*(2), 199-210.

Macgowan, J. (1909). Lights and Shadows of Chinese Life. North China Daily News & Herald Limited. Shanghai, China.

Macrì, A., Stazi, A. V., & Dojmi di Delupis, G. (1988). Acute toxicity of furazolidone on Artemia salina, Daphnia magna, and Culex pipiens molestus larvae. *Ecotoxicology and Environmental Safety, 16,* 90–94.

Maggi, F., & Daly, E. (2013). Use of human urine as a fertilizer for corn, potato, and soybean: A case-study analysis using a reactive model. In *International Congress on Modelling and Simulation 2013: Adapting to Change: the multiple roles of modelling* (pp. 614-620). ACTA Press. www.mssanz.org.au/modsim2013

Magnusson, B., & Örnemark, U. (2014). Eurachem Guide: The fitness for purpose of analytical methods: a laboratory guide to method validation and related topics, 2nd edn. 2014; www.eurachem.org

Maia, A. S., Ribeiro, A. R., Amorim, C. L., Barreiro, J. C., Cass, Q. B., Castro, P. M., & Tiritan, M. E. (2014). Degradation of fluoroquinolone antibiotics and identification of metabolites/transformation products by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1333*, 87-98.

Makaya, M. J., Somda, K. M, Savadogo, A., Dianou, D., Barro, N., & Traore, S. A. (2014). Survival of enteric bacteria in source-separated human urine used as fertilizer: Effects of temperature and ammonia. *African Journal of Environmental Science and Technology, 8*(9), 512-520.

Makaya, J. M., Aho, S., Wethé, J., Dianou, D., Barro, N., & Traoré, A. S. (2014). Skin Problems among Users of the Urine-Based Fertiliser in Ouagadougou Periurban Areas, Burkina Faso: A Prospective Study.

Malachowa, N., & DeLeo, F. R. (2010). Mobile genetic elements of *Staphylococcus aureus*. *Cellular and molecular life sciences, 67*, 3057–3071.

Marshall, E. K., & Davis, D. M. (1914). Urea: its distribution in and elimination from the body. *Journal of Biological Chemistry*, *18*, 53-80.

Mariwah, S., & Drangert, J. O. (2011). Community perceptions human excreta as fertilizer in peri-urban agriculrture in Ghana. *Waste Managment & Research, 29*(8), 815-822.

Marti, R., Scott, A., Tien, Y, Murray, R., Sabourin, L., Zhang, Y., & Topp, E. (2013). Impact of Manure Fertilization on the Abundance of Antibiotic-Resistant Bacteria and Frequency of Detection of Antibiotic Resistance Genes in Soil and on Vegetables at Harvest. *Applied and Environmental Microbiology,* 79(18), 5701–5709.

Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution, 157,* 2893–902.

Marx, K., 1993. Capital: A Critique of Political Economy, Vol. 3. Penguin Classics.

Maskell, R., Pead, L., & Allen, J. (1979). The Puzzle of "Urethral Syndrome": A Possible Answer? *Lancet, 1*, 1058–1059.

Massey, M. S., Davis, J. G., Sheffield, R. E., & Ippolito, J. A. (2007). Struvite production from dairy wastewater and its potential as a fertilizer for organic production in calcareous soils. In *International Symposium on Air Quality and Waste Management for Agriculture, 16-19 September 2007, Broomfield, Colorado* (p. 23). American Society of Agricultural and Biological Engineers.

Massey, M. S., Davis, J. G., Ippolito, J. A., & Sheffield R. E. (2009). Effectiveness of recovered magnesium phosphates as fertilizers in neutral and slightly alkaline soils. *Journal of Agronomy 101,* 323–329.

Mašlaňová, I., Doškař, J., Varga, M., Kuntová, L., Mužík, J., Malúšková, D., ... & Pantůček, R. (2013). Bacteriophages of S taphylococcus aureus efficiently package various bacterial genes and mobile genetic elements including SCC mec with different frequencies. *Environmental microbiology reports*, *5*(1), 66-73.

Mathew, M., Kingsbury, P., Takagi, S., & Brown, W. E. (1982). A New Struvite-Type Compound, Magnesium Sodium Phosphate Heptahy-drate. *Acta Crystallographica Section B: Structural Crystallography and Crystal, 38*, 40–44.

Mathew, M., & Schroeder, L. W. (1979). Crystal Structure of a Struvite Analogue, MgKPO4 .6H 2 O. *Acta Crystallographica Section B: Structural Crystallography and Crystal, 35*, 11–13.

Matson, P. A., Naylor, R., & Ortiz-Monasterio, I. (1998). Integration of environmental, agronomic, and economic aspects of fertilizer management. *Science, 280,* 112–115.

Maurer, M., Pronk, W., & Larsen, T. A. (2006). Treatment processes for source separated urine. *Water Research, 40*(17), 3151-3166.

Mazaheri Nezhad Fard, R., Barton, M. D., & Heuzenroeder, M. W. (2011). Bacteriophage-mediated transduction of antibiotic resistance in enterococci. *Letters in applied microbiology*, *52*(6), 559-564.

Mead, P. S., Slutsker, L., & Dietz, V. (1999). Food-related illness and death in the United States. *Emerging Infectious Disease,* ***5***(5), 607-625.

Mendonça, B. S., de Oliveira, W. R., Pereira, R. S., Santos, L. R., Rodrigues, L. B., Dickel, E. L., Daroit, L., & Pilotto, F. (2021). Research Note: The use of ammonia gas for Salmonella control in poultry litters. *Poultry Science, 100*, 314–318.

Migliore, L., Brambilla, G., Grassitellis, A., & Dojmi di Delupis, G. (1993). Toxicity and bioaccumulation of sulphadimethoxine in Artemia (Crustacea, Anostraca). *International Journal of Salt Lake Research, 2,* 141–152.

Migliore, L., Civitareale, C., Brambilla, G., & Dojmi di Delupis, G. (1997). Toxicity of several important agricultural antibiotics to Artemia. *Water Research, 31,* 801–1806.

Mihelcic, J. R. , Fry, L. M., & Shaw, R. (2011). Global potential of phosphorus recovery from human urine and feces. *Chemospere, 84,* 832–839.

Miller, E. L., Nason, S. L., & Karthikeyan, K. G. (2016). Pedersen J. A. Root Uptake of Pharmaceuticals and Personal Care Product Ingredients. *Environmental Science and Technology*, *50*, 525–541.

Ministry of Mines and Petroleum (http://www.mom.gov.et/Industrial.aspx?id=36)

Mnkeni, P. N. S., Cisneros, B. J., Phasha, M. C., & Austin, L. M. (2006). Use of human excreta from urine-diversion toilets in food gardens: Agronomic and health aspects. WRC Report № 1439/3/06. ISBN № 1-77005-455-3. *Water Resource Commission*, *3*, 1-40.

Mnkeni, P. N. S., & Austin, L. M. (2009). Fertiliser value of human manure from pilot urine-diversion toilets. *Water SA*, *35*(1).

Moe, C. L., & Rheingans, R. D. (2006). Global challenges in water, sanitation and health. *Journal of water and health*, *4*(S1), 41-57.

Morgan, P. (2003). Experiments using urine and humus derived from ecological toilets as a source of nutrients for growing crops. 3rd World Water Forum 16-23 March 2003. *Retrieved May*, *9*, 2008.

Moustafa, A., Li, W., Singh, H., Moncera, K. J., Torralba, M. G., Yu, Y., Manuel, O., Biggs, W., Venter, J. C., Nelson, K. E., Pieper, R., & Telenti, A. (2018). Microbial metagenome of urinary tract infection. *Scientific Reports, 8,* 4333.

Mugivhisa, L. L., & Olowoyo, J. O. (2015). An assessment of university students and staff perceptions regarding the use of human urine as a valuable soil nutrient in South Africa. *African Health Sciences, 15*(3), 999-1010.

Mullen, R. A., Wigginton, K. R., Noe-Hays, A., Nace, K., Love, N. G., Bott, C. B., & Aga, D. S. (2017). Optimizing extraction and analysis of pharmaceuticals in human urine, struvite, food crops, soil, and lysimeter water by liquid chromatography-tandem mass spectrometry. *Analytical Methods*, *9*(41), 5952-5962.

Mund, A., Diggle, S.P. & Freya Harrison, F. (2017). The Fitness of Pseudomonas aeruginosa Quorum Sensing Signal Cheats Is Influenced by the Diffusivity of the Environment. mBio. 8(3):e00353-17. doi: 10.1128/mBio.00353-17.

Munch, E. V., & Barr, K. (2001). Controlled struvite crystallisation for removing phosphorus from anaerobic digester side streams. *Water Research, 35,* 151–159.

Muniesa, M., Colomer-Lluch, M., & Jofre, J. (2013a). Potential impact of environmental bacteriophages in spreading antibiotic resistance genes. *Future Microbiology, 8*, 739–751.

Muniesa, M., Colomer-Lluch, M., & Jofre, J. (2013b). Could bacteriophages transfer antibiotic resistance genes from environmental bacteria to human-body associated bacterial populations? *Mobile Genetic Elements, 3*, e25847

Muniesa, M., García, A., Miró, E., Mirelis, B., Prats, G., Jofre, J., & Navarro, F. (2004). Bacteriophages and diffusion of β-lactamase genes. *Emerging infectious diseases*, *10*(6), 1134.

Munita, J. M., & Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Virulence mechanisms of bacterial pathogens*, 481-511.

Münch, E., & Winker, M. (2009). Overview of urine diversion components such as waterless urinals, urine diversion toilets, urine storage and reuse system. *Technol. Rev.* 1(5): 1-32.

Muskolus, A. (2008) A*nthropogenic plant nutrients as fertilizer. PhD thesis, Institut for plant research*. Humboldt-Universität zu Berlin, Berlin, Germany.

Nebiyat Nigusie Woldeyohannis and Adey Feleke Desta. Fate of antimicrobial resistance (ARG) and ARG carriers in struvite production process from human urine. Journal of Environmental Science and Health, Part A 2023; DOI: https://doi.org/10.1080/10934529.2023.2235246

Nelson, D. E., Van Der Pol, B., Dong, Q., Revanna, K. V., Fan, B., Easwaran, S., ... & Fortenberry, J. D. (2010). Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. *PloS one*, *5*(11), e14116.

Nelson, D. E., Dong, Q., Van Der Pol, B., Toh, E., Fan, B., Katz, B. P., ... & Fortenberry, J. D. (2012). Bacterial communities of the coronal sulcus and distal urethra of adolescent males. *PloS one*, *7*(5), e36298.

Nelson, N. O. (2000). *Phosphorus removal from anaerobic swine lagoon effluent as struvite and its use as a slow-release fertilizer* (Doctoral dissertation, North Carolina State University). NH3 wikipedia, browsed on December 05 at 7:30pm EAT

Nelson, D.E., Van Der Pol B., Dong Q, Revanna KV, Fan B, et al. (1954). Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. PLoS ONE. 2010;5:e14116. https://doi.org/10.1371/journal. pone.0014116.

Nordin, A., Nyberg, K., & Vinneras, B. (2009). In activation of Ascaris eggs in source separated urine and faeces by ammonia at ambient temperatures. *Applied Envirnmental Microbiology, 75*(3), 662-667.

Normal flora of human wikipedia, browsed on December 07 at 8:00pm EAT

Nygaard, K., Lunestad, B. T., Hektoncn, H., Berge, J. A., & Hormazabal, V. (1992). Resistance to oxytetracycline, oxolinic acid and furazolidonc in bacteria from marine sediments. *Aquaculture, 104,* 31-36.

OECD (2000). Environmental Indicators for Agriculture, Methods and Results, Executive Summary. Paris, France.

Ogawa, W., Onishi, M., Ni, R., Tsuchiya, T., & Kuroda, T. (2012). Functional study of the novel multidrug efflux pump KexD from Klebsiella pneumoniae. *Gene, 498*, 177–182.

Ohlinger, K. N., PE, Young, T. M., & Schroeder, E. D. (1999). Kinetics effects on preferential struvite accumulation in wastewater. Journal of Environmental Engineering, 125(8), 730-737.

Ohlinger, K. M., Young, T. M., & Schroeder, E. (1998). Predicting struvite formation in digestion. *Water Research, 26,* 2229–2232.

Okem, A. E., Xulu, E., Tilley, C. Buckley, C., & Roma, E. (2013). Assessing perceptions and willingness to use urine in agriculture: a case study from rural areas of eThekwini municipality, South Africa. *Journal of water, sanitation and hygiene for development 03*(4): 582-591.

Ostara, (2010). OSTARA https://ostara.com/project/people-fixing-the-world/ browsed at 15: 29 (EAT) on April 29, 2021

Oulton, R. L., Kohn, T., & Cwiertny, D. M. (2010). Pharmaceuticals and personal care products in effluent matrices: A survey of transformation and removal during waste water treatment and implications for waste water management. *Journal of Environmental Monitoring, 12*(11), 1956-1978.

Pan, L. J., Li, J., Li, C. X., Yu, G. W., & Wang, Y. (2018). Study of ciprofloxacin biodegradation by a Thermus sp. isolated from pharmaceutical sludge. *Journal of hazardous materials*, *343*, 59-67.

Pan, M., & Chu, L. M. (2016). Phyto toxicity of veterinary antibiotics to seed germination and root elongation of crops. *Ecotoxicology and Environmental Safety, 126,* 228–237.

Parashar, U. D., Holman, R. C., Clarke, M. J., Bresee, J. S., & Glass, R. I. (1998). Hospitalizations associated with rotavirus diarrhoea in the United States, 1993 through 1995-surveillance based on the new ICD-9-CM rotavirus-specific diagnostic code. *Journal of Infectious Diseaseses, 177*(1), 13-17.

Park, S., & Choi, K. (2008). Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology, 17, 526–538.*

Patyra, E., Osiński, Z., & Kwiatek, K. (2024). The Identification and Quantification of 21 Antibacterial Substances by LC-MS/MS in Natural and Organic Liquid Fertilizer Samples. *Molecules*, *29*, 1644.

Pecson, B. M., & Nelson, K. L. (2005). Inactivation of Ascaris suum eggs by ammonia. *Environmental Science & Technology, 39,* 7909-7914.

Pehrsson, E. C, Tsukayama, P., Patel, S., Mejía-Bautista, M, Sosa-Soto, G., Navarrete, K. M., Calderon, M., Cabrera, L., Hoyos-Arango, W., Bertoli, M. T., Berg, D. E., Gilman, R. H., & Dantas G. (2016). Interconnected microbiomes and resistomes in low-income human habitats. *Nature,* 5 3 3, 212-228.

Pearce, M. M., Hilt, E. E., Rosenfeld, A. B., Zilliox, M. J., Thomas-White, K., Fok, C., ... & Wolfe, A. J. (2014). The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. *MBio*, *5*(4), 10-1128.

Peng, Q., & Yuan, Y. (2018). Characterization of a newly isolated phage infecting pathogenic Escherichia coli and analysis of its mosaic structural genes. Sci. Rep. 8:8086. doi: 10.1038/s41598-018-26004-26004

Perala, P., Kapuinen, P., Esala, M., Tyynela, S., & Regina, K. (2006). Influence of slurry and mineral fertiliser application techniques on N_2O and CH_4 fluxes from a barley field in southern Finland. *Agriculture, Ecosystem & Environment, 117,* 71–78.

Perez-Carrasco, V., Soriano-Lerma, A., Soriano, M., Gutiérrez-Fernández, J., & Garcia-Salcedo, J. A. (2021). Urinary microbiome: yin and yang of the urinary tract. *Frontiers in cellular and infection microbiology*, *11*, 617002.

Petrovski, S., Seviour, R. J., & Tillett, D. (2011). Characterization of the genome of the polyvalent lytic bacteriophage GTE2, which has potential for biocontrol of Gordonia-, Rhodococcus-, and Nocardia-stabilized foams in activated sludge plants. *Applied and environmental microbiology*, *77*(12), 3923-3929.

Phosphrous plat form: https://www.phosphorusplatform.eu/scope-in-print/enews/2100-espp-enews-no-57-augus2021#_Toc77662966

Piddock, L. J. V. (2016). Reflecting on the final report of the O'Neill Review on Antimicrobial Resistance. *Lancet Infectious Diseases, 16*(7), 767−768.

Poirel, L., Bonnin, R. A., & Nordmann, P. (2012). Genetic support and diversity of acquired extended-spectrum β-lactamases in Gram-negative rods. *Infection, Genetics and Evolution, 12*, 883–893 .

Poulsen, P. H. B., Abu Al-Soud, W., Bergmark, L., Magid, J., Hansen, L. H., & Sørensen, S. J. (2013). Effects of fertilization with urban and agricultural organic wastes in a field trial - Prokaryotic diversity investigated by pyrosequencing. *Soil Biology & Biochemistry, 57,* 784-793.

Pradhan, S. K., Nerg, A. M., Sjöblom, A., Holopainen, J. K., & Heinonen-Tanski, H. (2007). Use of human urine fertilizer in cultivation of cabbage (*Brassica oleracea*): Impacts on chemical, microbial and flavor quality. *Journal of Agriculture & Food Chemistry, 55,* 657-663.

Prakash, P., Hoskins, D., & SenGupta, A. K. (2004). Application of homogenous and heterogenous cation-exchange membranes in coagulant recovery from water treatment plant residuals using Donnan membrane process. *Journal of Membrane Science, 237,* 131–144.

Prüss, A., Kay, D., Fewtrell, L., & Bartram, J. (2002). Estimating the burden for disease for water, sanitation, and hygiene at a global level. *Environmental Health Perspective, 110*(5), 537–542.

Pryor, W. M. (1987). Acute diarrhoea in adults: a prospective study. *Medical journal of Australia, 147*(10), 490-493.

Putnam, D. F. (1971). *Composition and concentrative properties of human urine* (No. NASA-CR-1802). NASA.

Pynnönen, S., & Tuhkanen, T. (2012, August). Environmental impact of micropollutants present in urine. In 4th International Dry Toilet Conference (pp. 22-24).

Qiu, Y., Zhou, Y., Chang, Y., Liang, X., Zhang, H., Lin, X., Qing, K., Zhou, X., & Luo, Z. (2022). The Effects of Ventilation, Humidity, and Temperature on Bacterial Growth and Bacterial Genera Distribution. *International Journal of Environmental Research and Public Health*, *19*, 15345.

Qu, Y., Daley, A. J., Istivan, T. S., Garland, S. M., & Deighton, M. A. (2010). Antibiotic susceptibility of coagulase-negative staphylococci isolated from very low birth weight babies: comprehensive comparisons of bacteria at different stages of biofilm formation. *Annals of clinical microbiology and antimicrobials, 9,* 16.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J., & Ziebuhr, W. (2000). Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesion expression in biofilm-forming *Staphylococcus epidermidis*. *Antimicrobial agents and chemotherapy, 44,* 3357–3363.

Radkau, J., (2008). Nature and Power: A Global History of the Environment. Cambridge: Cambridge University Press.

Radovanovic, M., Day, O. R., Jones, R. D. G., Galettis, P., & Norris, G. L. R. (2022). LC–MS/MS method for simultaneous quantification of ten antibiotics in human plasma for routine therapeutic drug monitoring. *Journal of Mass Spectrometry and Advances in the Clinical Lab*, 26, 48–59.

Rahman, M. M., Liu, Y. H., Kwag, J. H., & Ra, C. S. (2011). Recovery of struvite from animal wastewater and its nutrient leaching loss in soil. *Journal of Hazardous Materials, 186,* 2026–2030.

Rahman, M., Salleh, M. A. M., Rashid, U., Ahsan, A, Hossain, M. M. & Ra, S. C. (2014) Production of slow release crystal fertilizer from wastewaters through struvite crystallization – A review. *Arabian Journal of Chemistry, 7,* 139-155.

Randall, D. G., & Naidoo, V. (2018). Urine: The liquid gold of wastewater. *Journal of Environmental Chemical Engineering*, *6*(2), 2627-2635.

Richert, A., Gensch, R., Jönsson, H., Stenström, T. A., & Dagerskog, L. (2010). Practical guidance on the use of urine in crop production. *EcoSanRes Publications Series*, (2010-1).

Richert Stintzing, A., Rodhe, L., Åkerhielm, H., & Steineck, S. (2001). Human urine as fertilizer–plant nutrients, application technique and environmental effects. *JTI-Rapport Lantbruk & Industri*, *278*.

Rico, A., Dimitrov, M. R.,Van Wijngaarden, R. P. A., Satapornvanit, K., Smidt, H., & Brink, P. J. V. (2014). Effects of the antibiotic enrofloxacin on the ecology of tropical eutrophic fresh water microcosms. *Aquatic Toxicology, 147,* 92–104.

Rizzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C., & Gasbarrini, A. J. B. R. I. (2017). Proteobacteria: a common factor in human diseases. *BioMed research international*, *2017*(1), 9351507.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., & Ploy, M. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Science of the Total Environment, 447,* 345–60.

Rodgers-Gray, Trevor P., Jobling, Susan, Morris, Steven, Kelly, Carole, Kirby, Sonia, Janbakhsh, Afsaneh, Harries, Jule E., Waldock, Michael J., Sumpter, John P., and Tyler, Charles R. (2000). Long-Term Temporal Changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environ. Sci.Technol*.34:8.

Rodhe, L., Stintzing, A. R., & Steineck, S. (2004). Ammonia emissions after application of human urine to clay soil for barley growth. *Nutrient Cycling in Agroecosystems*, *68,*191-198.

Ronteltap, M, Maurer, M., & Gujer, W. (2007). The behaviour of pharmaceuticals and heavy metals during struvite precipitation in urine. *Water Research, 41,* 1859-1868.

Rossi, L., Lienert, J., & Larsen, T. A. (2009). Real-life efficiency of urine source separation. *Journal of Environmental Management, 90*(5), 1909-1917.

Ryu, H. D., Lim, C. S., Kang, M. K., & Lee, S. I. (2012). Evaluation of Struvite Obtained from Semiconductor Wastewater as a Fertilizer in Cultivating Chinese Cabbage. *Journal of hazardous materials, 221-222*, 248–255.

Sanderson, H., Johnson, D.J., Reitsma, T., Brain, R.A., Wilson, C.J., Solomon, K.R., (2004). Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. Regul. Toxicol. Pharmacol. 39, 158–183. https://doi.org/10.1016/j.yrtph.2003.12.006

Salvatore, S. R., Vitturi, D. A., Baker, P. R., Bonacci, G., Koenitzer, J. R., Woodcock, S. R., ... & Schopfer, F. J. (2013). Characterization and quantification of endogenous fatty acid nitroalkene metabolites in human urine. *Journal of lipid research*, *54*(7), 1998-2009.

Salyers, A. A., Shoemaker, N. B., Stevens, A. M., & Li, L. Y. (1995). Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiological reviews, 59*, 579–590.

Schadow, K. H., Simpson, W. A., & Christensen, G. D. (1988). Characteristics of adherence to plastic tissue culture plates of coagulase-negative staphylococci exposed to subinhibitory concentrations of antimicrobial agents. *Journal of Infectious Diseaseses, 157,* 71–77.

Schonning, C., Leeming, R., & Stenstrom, T. A. (2002). Faecal contamination of source-separated human urine based on the content of faecal sterols. *Water Research, 36,* 1965-1972.

Shu, L., Schneider, P., Jegatheesan, V., & Johnson, J. (2006). An economic evaluation of phosphrous recovery as struvite from digester supernatant. *Bioresource technology, 97*(17), 2211-2216.

Sailer, F. C., Meberg, B. M., & Young, K. D. (2003). Beta-Lactam induction of colanic acid gene expression in *Escherichia coli*. *FEMS Microbiology Letters, 226,* 245–249.

Sakthivel, S. R., Tilley, E., & Udert, K. M. (2012). Wood ash as a magnesium source for phosphorus recovery from source separated urine. *Science of the Total Environment, 419,* 68-75.

Salcedo, D. E., Lee, J. H., Ha, U. H., & Kim, S. P. (2015). The effects of antibiotics on the biofilm formation and antibiotic resistance gene transfer. *Desalination and Water Treatment*, *54*(13), 3582-3588.

Salgado, R., Marques, R., Noronha, J. P. Carvalho, G., Oehmen, A., & Reis, M. A. M. (2012). Assessing the removal of pharmaceuticals and personal care products in a full scale activated sludge plant. *Environmental Science Pollution Research, 19*(5), 1818-1827.

Sanderson, H., Johnson, D.J., Reitsma, T., Brain, R.A., Wilson, C.J., Solomon, K.R., 2004. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. Regul. Toxicol. Pharmacol. 39, 158–183. https://doi.org/10.1016/j.yrtph.2003.12.006

Sangare, D., Dakoure, S. M., Hijikata, N., Lahmar, R., Yacouba, H., Coulibaly, L., & Funamizu, N. (2015). Toilet compost and human urine used in agriculture: fertilizer value assessment and effect on cultivated soil properties. *Environmental Technology, 36*(10), 1291–1298.

Schuch, R., & Fischetti, V.A. (2006). Detailed genomic analysis of the beta and gamma phages infecting Bacillus anthracis: implications for evolution of environmental fitness and antibiotic resistance. *Journal of Bacteriology, 188*, 3037–3051.

Schwaiger, J. Mallow, U., Ferling, H., Braunbeck, T., Kalbfus, W., & Negele, R. D. (2002). How estrogenic is nonylphenol? A transgenerational study using rainbow trout *(Oncorhynchus mykiss*) as a test organism. *Aquatic Toxicology, 59,* 177–189.

Schmieger, H., & Schicklmaier, P., (1999). Transduction of multiple drug resistance of *Salmonella enterica serovar typhimurium* DT104. *FEMS Microbiology Letters, 170*, 251–256.

Scope Newsletter (2003). Struvite shows to be good fertilizer. March, pp. 3.

Seki, M., Yokota, H., Matsubara, H., Maeda, M., Tadokoro, H., & Kobayashi, K. (2003). Fish full life-cycle testing for the weak estrogen 4-*tert*-pentylphenol on medaka (*Oryzias latipes*). *Environmental Toxicology & Chemistry, 22,* 1487–1496.

Sene, M., Hijikata, N., Ushijima, K., & Funamizu, N. (2019). Application of human urine in agriculture. *Resource-Oriented Agro-sanitation Systems: Concept, Business Model, and Technology*, 213-242.

Senecal, J., & Vinneras, B. (2017). Urea stabilization and concentration for urine diverting dry toilet: Urine dehydration in ash. *Science of the Total Environment, 586*, 650-657.

Shaw, R. (2010). *The use of human urine as crop fertilizer in Mali, West Africa* (Doctoral dissertation, Michigan Technological University).

Sherratt, D. J (1974). Bacterial plasmids.*Cell, 3*, 189-195.

Shih, K., & Yan, H. (2016). The Crystallization of Struvite and Its Analog (K-Struvite) From Waste Streams for Nutrient Recycling. *Environmental Materials and Waste*, 665–686.

Shu, L., Schneider, P., Jegatheesan, V., & Johnson, J. (2006). An economic evaluation of phosphorus recovery as struvite from digester supernatant. *Bioresource Technology, 97,* 2211–2216.

Siciliano, A. (2016). Assessment of Fertilizer Potential of the Struvite Produced from the Treatment of Methanogenic Landfill Leachate Using Low-Cost Reagents. *Environmental Science and Pollution Research, 23*, 5949–5959.

Siddiqui, H., Nederbragt, A. J., Lagesen, K., Jeansson, S. L., & Jakobsen, K. S. (2011). Assessing Diversity of the Female Urine Microbiota by High Throughput Sequencing of 16S rDNA Amplicons. *BMC Microbiology, 11*, 244.

Siddiqui, H., Lagesen, K., Nederbragt, A. J., Jeansson, S. L., & Jakobsen, K. S. (2012). Alterations of Microbiota in Urine from Women with Interstitial Cystitis. *BMC Microbiology, 12*, 205.

Simlai, A., Mukherjee, K., Mandal, Bhattacharya, K., Samanta, A., & Roy, A. (2016). Partial purification and characzerization of antimicrobial activity from the wood excreta of mangroove plant *Ceriops decandra*. *EXCLI. Journal, 15*, 103-112.

Simons, J., & Clemens, J. (2003, April). The use of separated human urine as mineral fertilizer. In 2nd International Symposium on Ecological Sanitation “ecosan–closing the loop"(pp. 595-600).

Singer, A. C., Shaw, H., Rhodes, V., & Hart, A. (2016). Review of Antimicrobial Resistance in the Environment and Its Relevance to Environmental Regulators. *Frontiers in Microbiology 1728*(7), 1-22.

Silva, M. A. L. D., Medeiros, Z., Soares, C. R. P., Silva, E. D. D., Miranda-Filho, D. B., & Melo, F. L. D. (2014). A comparison of four DNA extraction protocols for the analysis of urine from patients with visceral leishmaniasis. Revista da sociedade brasileira de medicina tropical, 47, 193-197.

Smith, A. W. (2005). Biofilm and antibiotic therapy: Is there a role for combating bacterial resistance by the use of novel drug delivery systems? *Advanced drug delivery reviews, 57,* 1539–1550.

Snowdon, J. A., Cliver, D. O., & Converse, J. C. (1989a). Land disposal of mixed human and animal wastes: A review. *Waste Management and Research, 7,* 121-134.

Sohn, W., Jiang, J., Phuntsho, S., Choden, Y., & Shon, H. K. (2023). Nutrients in a circular economy: role of urine separation and treatment. *Desalination*, *560*, 116663.

Song, Yuanming 宋元明, (2017). Kuaguo de Nongxue Zhishi Hudong – Yi Meiguo Turang Xuejia Fulankelin Jin Wei Zhongxin de Kaocha 跨国的农学知识互动——以美国土壤学家富兰克林·金为中心的考察 America and China –a case study of Franklin King and his中心的考察 [The exchanges of agricultural knowledge between works]. Zhongguo Nongshi 中 国 农 史 [Agricultural History of China] 1, 30–37.

Soto, S. M. (2013). Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence, 4,* 223–229.

Souza, K. A., Ginoza, H. S., & Haight, R. D. (1972). Isolation of a polyvalent bacteriophage for Escherichia coli, Klebsiella pneumoniae, and Aerobacter aerogenes. *Journal of Virology, 9*, 851–856.

Sridevi, G., Surendran, U., & Srinivasamurthy, C. A. (2016). Influence of human urine combined with farm yard manure and chemical fertilizers on french bean and maize cropping sequence in lateritic soils of Karnataka, India. *International Journal of Plant Production*, *10*(3), 1735-8043.

Sridhar, M. K. C., Coker, A. O., Adeoye, G. O., & Akinjogbin, I. O. (2005, May). Urine harvesting and utilization for cultivation of selected crops: Trials from Ibadan, South West Nigeria. In *3rd international ecological sanitation conference* (pp. 23-26).

Srinivasamurthy, C. A., & Yogeeshappa, M. H. (2012, August). Human urine an alternative to chemical fertilizers in crop production. In 4th International Dry Toilet Conference (pp. 22-24).

Starliper, C. E., Watten, B. J., Iwanowicz, D. D., Green, P. A., Bassett, N. L., & Adams, C. R. (2015). Efficacy of pH Elevation as a Bactericidal Strategy for Treating Ballast Water of Freight Carriers. *Journal of Advanced Research, 6*, 501–509.

Stenström, T. A. (1996). Pathogenic microrganisms in wastewater systems-Risk assessment of traditional and alternative wastewater systems.(Sjukdomsframkallande mikroorganismer i avloppssystem-Riskvärdering av traditionella och alternativa avloppslösningar). Report 4683, Swedish Environmental Protection Agency and National Board of Health and Welfare, Stockholm, Sweden.

Stephanie, K. L. I., & Treavor, H. B. (2015). Life cycle comparison of centralized wastewater treatment and urine source separation with struvite precipitation: Focus on urine nutrient management. *Water Research, 79,* 88-103.

Stratful, I., Scrimshaw, M., & Lester, J. (2001). Conditions influencing the precipitation of magnesium ammonium phosphate. *Water Research, 35,* 4191–4199.

Sun, Y., Snow, D., Walia, H., & Li, X. (2021). Transmission Routes of the Microbiome and Resistome from Manure to Soil and Lettuce. *Environmental Science & Technology, 55*, 11102−11112.

Sutherland, W. (2001). Biofilm exopolysaccharides: A strong and sticky framework. *Microbiology, 147,* 3–9.

Svanteson, B., Thorén, A., & Castor, B. (1988). Acute diarrhoea in adults: aetiology, clinical appearance and therapeutic aspects. *Scandinavian journal of infectious diseases, 20*(3), 303-314.

Talboys, P. J., Heppell, J., Roose, T., Healey, J. R., Jones, D. L., & Withers, P. J. (2016). Struvite: a slow-release fertiliser for sustainable phosphorus management?. *Plant and soil*, *401*, 109-123.

Tanoue, R., Sato, Y., Motoyama, M., Nakagawa, S., Shinohara, R., & Nomiyama, K. (2012). Plant Uptake of Pharmaceutical Chemicals Detected in Recycled Organic Manure and Reclaimed Wastewater. *Journal of Agriculture & Food Chemistry, 60*(*41*), 10203–10211.

Tasho, R. P., & Cho, J. Y. (2016).Veterinary antibiotics in animal waste, its distribution in soil and uptake by plants: a review. *Science of the Total Environment, 56,* 366–376.

Tauxe, R. V., & Cohen, M. L. (1995). Epidemiology of diarrheal diseases in developed countries. *Infections of the gastrointestinal tract. Raven Press, Ltd., New York, NY, USA*, 37-51.

Tetz, G. V., Artemenko, N. K., & Tetz, V. V. (2009). Effect of Dnase and antibiotics on biofilm characteristics. *Antimicrobial Agents and Chemotherapy, 53,* 1204–1209.

Timmer, L., & Visker, C. (1998). Possibilities and Impossibilities of the use of human excreta as fertiliser in agriculture in sub-Saharan Africa. *Royal Tropical Insitute and University of Amsterdam, The Netherlands*, *36*, 449-452.

Tirado, R., & Allsopp, M. (2012). Phosphorus in agriculture: problems and solutions. *Greenpeace Research Laboratories Technical Report (Review)*, *2*.

Thorup-Kristensen, K. (2001). Root growth and soil nitrogen depletion by onion, lettuce, early cabbage and carrot. *Acta Horticulturae, 563,* 201-206.

Toala, P., Wilcox, C., & Finland, M. (1970). Effect of p H of Medium and Size of Inoculum on Activity of Antibiotics Against Group D Streptococcus (Enterococcus). *Applied Microbiology*, *19*(4), 629-637.

Topaz, T., Boxall, A., Suari, Y., Egozi, R., Sade, T. and Chefetz, B. (2020). Ecological Risk Dynamics of Pharmaceuticals in Micro-Estuary Environments. *Environ. Sci. Technol.* 54(18): 11182-11190.

Truog, E., & Jones, R. J. (1938). Fate of soluble potash applied to soils. *Industrial & Engineering Chemistry*, *30*(8), 882-885.

Tsai, Y.L, Olson, B.H. (1992). Rapid Method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Appl. Environ Microbiol*. 58:2292-5.

Uddin, S. M. N., Muhandiki, V. S., Fukuda, J. Nakamura, M., & Sakai, A. (2012). Assessment of social acceptance and scope of scaling up urine diversion dehydration toilets in Kenya. *Journal of water Sanitation and Hygiene for development, 02*(3), 182-189.

Udert, K. M., Larsen, T. A., Biebow, M., & Gujer, W. (2003). Urea hydrolysis and precipitation dynamics in a urine-collecting system. *Water research*, *37*(11), 2571-2582.

Ubukata, K., Konno, M., & Fujii, R. (1975). Transduction of drug resistance to tetracycline, chloramphenicol, macrolides, lincomycin and clindamycin with phages induced from Streptococcus pyogenes. *The Journal of Antibiotics, 28*, 681–688.

Uhnoo, I., Wadell, G., Svensson, L., Olding-Stenkvist, E., Ekwall, E., & Möllby, R. (1986). Aetiology and epidemiology of acute gastro-enteritis in Swedish children. *Journal of Infection, 13*(1), 73-89.

Upadhyay, S. K., Srivastava, A. K., Rajput, V. D., Chauhan, P. K., Bhojiya, A. A., Jain, D., ... & Minkina, T. (2022). Root exudates: mechanistic insight of plant growth promoting rhizobacteria for sustainable crop production. *Frontiers in microbiology*, *13*, 916488.

Utter, B., Deutsch, D. R., Schuch, R., Winer, B. Y., Verratti, K., Bishop-Lilly, K., ... & Fischetti, V. A. (2014). Beyond the chromosome: the prevalence of unique extra-chromosomal bacteriophages with integrated virulence genes in pathogenic Staphylococcus aureus. *PLoS One*, *9*(6), e100502.

Uysal, A., Demir, S., Sayilgan, E., Eraslan, F., & Kucukyumuk, Z. (2014). Optimization of Struvite Fertilizer Formation from Baker's Yeast Wastewater: Growth and Nutrition of Maize and Tomato Plants. *Environmental Science and Pollution Research, 21*, 3264–3274.

Uysal, A., Yilmazel, Y. D., & Demirer, G. N. (2010). The Determination of Fertilizer Quality of the Formed Struvite from Effluent of a Sewage Sludge Anaerobic Digester. *Journal of hazardous materials*, *181*, 248–254.

van der Graaf-Van Bloois, L., Wagenaar, J. A., & Zomer, A. L. (2021). RFPlasmid: predicting plasmid sequences from short-read assembly data using machine learning. *Microbial Genomics*, *7*(11), 000683.

Vázquez-Rosas-Landa, M., Ponce-Soto, G. Y., Eguiarte, L. E., & Souza, V. (2017). Comparative genomics of free-living Gammaproteobacteria: pathogenesis-related genes or interaction-related genes?. *Pathogens and Disease*, *75*(5), ftx059.

Venkatesh, P., Kumar, A. N., Prasad, R. H., Krishnamoorthy, K. B., Prasath, K. H., & Soumya, V. (2013) LC –MS/MS analysis of tetracycline antibiotics in prawns (Penaeus monodon) from south India coastal region. *Journal of pharmacy research*, *6*, 48–52.

Vermeulen, A., Gysemans, K.P.M., Bernaerts, K., Geeraerd, A., Van Impe, J.F.M, Debevere, J. & Devlieghere, F. (2007). Influence of pH, water activity and acetic acid concentration on Listeria monocytogenes at 7 °C: Data collection for the development of a growth/no growth model. *International Journal of Food Microbiology*, 114(3):332-341.

Vetting, M. W., Hegde, S. S., Wang, M., Jacoby, G. A., Hooper, D. C., & Blanchard, J. S. (2011). Structure of QnrB1, a plasmid-mediated fluoroquinolone resistance factor. *Journal of Biological Chemistry*, *286*(28), 25265-25273.

Vinneras, B., Nordin, A., Niwagaba, C., & Nyberg, K. (2008). Inactivation of bacteria and viruses in human urine depending on temperature and dilution rate. *Water Reasearch, 42,* 4067-4074.

Vinneras, B., & Jonsson, H. (2006). The performance and potential of faecal separation and urine diversion to recycle plant nutrients in household waste water. *Bioresource Technology, 84,* 275-282.

Vinnerås, B. (2001). Faecal separation and urine diversion for nutrient management of household biodegradable waste and wastewater. Swedish University of Agricultural Sciences Department of Agricultural Engineering, Uppsala, Sweden.

Voulgari, E., Poulou, A., Koumaki, V., & Tsakris, A. (2013). Carbapenemase-producing Enterobacteriaceae: now that the storm is finally here, how will timely detection help us fight back? *Future Microbiology, 8*, 27–39.

Wang, Q., Ma, M., Jiang, X., Guan, D., Wei, D., Cao, F., ... & Li, J. (2020). Influence of 37 years of nitrogen and phosphorus fertilization on composition of rhizosphere arbuscular mycorrhizal fungi communities in black soil of northeast China. *Frontiers in Microbiology*, *11*, 539669.

Wang, C., Zheng, X., Guan, Z., Zou, D., Gu, X., Lu, H., ... & Zhou, P. (2022). Quantified detection of Treponema pallidum DNA by PCR assays in urine and plasma of syphilis patients. *Microbiology Spectrum*, *10*(2), e01772-21.

Wang, Y., Ji, H., Wang, R., Hu, Y., & Guo, S. (2020). Synthetic Fertilizer Increases Denitrifier Abundance and Depletes Subsoil Total N in a Long-Term Fertilization Experiment. *Frontiers in Microbiology, 11***(**2026), 1-11.

Wang, Q., Sun, F. J., Liu, Y., Xiong, L. R., Xie, L. L., & Xia, P. Y. (2010). Enhancement of biofilm formation by subinhibitory concentrations of macrolides in icaADBC-positive and-negative clinical isolates of Staphylococcus epidermidis. *Antimicrobial agents and chemotherapy*, *54*(6), 2707-2711.

Warren, K. S. (1962). Ammonia toxicity and pH. *Nature, 195,* 47-49.

Waters, J. L., & Salyers, A. A. (2013). Regulation of CTnDOT conjugative transfer is a complex and highly coordinated series of events. MBio, *4*(6), 10-1128.

Westerman, P. W., Zering, K. D., & Rashash, D. (2009). Struvite crystallizer for recovering phosphorous from lagoon and digester liquid. Website: http://www. bae. ncsu. edu/programs/extension/manure/lagoon/ag-724w_struvite_crystallizer. pdf.

Wetzel, D., & McBride, S. M. (2020). The impact of pH on Clostridioides difficile sporulation and physiology. *Applied and environmental microbiology*, *86*(4), e02706-19.

Wheeler, D., & Carroll, R. F. (1989). The minimisation of microbiological hazards associated with latrine wastes. *Water Science and Technology, 21*(3), 35-42.

Wiedner, K.; Schneeweiß, J.; Dippold, M.A.; Glaser, B. (2015). Anthropogenic dark earth in Northern Germany-The Nordic Analogue to terra preta de Índio in Amazonia. Catena, 132: 114-125.

Wilsenach, J.A.; Schuurbiers, C.A.H.; van Loosdrecht, M.C.M. (2007). Phosphate and potassium recovery from source separated urine through struvite precipitation. *Water Res.* 41: 458–466.

Wilsenach, J. and van Loosdrecht, M. (2003). Impact of Separate Urine Collection on Wastewater Treatment Systems. *Water Science & Technology* 48(1):103-110. DOI:10.2166/wst.2003.0027

Willi, K., Sandmeier, H., Kulik, E. M., & Meyer, J. (1997). Transduction of antibiotic resistance markers among Actinobacillus actinomy-cetemcomitans strains by temperate bacteriophages Aa phi 23. *Cellular and Molecular Life Sciences, 53*, 904–910.

Winker, M., Vinneras, B., Muskolus, A., Arnold, U., & Clemens, J. (2009). Fertilizer products from new sanitation systems: their potential values and risks. *Bioresource Technology, 100*(18), 4090-4096.

Witte, W. (2004) International dissemination of antibiotic resistant strains of bacterial pathogens. *Infection, Genetics and Evolution, 4*, 187–191.

Wollenberger, L., Halling-Sørensen, B., & Kusk, K. O. (2000). Acute and chronic toxicity of veterinary antibiotics to Daphnia magna. *Chemosphere, 40,* 723–730.

Wojciuk, B., Salabura, A., Grygorcewicz, B., Kędzierska, K., Ciechanowski, K., & Dołęgowska, B. (2019). Urobiome: in sickness and in health. *Microorganisms*, 7(11), 548.

World Health Organization (WHO) (1993) Guidelines for drinking-water quality. Volume 1 - Recommendations. 2nd ed. World Health Organization, Geneva, Switzerland.

World Health Organization (2006). Guidelines for the safe use of wastewater, Excreta and Greywater. Volume 4. Waste water use in Agriculture.

World Health Organization (2012). The evolving threat of antimicrobial resistance: options for action. Geneva.

World Health Organization (2014). Antimicrobial resistance global report on surveillance. Geneva: Switzerland.

World Health Organization (WHO) (2019). https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-avert-antimicrobial-resistance-crisis

Wozniak, A., Villagra, N. A., Undabarrena, A., Gallardo, N., Keller, N., Moraga, M., ... & García, P. (2012). Porin alterations present in non-carbapenemase-producing Enterobacteriaceae with high and intermediate levels of carbapenem resistance in Chile. *Journal of medical microbiology*, *61*(9), 1270-1279.

Wozniak, R. A. F., & Waldor, M. K. (2010). Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nature Reviews Microbiology*, *8*, 552–563.

Wu, S., Li, X., Gunawardana, M., Maguire, K., Guerrero-Given, D., Schaudinn, C., ... & Webster, P. (2014). Beta-lactam antibiotics stimulate biofilm formation in non-typeable Haemophilus influenzae by up-regulating carbohydrate metabolism. *PLoS One*, *9*(7), e99204.

Xu, K., Lu, J., Hu, L., Li, J., Cheng, S., Zheng, M., & Wang, C. (2022). Pathogens inactivation in nutrient recovery from urine: A review. *Frontiers in Environmental Science*, *10*, 1056019.

Xu, K., Li, J., Zheng, M., Zhang, C., Xie, T., & Wang, C. (2015). The Precipitation of Magnesium Potassium Phosphate Hexahydrate for P and K Recovery from Synthetic Urine. *Water Research, 80*, 71–79.

Xu, K., Wang, C., Liu, H., & Qian, Y. (2011). Simultaneous Removal of Phosphorus and Potassium from Synthetic Urine through the Precipitation of Magnesium Potassium Phosphate Hexahydrate. *Chemosphere, 84*, 207–212.

Xuan, R., Arisi, L., Wang, Q., Yates, S. R., & Biswas, K. C. (2009). Hydrolysis and photolysis of oxytetracycline in aqueous solution. *Journal of Environmental Science and Health, Part B, 45*, 73–81.

Yamashita, N., Yasojima, M., Nakada, N., Miyajima, K., Komori, K., Suzuki, Y., & Tanaka, H. (2006). Effects of antibacterial agents, levofloxacin and clarithromycin, on aquatic organisms. *Water Science & Technology, 53,* 65–72.

Yang, Q., Gaoa, Y., Ke, J., Show, P. L., Ge, Y., Liu, Y., Guo, R., & Chen, J. (2021). Antibiotics: An overview on the environmental occurrence, toxicity, degradation, and removal methods *Bioengineered, 12*(1), 7376–7416.

Yang, C. W., Hsiao, W. C., & Chang, B. V. (2016). Biodegradation of sulfonamide antibiotics in sludge. *Chemosphere,* 150, 559–565.

Yang, F. X., Xu, Y., & Hui, Y. (2006). Reproductive effects of prenatal exposure to nonylphenol on zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology Part C, 142,* 77–84.

Yang, Y., Liu, J., Wang, B., Liu, R., & Zhang, T. A (2019). Thermodynamic Modeling Approach for Solubility Product from Struvite-k. *Computational materials science, 157*, 51–59.

Yetilmezsoy, K., & Zengin, Z. S. (2009). Recovery of ammonium nitrogen from the effluent of UASB treating poultry manure wastewater by MAP precipitation as a slow release fertilizer. *Journal of Hazardous Materials, 166,* 260–269.

Żaczek, M., Weber-Dąbrowska, B., Międzybrodzki, R., & Górski, A. (2020). Phage prevalence in the human urinary tract-Current knowledge and therapeutic implications. *Microorganisms*, 8(11), 1802.

Zhou, S., Zhu, D., Giles, M., Yang, X., Daniell, T., Neilson, R., & Zhu, Y. (2019). Phyllosphere of staple crops under pig manure fertilization, a reservoir of antibiotic resistance genes. *Environmental Pollution 252*, 227-235.

Zhao, W. H., & Hu, Z. Q. (2013). Epidemiology and genetics of CTX M extended-spectrum β-lactamases in Gram-negative bacteria. *Critical reviews in microbiology,* 39, 79–101.

Zhang, J., Giannis, A., Chang, W. C. V., Bernard, J. H. N., & Wang, J. Y. (2013). Adaptation of urine source separation in tropical cities: Process optimization and odour mitigation. *Journal of the Air & Waste Management Association, 63*(4), 472-481.

Zhang, W., Qiu, L., Gong, A., & Yuan, X. (2017). Isolation and characterization of a high-efficiency erythromycin A-degrading Ochrobactrum sp. strain. *Marine Pollution Bulletin*, *114*(2), 896-902.

Zhang, Y. J., Hu, H. W., Chen, Q. L., Singh, B. K., Yan, H., Chen, D., & He, J. Z. (2019). Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. *Environment international*, *130*, 104912.

Zhu, M., Wang, Z., Chen, J., Xie, H., Zhao, H., & Yuan, X. (2020). Bioaccumulation, biotransformation and multi-compartmental toxicokinetic model of antibiotics in sea cucumber (Apostichopus japonicus). *Environmental Science & Technology, 54*(20), 13175–13185.

Zinder, N. D., & Lederberg, J. (1952). Genetic exchange in Salmonella. *Journal of bacteriology, 64*(5), 679–99.

Zseni, A. and Nagy, J. (2016). Environmental impacts and the possibility for sustainable development of human excreta. *Environmental Impact* 2016 ,203:49-60. DOI:10.2495/EID160051

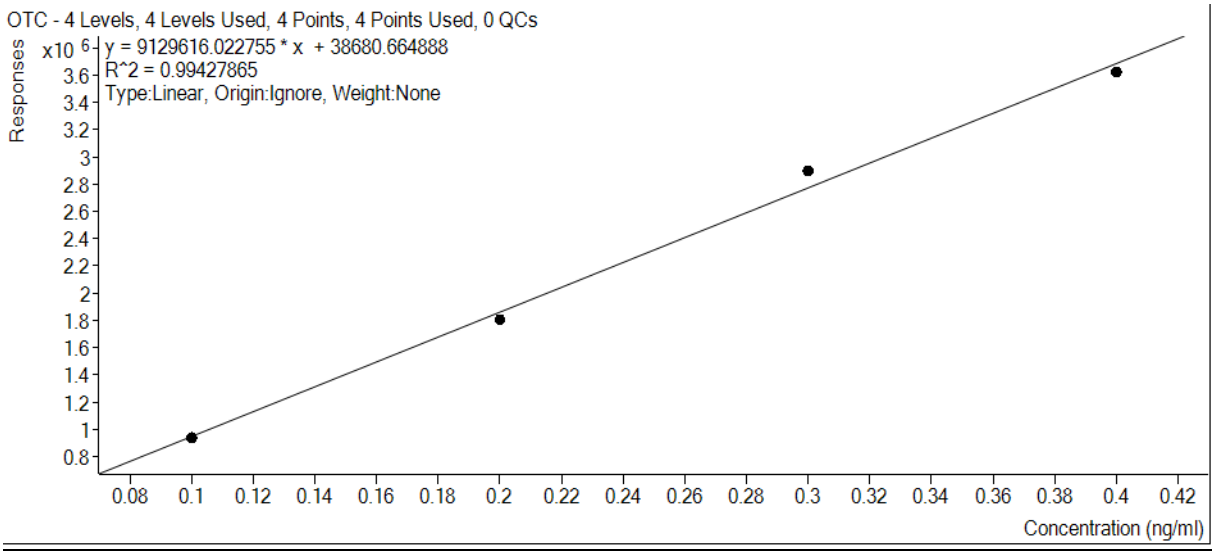
Zuber, A., Peric, A., Pluchino, N., Baud, D., & Stojanov, M. (2023). Human Male Genital Tract Microbiota. *International Journal of Molecular Sciences,* 24, 6939.

Zuccato, E., Calamari, D., Natangelo, M., & Fanelli, R. (2000). Presence of therapeutic drugs in the environment. *Lancet, 355,* 1789-1790.

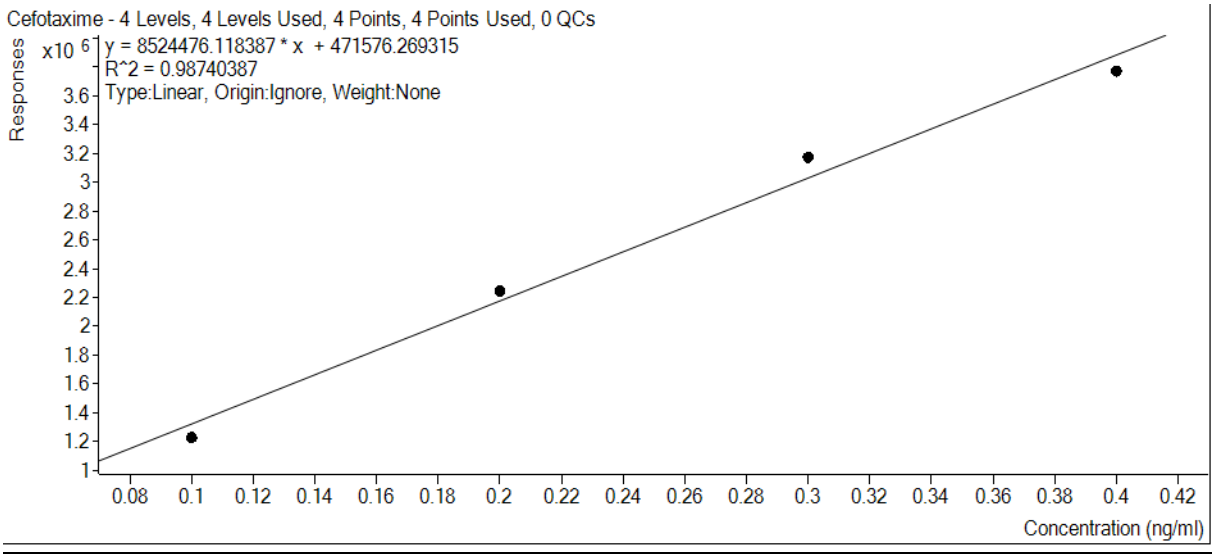
8. APPENDICES

Appendix 1. Calibration curves for the various antimicrobial drug analytes generated by LCMS/MS machine

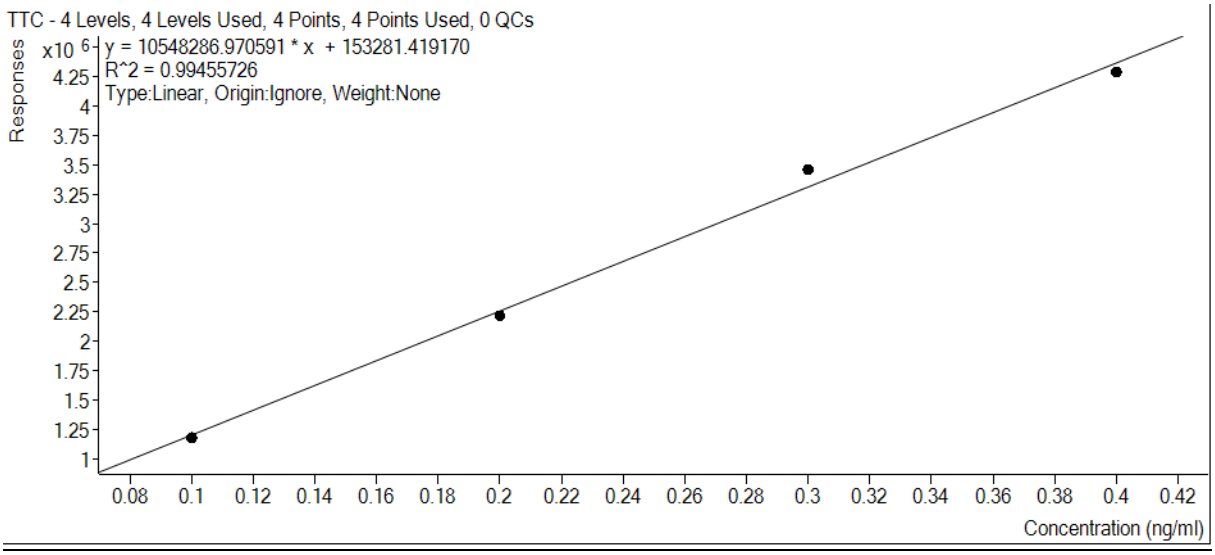
1. Oxytetracycline



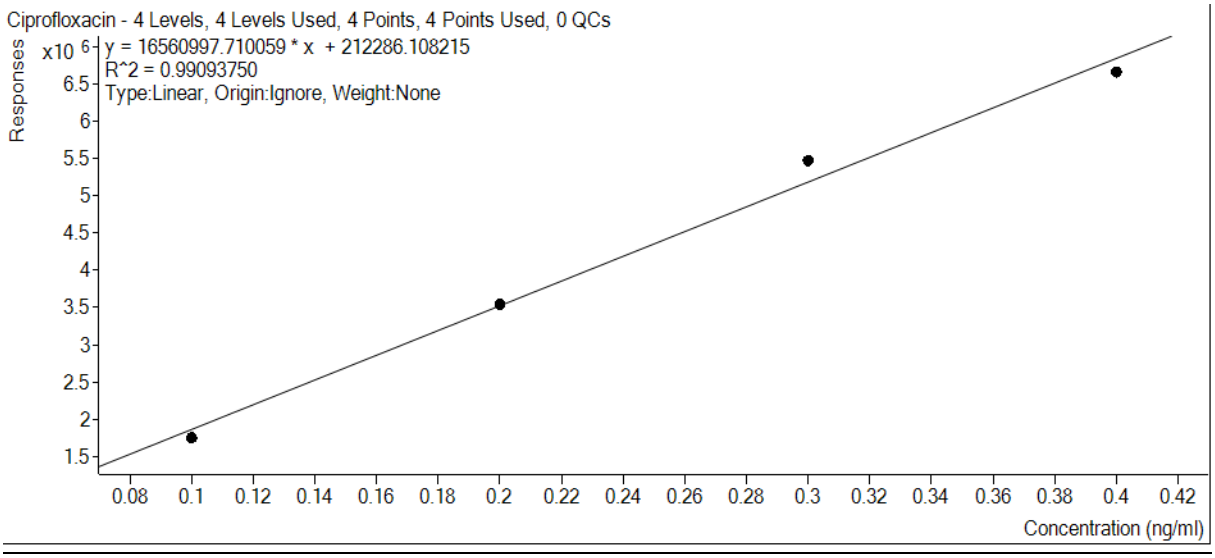
2. Cefotaxime



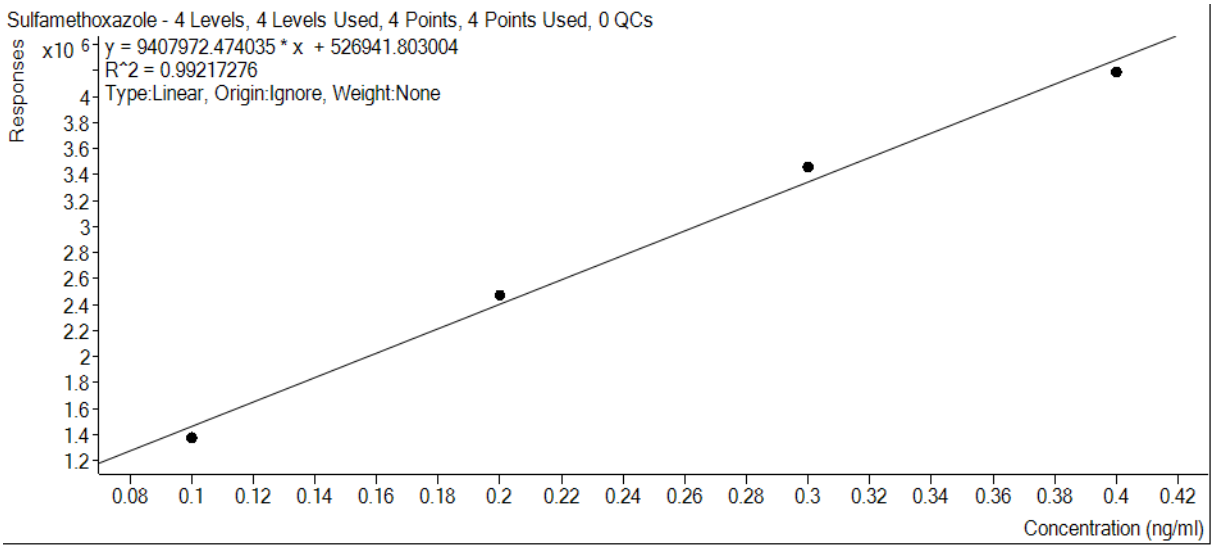
3. Tetracycline



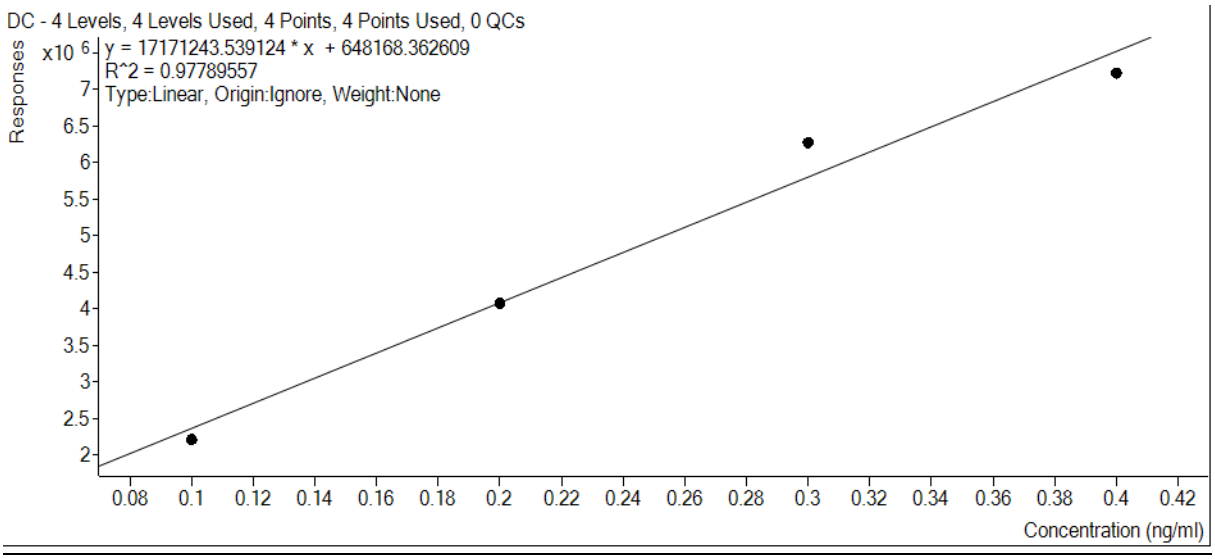
4. Ciprofloxacin



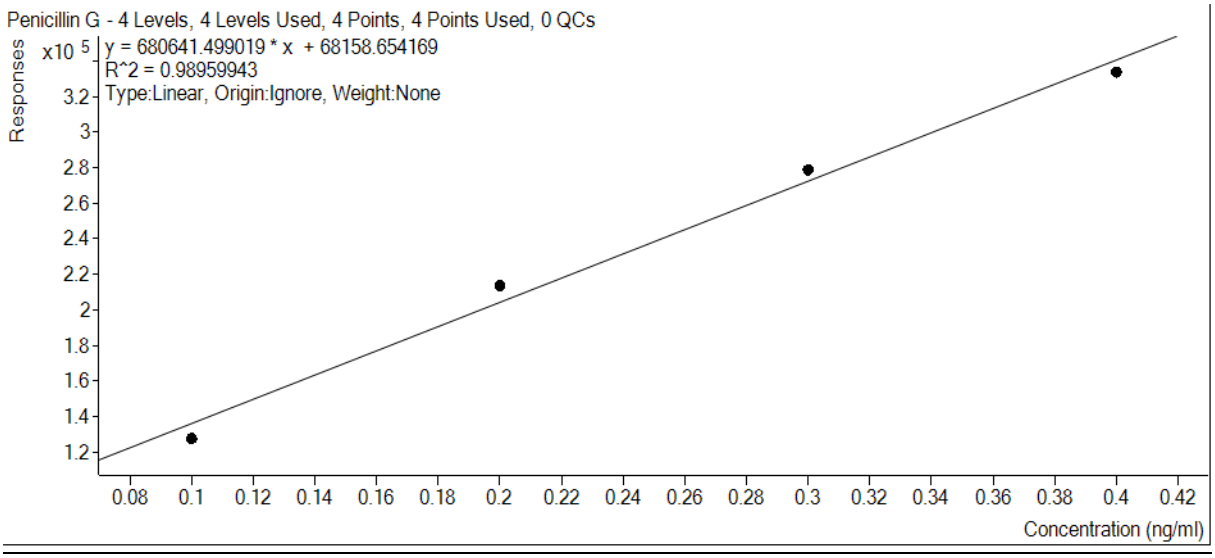
5. Sulfamethoxazole



6. Doxycycline



7. Pencillin G



Appendix 2. Identified Gamma proteobacteria and their abundance in Fresh urine, stored urine and

OUT	Phylum	Class	Order	Family	Genus	Fresh	Stored	Struvite
23	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	993	356	229
24	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	19	18	9
25	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	48	44	22
26	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	885	103	46
27	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	69	57	29
28	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	231	58	52
29	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	50	39	27
30	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	776	310	174
31	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	8	3	1
32	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Actinobacillus	19	40	40
33	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Actinobacillus	0	68	0
34	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Actinobacillus	93	85	104
35	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Actinobacillus	48	71	75
36	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Actinobacillus	245	83	23
45	Pseudomonadota	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	11	30	2
46	Pseudomonadota	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	1324	133	73
47	Pseudomonadota	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	771	132	88
49	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Aggregatibacter	35	39	27
50	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Aggregatibacter	24	43	43
51	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Aggregatibacter	14	21	8
58	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	Alcanivorax	73	145	145
59	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	Alcanivorax	39	88	86
63	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Aliivibrio	26	27	42
65	Pseudomonadota	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	Alkalilimnicola	79	240	151
68	Pseudomonadota	Gammaproteobacteria	Chromatiales	Chromatiaceae	Allochroma-tium	328	149	48
70	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Unassigned	Unassigned	132	21	14
71	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Alteromonas	12	28	23
107	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Avibacterium	0	1	2
111	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Azotobacter	531	268	761
161	Pseudomonadota	Gammaproteobacteria	Gammaproteobacteria	Candidatus	Baumannia	2	7	4
163	Pseudomonadota	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Beggiatoa	17	50	30
165	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Bermanella	22	40	38
216	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Buchnera	14	18	16

264	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Candidatus Blochmannia	5	2	0
265	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Candidatus Blochmannia	1	6	1
269	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Candidatus Hamiltonella	35	104	59
281	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Candidatus Regiella	1	1	3
282	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Candidatus Riesia	8	10	16
283	Pseudomonadota	Gammaproteobacteria	Unassigned	Unassigned	Candidatus Ruthturnera	71	148	61
285	Pseudomonadota	Gammaproteobacteria	Unassigned	Unassigned	Candidatus Vesicomyosocius	8	20	11
292	Pseudomonadota	Gammaproteobacteria	Cardiobacteriales	Cardiobacteriaceae	Cardiobacterium	38	74	35
324	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Chromohalobacter	111	170	124
328	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Citrobacter	8	5	2
329	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Citrobacter	244	84	36
330	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Citrobacter	85	22	14
331	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Citrobacter	68	28	15
332	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Citrobacter	38	16	4
371	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Colwelliaceae	Colwellia	45	106	88
375	Pseudomonadota	Gammaproteobacteria	Cellvibrionales	Halieaceae	Congregibacter	42	98	31
401	Pseudomonadota	Gammaproteobacteria	Legionellales	Coxiellaceae	Coxiella	22	51	25
404	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Cronobacter	240	91	42
405	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Cronobacter	55	25	3
452	Pseudomonadota	Gammaproteobacteria	Cardiobacteriales	Cardiobacteriaceae	Dichelobacter	50	74	60
453	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Pectobacteriaceae	Dickeya	214	110	56
454	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Pectobacteriaceae	Dickeya	66	31	22
462	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Hafniaceae	Edwardsiella	84	26	24

463	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Hafniaceae	Edwardsiella	100	55	15
470	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacter	34	18	6
471	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacter	242	103	43
472	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacter	266	91	39
479	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Erwinia	16	10	9
480	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Erwinia	81	39	12
481	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Erwinia	31	15	7
482	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Erwinia	82	35	26
487	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Escherichia	11	9	12
488	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Escherichia	867	397	326
489	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Escherichia	73	31	15
490	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Escherichia	23	15	5
508	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Ferrimonadaceae	Ferrimonas	44	57	31
518	Pseudomonadota	Gammaproteobacteria	Legionellales	Legionellaceae	Fluoribacter	0	1	0
519	Pseudomonadota	Gammaproteobacteria	Thiotrichales	Francisellaceae	Francisella	31	45	52
520	Pseudomonadota	Gammaproteobacteria	Thiotrichales	Francisellaceae	Francisella	13	31	32
521	Pseudomonadota	Gammaproteobacteria	Thiotrichales	Francisellaceae	Francisella	22	60	38
549	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola	2	4	2
560	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Grimontia	26	19	11
561	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	20	30	18
562	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	121	157	125
563	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	67	84	66
564	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	70	109	114
565	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Hahellaceae	Hahella	148	240	144
567	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	53	111	98
568	Pseudomonadota	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	Halorhodospira	45	115	50
570	Pseudomonadota	Gammaproteobacteria	Chromatiales	Halothiobacillaceae	Halothiobacil-lus	36	71	33
602	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Alcanivoraceae	Kangiella	45	88	44
606	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Klebsiella	468	187	98
607	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Klebsiella	24	7	12
608	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Klebsiella	43	16	7

652	Pseudomonadota	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	10	29	13
653	Pseudomonadota	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	18	36	19
654	Pseudomonadota	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	66	121	79
676	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Listonella	1	1	5
686	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Mannheimia	29	60	40
687	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Mannheimia	54	95	47
690	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter	52	72	87
691	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter	233	255	331
692	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter	147	297	242
707	Pseudomonadota	Gammaproteobacteria	Methylococcales	Methylococcaceae	Methylobacter	34	93	40
715	Pseudomonadota	Gammaproteobacteria	Methylococcales	Methylococcaceae	Methylococcus	78	252	79
716	Pseudomonadota	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	Methylophaga	26	46	23
733	Pseudomonadota	Gammaproteobacteria	Moraxellales	Moraxellaceae	Moraxella	5	3	1
734	Pseudomonadota	Gammaproteobacteria	Moraxellales	Moraxellaceae	Moraxella	142	95	29
735	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Moritellaceae	Moritella	23	23	27
788	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Neptuniibacter	33	59	58
793	Pseudomonadota	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	Nitrococcus	45	131	50
794	Pseudomonadota	Gammaproteobacteria	Chromatiales	Chromatiaceae	Nitrosococcus	51	97	61
795	Pseudomonadota	Gammaproteobacteria	Chromatiales	Chromatiaceae	Nitrosococcus	86	223	192
796	Pseudomonadota	Gammaproteobacteria	Chromatiales	Chromatiaceae	Nitrosococcus	18	60	51
834	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Pantoea	81	35	20
835	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Pantoea	137	79	40
836	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Pantoea	86	34	8
848	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Pasteurella	24	26	15
849	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Pasteurella	84	125	94
850	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Pectobacteriaceae	Pectobacterium	446	148	62
851	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Pectobacteriaceae	Pectobacterium	131	63	33
852	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Pectobacteriaceae	Pectobacterium	76	35	17
873	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium	48	42	39
874	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium	27	29	39
875	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium	166	154	165
876	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Photobacterium	9	14	11

877	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Photorhabdus	797	58	26
878	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Photorhabdus	2326	164	112
918	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Proteus	2869	385	81
919	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Proteus	676	174	19
920	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Proteus	9	1	0
921	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Providencia	781	45	18
922	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Providencia	6447	92	30
923	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Providencia	705	46	18
924	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Providencia	720	80	33
925	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Pseudoalteromona-daceae	Pseudoalter-omonas	83	160	65
926	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Pseudoalteromona-daceae	Pseudoalter-omonas	77	117	137
927	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Pseudoalteromona-daceae	Pseudoalter-omonas	16	39	43
928	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Pseudoalteromona-daceae	Pseudoalter-omonas	31	52	39
929	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	2193	1027	2592
930	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	645	223	383
931	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	2753	2686	1888
932	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	2	1	0
933	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1437	415	1876
934	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	2449	684	1230
935	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	4	2	2
936	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	103	36	109
937	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	12	9	3
938	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1280	431	1694
939	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1238	641	1177
942	Pseudomonadota	Gammaproteobacteria	Moraxellales	Moraxellaceae	Psychrobacter	1243	273	125
943	Pseudomonadota	Gammaproteobacteria	Moraxellales	Moraxellaceae	Psychrobacter	1910	342	202
944	Pseudomonadota	Gammaproteobacteria	Moraxellales	Moraxellaceae	Psychrobacter	493	280	168
946	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas	50	70	99

947	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Psychromonadaceae	Psychromonas	30	50	45
954	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Saccharospirillaceae	Reinekea	32	77	59
985	Pseudomonadota	Gammaproteobacteria	Legionellales	Coxiellaceae	Rickettsiella	5	9	2
1013	Pseudomonadota	Gammaproteobacteria	Cellvibrionales	Cellvibrionaceae	Saccharophagus	75	152	97
1019	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Salmonella	573	212	152
1029	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Serratia	11	1	1
1030	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Serratia	74	80	32
1031	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Serratia	717	177	85
1032	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	43	64	38
1033	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	104	122	119
1034	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	13	14	17
1035	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	28	48	45
1036	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	49	71	60
1037	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	28	23	45
1038	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	33	51	51
1039	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	62	57	45
1040	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	45	69	57
1041	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	14	32	17
1042	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	50	63	76
1043	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	46	51	75
1044	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	230	139	165
1045	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	18	25	16
1046	Pseudomonadota	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella	30	48	29
1047	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Shigella	11	19	9
1048	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Shigella	37	24	8
1049	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Shigella	58	17	11
1050	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Shigella	24	15	11
1051	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Shigella	4	1	2
1090	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomo-nas	201	316	74
1091	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomo-nas	70	105	28

1154	Pseudomonadota	Gammaproteobacteria	Cellvibrionales	Cellvibrionaceae	Teredinibacter	34	62	40
1191	Pseudomonadota	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	Thioalkalivibrio	121	359	100
1192	Pseudomonadota	Betaproteobacteria	Nitrosomonadales	Thiobacillaceae	Thiobacillus	45	197	88
1193	Pseudomonadota	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	Thiomicrospira	64	80	62
1194	Pseudomonadota	Betaproteobacteria	Burkholderiales	Burkholderiales genera incertae sedis	Thiomonas	30	98	47
1195	Pseudomonadota	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Tolumonas	104	83	79
1196	Pseudomonadota	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Treponema	67	465	815
1197	Pseudomonadota	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Treponema	6	26	35
1198	Pseudomonadota	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Treponema	13	57	93
1216	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	34	27	40
1217	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	8	17	5
1218	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	246	162	172
1219	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	22	20	14
1220	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	95	82	105
1221	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	16	19	20
1222	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	74	72	70
1223	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	22	20	30
1224	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	17	12	23
1225	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	13	6	10
1226	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	77	76	54
1227	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	19	23	16
1228	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	77	80	77
1229	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	46	51	49
1230	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	2	3	4
1231	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	92	88	68
1232	Pseudomonadota	Gammaproteobacteria	Vibrionales	Unassigned	Unassigned	11	11	9
1236	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Wigglesworthia	4	5	0
1241	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas	28	75	14
1242	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas	74	193	42
1243	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas	261	538	92
1244	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas	0	2	2

1245	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas	17	47	17
1246	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas	84	189	45
1247	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Xenorhabdus	490	45	29
1248	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Morganellaceae	Xenorhabdus	484	42	36
1250	Pseudomonadota	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xylella	60	130	52
1251	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	9	10	5
1252	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	69	15	13
1253	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	493	96	48
1254	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	87	21	9
1255	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	101	42	31
1256	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	11	19	10
1257	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	51	23	13
1258	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	440	124	60
1259	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	277	81	53
1260	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	15	23	7
1261	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Yersinia	20	20	19
1264	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Actinobacillus	1	0	0
1269	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Enterobacter	6	0	0
1271	Pseudomonadota	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	1	0	0
1274	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Pantoea	1	0	0
1275	Pseudomonadota	Gammaproteobacteria	Moraxellales	Moraxellaceae	Moraxella	2	0	0
1276	Pseudomonadota	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Mannheimia	2	0	0
1277	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Marinomonas	171	0	0
1279	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Klebsiella	3	0	0
1283	Pseudomonadota	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	1	0	38
1288	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Erwiniaceae	Erwinia	0	0	1
1289	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Hafniaceae	Hafnia	0	0	1
1291	Pseudomonadota	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	Marinomonas	0	0	261
1296	Pseudomonadota	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0	0	1
1299	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Yersiniaceae	Serratia	0	0	1
1300	Pseudomonadota	Gammaproteobacteria	Enterobacterales	Bruguierivoracaceae	Sodalis	1	0	3