QUANTITATIVE DETERMINATION OF SELECTED ANTIBIOTIC RESIDUES IN WATER FROM RIVERS, DAM AND WASTEWATER IN MOROGORO, TANZANIA

Ally, Mohamed Hamed Sango
Department of Veterinary Physiology, Biochemistry and Pharmacology, Sokoine University of Agriculture, P. O. Box 3000, Chuo Kikuu, Morogoro, Tanzania
Corresponding Author Email: ally2005mood@yahoo.com: ally.mohamed@sua.ac.tz

ABSTRACT
The anthropogenic load of antibiotics in Morogoro Municipality water bodies was estimated using the ELISA technique. Commercially available enzyme-linked immunosorbent assay (ELISA) kits commonly used for detection of tetracyclines, sulfonamides and quinolones residues in meat, milk, eggs and honey were adopted for analysis of these antibiotics in surface waters. Thirty-three sampling points were selected, including two rivers, one dam and one wastewater treatment plant. Results showed that there were detectable levels of the selected antibiotics in all the sampling sites. In rivers there were slightly higher mean concentration of antibiotics in the downstream rivers in close vicinity to high human activities. High mean concentrations of antibiotics were also found in wastewater treatment plant. In rivers, the maximum mean concentrations detected were 7.74±2.45, 8.76±2.65 and 8.94±3.83µg/l quinolones, tetracyclines and sulfonamides, respectively. At Mindu Dam, mean concentrations were, 1.61±0.17, 4.84±1.23 and 3.65±1.37µg/l, quinolones, tetracyclines and sulfonamides, respectively, while at Wastewater Treatment Plant mean concentrations were 31.55±11.23, 48.89±21.82 and 37.94±14.05µg/l quinolones, tetracyclines and sulfonamides, respectively. Differences in mean concentrations in Rivers, Dam and Wastewater Plant were attributed to the fact that in wastewater treatment plant antibiotic residues are continuously poured through human domestic and hospital waste, while at Mindu Dam and Rivers antibiotic residues are poured mainly through run-off during rainy season, hence high mean concentrations in Wastewater Treatment Plant than in rivers and dam. The presence of antibiotics residues in rivers, Mindu Dam and Wastewater Treatment Plant although in very low concentrations poses risks to population who take antibiotics at low doses. This can be through drinking water and consumption of crops produced through irrigation using water from these sources; this can lead to acceleration of antibiotic resistance through selection pressure; also can harm organisms in water ecosystem in long time exposure.
Keywords. Antibiotics, ELISA, Water bodies, Morogoro, Tanzania

INTRODUCTION

Antibiotics are routinely used in human and veterinary medicine for the therapeutic treatment of infectious diseases, as well as for animal growth promoters (Zhu *et al.,* 2013). However; antibiotics are excreted as the parent compounds or metabolites due to poor gut absorption or incomplete metabolism (Chen *et al.,* 2017, Yu *et al.,* 2017). The excretion of antibiotics as a parent compounds or metabolites result to environment contamination including aquatic environment due to incomplete removal of antibiotics during wastewater treatment, run-off and their continued release into the environment (Ma *et al.,* 2016).

Over the past decades, a high number of antibiotics have been detected in surface, ground and drinking waters. This contamination comes from domestic sewage, livestock manure, hospitals and chemical pharmaceutical industries. Typical examples of these contaminants are tetracyclines, sulfonamides and quinolones, powerful antibiotic used in human and veterinary medicine. Presence of these antibiotics in the environment can pose a serious threat to the ecosystem and human health due to their high consumption rate (Veronica *et al.,* 2014). The concentrations and detection frequencies of antibiotics in aquatic environment, could lead to selective pressure on the water bacteria and induce the formation of antibiotic resistant bacteria, reducing their therapeutic potential against human and animal pathogens (Carvalho and Santos 2016, Quiao *et al.,* 2017), hence, the antibiotic residues in aquatic environment could pose a potential threat to environment and human health.

More than 22.7 million kilograms of antibiotics are being produced each year in the USA. Although most of these antibiotics are used for treatment of infections in humans and animals, a significant portion is used as animal growth promoters in form of feed supplements in livestock. More than 40% of the antibiotics produced in USA are used as feed supplements (Environmental Media Services, 2000). The use of antibiotics in animal feeds helps to increase the animal's ability to absorb feeds and reach market weight on time. It may also counteract the effects of crowded living conditions and poor hygiene in intensive animal husbandry systems (Environmental Media Services, 2000). Antibiotics

commonly used as feed additives for animals include chlortetracycline, oxytetracycline, penicillin and tylosin (Church and Pond, 1982).

Another area where there is heavy use of antibiotics is aquaculture. Aquaculture has rapidly grown in the worldwide as a major industry, providing not only economic income and high-quality food product, but also provides employment to hundreds of thousand skilled and unskilled workers. It has been identified that all animal production system has challenges associated with disease and the best way to solve this is often through effective management practices via management of stock, soil, water, nutrition, and environment (Ringo *et al.*, 2014).

In oral administered antibiotics, as much as 80% of antibiotics administered may pass through the animal unchanged depending on the nature of the antibiotics (Levy, 1992). Once excreted in urine feaces, antibiotics may enter surface and/or ground waters through nonpoint source pollution. Land application of manure is a common practice in many parts of the world. Manure is applied to land in order to enhance fertility of the farms used for growing of crops but also as means of disposing unwanted waste, the antibiotic residues present in manure may enter surface and underground water. The United States Geologic Survey reported the presence of several antibiotics in 139 streams across 30 states in United States (Koplin *et al*., 2002). Increased antimicrobial resistance in bacteria affecting humans and animals in recent decades is primarily influenced by an increased in usage of antibiotics for a variety of purposes, including therapeutic and non-therapeutics in animal production

The extent and persistence of antibiotic residues in aquatic systems is unknown and current evidence is conflicting. Furthermore, no international guidelines currently exist for maximum antibiotic residues limits in water. Water is important vehicle for the spread of both antibiotic residues and resistance determinants, since contaminated water can be consumed directly by humans and livestock and used to irrigate crops (FAO, 2016). Tetracycline, constitute the most extensively used antibiotics classes due to their low cost, ease of use and relatively minor side effects (Li *et al*, 2016, Ahmed *et al.*, 2017). But the main risks associated with Quinolones, Tetracyclines and Sulphonamidde exposure humans and animals includes, drug resistance and allergic reactions, while in ecosystem toxic effects may occur in non-target organisms. Antibiotic agents may disturb the microflora of human intestinal tract and increased risk for certain infections. When people taking an antibiotic for any reason, increased the risk for infections due to particular pathogens become resistant to that antibiotic (Pham *et al*. *2015).*

The ELISA technique has been introduced into environmental chemistry research for determination of herbicides in surface and ground water samples (Thurman *et al*., 1990; Aga and Thurman, 1993). Radioimmunoassay procedure for quantification of tetracycline antibiotics in wastewater has also being developed (Meyer *et al.,* 2000). The advantages of ELISA compared with high performance liquid chromatography (HPLC) and LC-MS have been recognized by the USEPA (1992). This study examined the use of ELISA method for estimating the concentrations of tetracyclines, quinolones and sulfonamides in water samples. There is scant information on antibiotic residues in water bodies in Tanzania; hence the objective of this study was to investigate the presence of antibiotics residues in rivers passing through Morogoro Municipality, Mindu Dam and Mafisa Wastewater Treatment Plant.

MATERIALS AND METHODS

Sampling and Solid Phase Eextraction

Morogoro is a town with 2,218,492 populations, according to 2012 census (National Bureau of Statistics Ministry of Finance Dar es Salaam, 2013)**.** Thirty-three sampling points were identified and sampled in duplicates. A total of sixty-six grab samples were collected in river Ngerengere, Morogoro, Mindu dam and Mafisa wastewater treatment plant. Water samples collected using 2.5-liter amber bottles; pH adjusted to 3.0 by using Hydrochloric acid (HCL) (Carl-erbar) at site. Indicator strips were used to measure pH. Samples were transported to ecotoxicology laboratory in cool boxes within one hour. Samples were filtered twice. The first filtration was through a grade 5 filter paper from Munktell Company with particle retention size of 20µm. The second filtration through a grade 120H filter paper also from Munktell with particle retention size of 1-2µm. Samples were divided into 2x 800ml amber bottle. 800ml water samples was loaded into Oasis®HLB 6m³ 200mg (30µm) cartridges from Waters (Milford, MA, USA) using a vacuum pump and manifold. Manifold was a Vac Master from IST (Sweden) and pump was from ScanVac (Denmark). Drop rate was adjusted to 1.5ml/min. Prior to loading cartridges were preconditioned with 2ml MeOH followed by 2ml distilled water. After loading and running the solid phase extraction, cartridges were air-dried using a vacuum and stored at -18°C before analysis. The kits were for the analysis of these antibiotics' residues in eggs and meat. The modifications which were done were twice filtration of samples, the use MeOH and pH.

Prior to analysis, antibiotics were eluted from cartridges with 5ml formic acid in MeOH after washing with 2ml 5% MeOH in water. The eluent was evaporated to dryness under a gentle stream of nitrogen at 33^{o}c. Samples were reconstituted to 100µl (0.01% formic acid in MeOH) and 900µl water. Elution and evaporation were done in 12ml amber tubes. Samples were transferred to Eppendorf tubes, centrifuged and the supernatant were used for analysis.

Chemicals and Standards

Pure antibiotic salts of sulfonamides, tetracyclines and quinolones were purchased from Sigma-Aldrich (Augsburg, Germany) were used as positive controls. RIDASCREEN kits Ridascreen sulfonamides (R3004), Ridascreen tetracycline (R3505) and Ridascreen quinolones/chinolones (R3113) were purchased from R-Biopharm AG, (Darmstadt, Germany). Analytical grade methanol was purchased from Sigma-Aldrich (Augsburg, Germany) and HPLC (High performance Liquid chromatography) grade water was purchased from Sigma-Aldrich (Augsburg, Germany).

Enzyme-Linked Immunosorbent Assay Basis

Sulfonamides

The basis of the ELISA test was the antigen-antibody reaction. The microtiter wells were coated with capture antibodies directed against anti-sulfonamide antibodies. Standard or sample sulfonamide conjugate and anti-sulfonamide antibodies were added. Free sulfonamides and sulfonamide conjugate compete for the sulfonamide antibody binding sites (competitive enzyme immunoassay). At the same time, the anti-sulfonamide antibodies were also bound by the immobilized capture antibodies. Any unbound conjugate was removed in washing step. Substrate/chromogen was added to the wells followed by incubated. Bound conjugate converts the chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement was made photometrically at 450nm. The absorption was inversely proportional to the sulfonamide concentration in the sample.

Tetracyclines

The basis of the test was the antigen-antibody reaction. The microtiter wells were coated with tetracycline-protein-conjugate. Tetracycline standards or sample solutions and anti-tetracycline antibodies were added. Free tetracycline and immobilized tetracycline compete for tetracycline antibody binding sites (Competitive Enzyme immunoassay). Any unbound antibody was removed in washing step and enzyme labeled secondary antibody, directed against the anti-tetracycline antibody, was added. After removing unbound enzyme labeled antibodies by a washing step, substrate/chromogen was added to the wells followed by incubation. Bound conjugate converts the chromogen into a blue product. The addition of the stop solution leads to a color change from blue to a yellow. Measurement was made photometrically at 450nm.The absorption was inversely proportional to the tetracycline concentration in sample.

Figure 1. Mafisa Wastewater Treatment Plant Map

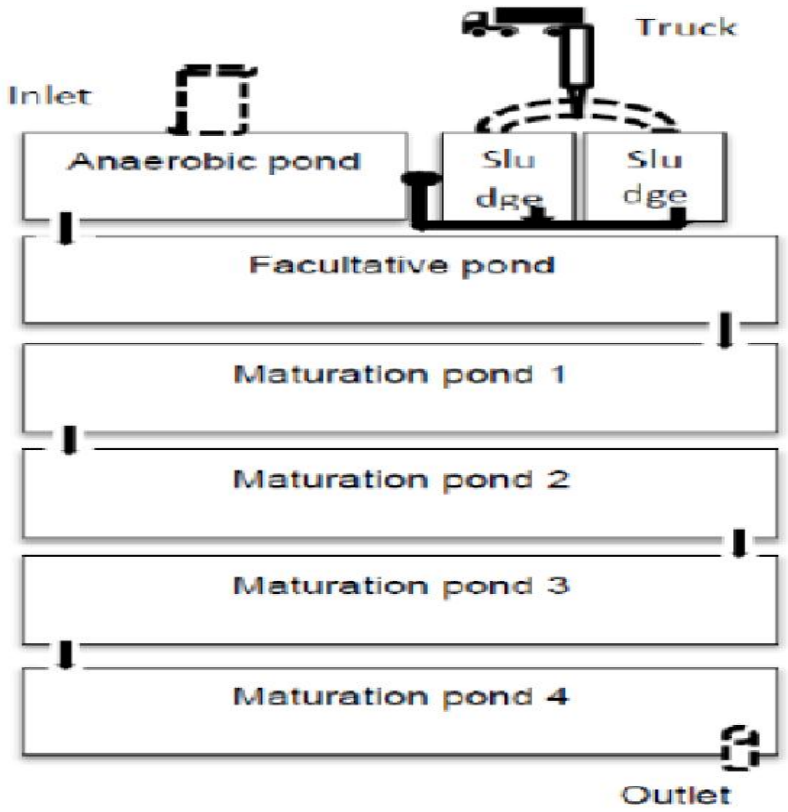

Figure 2. Mafisa wastewater sampling points, also showing the flow of water through the ponds

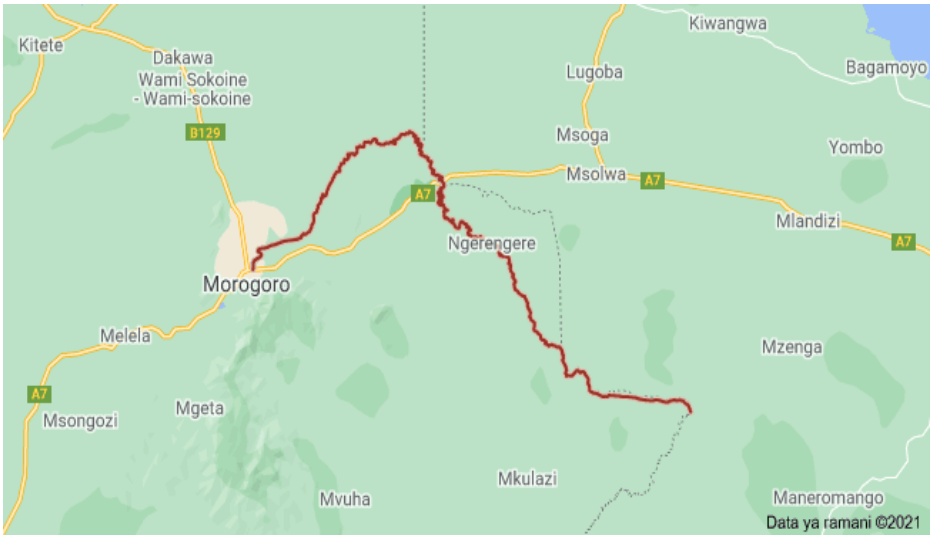

Figure 3. River Ngerengere map, red color showing river and sampling points

Quinolones

The basis of the test was antigen-antibody reaction. The wells were coated with a capture antibody directed against anti-quinolone antibodies. Standards or sample solutions of ciprofloxacin enzyme conjugate and anti-quinolones antibodies were added. Free quinolones and ciprofloxacin conjugate compete for the quinolone antibody binding sites. (Competitive enzyme immunoassay). At the same time the anti-quinolone antibodies were also bound by the immobilized capture antibodies. Any unbound conjugate was removed in washing step. Substrate/chromogen was added to the

wells followed by incubation. Bound conjugate converts the chromogen into a blue product. The addition of stop solution leads to a color change from blue to yellow. Measurement was done photometrically at 450nm; the absorption was inversely proportional to the quinolone's concentration in the sample.

Analytical Procedure

Standard solutions were used to plot standard curves for sulfonamides and quinolones included. They were diluted to final concentrations ready for use. They were 0,1,3,10,30 100µg/l and 0, 0.5, 1.5, 3, 6 and 18µg/l, respectively. Standard solutions for tetracyclines were provided as concentrates. In order to produce tetracycline standards ready for use, 50µl standard concentrate was diluted with 450µl sample buffer, all were included in kits. Each 50µl of the following standard concentrates 0, 0.5, 1.5, 3, 6, 18µg/l were diluted with 450µl sample buffer to make 0, 0.05, 0.15, 0.3, 0.6 and 1.8µg/l final concentrations. The r^2 for the standard curves were 0.994, 0.944 and 0.991 sulfonamide, quinolones and tetracycline, respectively.

Sulfonamides

Fifty microliters of each standard or prepared sample were added to microplate wells of ELISA plate in duplicate. Fifty microliters of conjugate were added to each well. Then fifty microliters of antibody were added to each well, incubated for one hour at room temperature. The solution in the wells was discarded and the microplate was tapped three times on blotting paper to ensure complete removal of solution from wells. The wells were filled with 250µl washing buffer. The liquid again was poured out and the wash step was repeated three times. One hundred microliters of substrate/chromogen were added to each well, incubated for 15minutes at room temperature in the dark. One hundred microliters of stop solution was added and incubated for 15 minutes. Absorbance was read at 450nm, thirty minutes after adding the stop solution.

Results were expressed in percentages of the maximum absorbance (B/B0%) using the following equation.

$$\frac{B}{B0}\% = (Absorbance\ standard\ (or\ sample)/Absorbace\ at\ zerostandard)\ X100$$

In order to obtain the sulfonamides concentrations in the sample, the B/B0% values were interpolated on the calibration curve built with sulfonamide standard solutions (0, 1, 3, 10, 10 and 100µg/l) with r^2= 0. 991 multiplied by dilution factor to obtain final concentration.

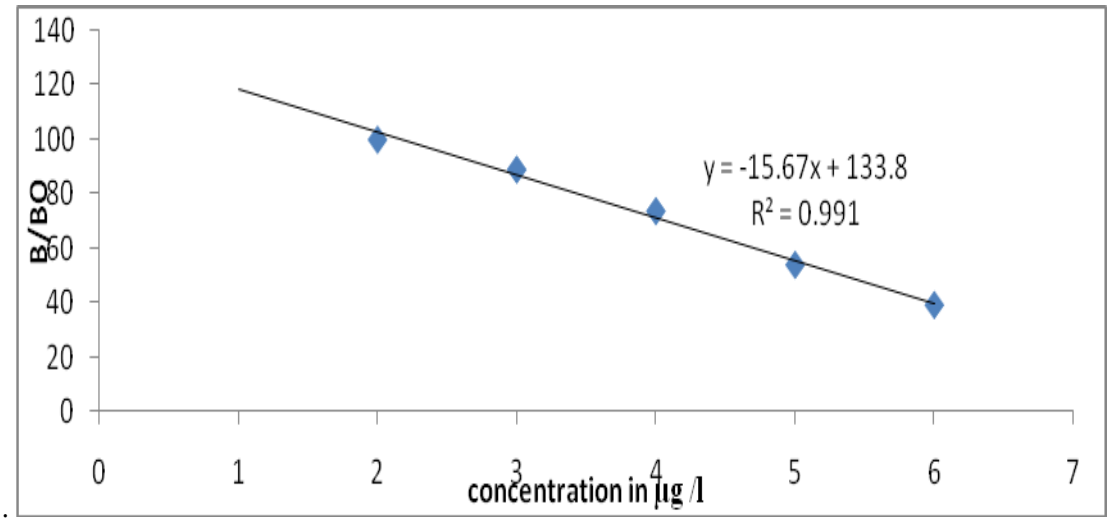

Figure 4. Standard curve for sulfonamide

Tetracyclines

In order to obtain tetracycline concentrations in the samples, the B/B0% values were interpolated on the calibration curve built with six tetracyclines standard solutions (0, 0.05, 0.3, 0.6 and 1.8µ/l) with r^2= 0.961. Multiplied by dilution factor to obtain the final concentration of tetracyclines.

Quinolones

In order to obtain the quinolones concentrations in the samples, the B/B0% values were interpolated on the calibration curve built with six quinolones standard solutions (0,0.5, 1.5,3,6,18µg/l) with r^2= 0.944. Multiplied by dilution factor to obtain the final concentration of quinolones.

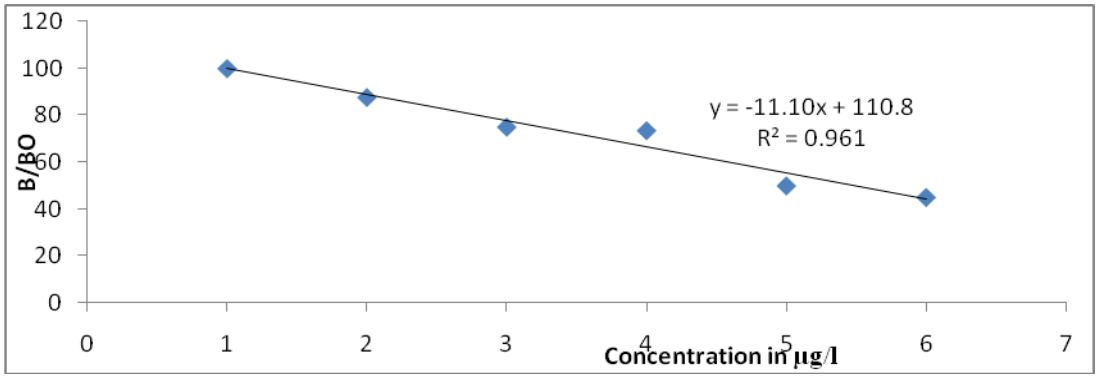

Figure 5. Standard curve for tetracycline

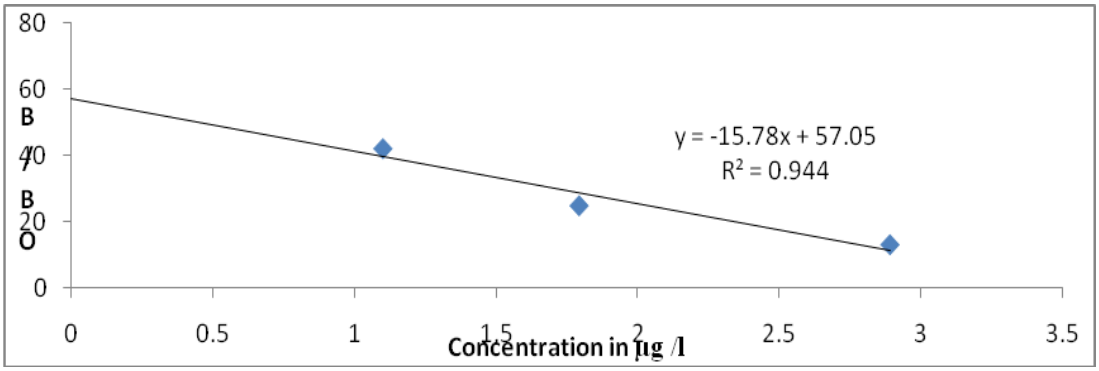

Figure 6. Standard curve for quinolone

RESULTS

Recovery and Limits of Detection

Recovery rates were 70-87%, 75-96% and 80-92% for sulfonamides, tetracyclines and quinolones, respectively, the rates were within the range stated in ELISA kits used. Limits of detection of the method ranged from 1.5µg/l, 1.2µg/l and 0.5µg/l sulfonamides, tetracyclines and quinolones, respectively. Mean concentration of quinolones in water samples from Mindu Dam was high compared to other antibiotics and the lowest mean concentration was that of tetracycline (Figure 8). At Mafisa Wastewater Treatment Plant, mean concentrations of all selected antibiotics were high compared to Mindu Dam and river waters (Figure 8, 9, 10, 11 and 12), but the mean concentration of tetracyclines was higher than quinolones and sulfonamides, although not statistically significant. Also, there was generally reduction of antibiotic concentrations from influent to effluent, although the reduction was not statistically significant (Figures 9, 10 and 11). In rivers, the mean concentrations of antibiotics were generally low at upstream and increased downstream where the human activities were also high, human activities like farming, washing of clothes and utensils (Figure 12).

(Cluster 1-Inlet pond,Anaerobic pond and Facultative pond, Cluster 2-Maturation ponds 4, 5 and 6, Cluster 3-Maturation ponds 7, 8 and exit point). There was slight decrease of antibiotics concentrations from influent (cluster 1) to effluent (cluster 3), although the decrease was not statistically significant.

(Custer 1- Inlet pond, Anaerobic pond and Facultative pond, Cluster 2- Maturation ponds 4, 5 and 6, Cluster 3- Maturation pond 7, 8 and exit point). There was decrease of tetracycline concentrations from influent(cluster 1) to effluent (cluster 3), the decrease was not statistically significant**.**

(Cluster 1- Inlet pond, Anaerobic pond and Facultative pond, Cluster 2- Maturation ponds 4,5 and 6, Cluster 3-Maturation ponds 7, 8 and exit point). There was a slight decrease of sulfonamides from influent (cluster 1) to effluent (cluster 3).

(Cluster one - upstream, Cluster two - middle and Cluster three - downstream of the rivers) (TET-Tetracycline, SULP-Sulfonamide and QUIN-Quinolones).

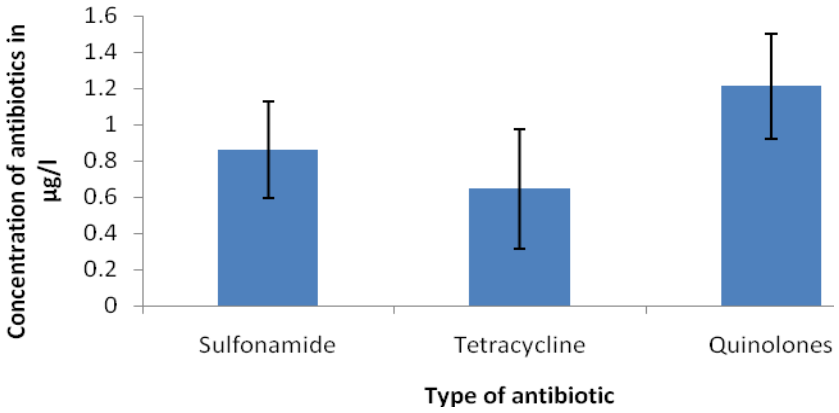

Figure 9: Mean concentrations of antibiotics at Mindu Dam

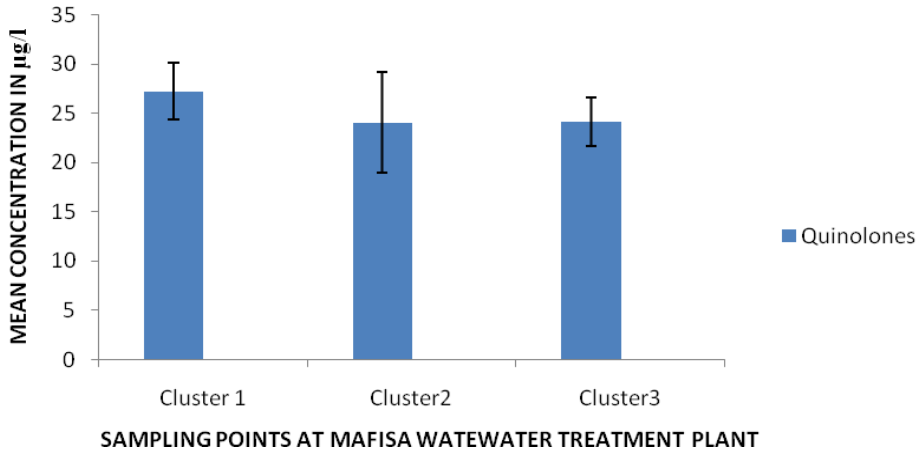

Figure 7: Mean concentrations of quinolones at sampling sites of Mafisa Wastewater Treatment Ponds.

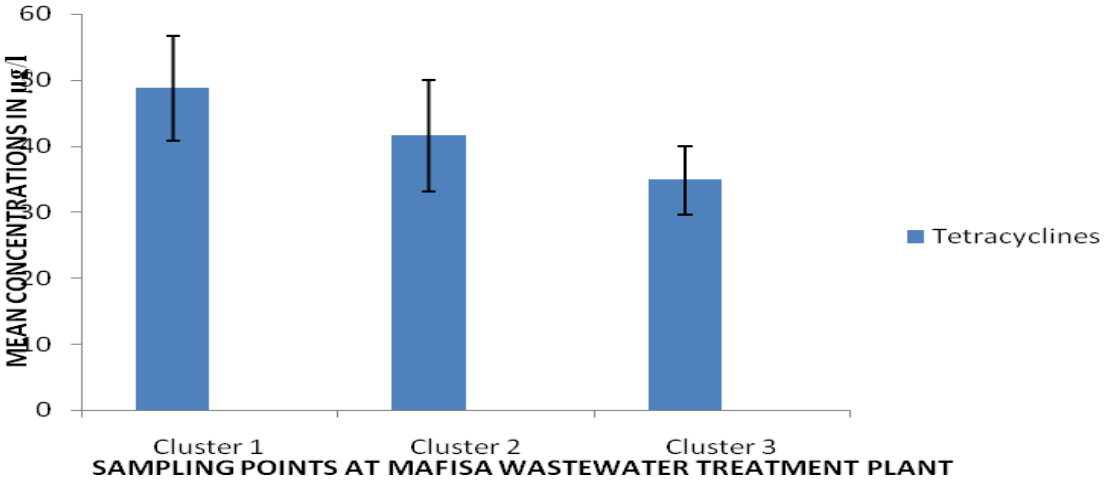

Figure 8: Mean concentrations of tetracyclines at sampling sites at Mafisa Wastewater Treatment Ponds.

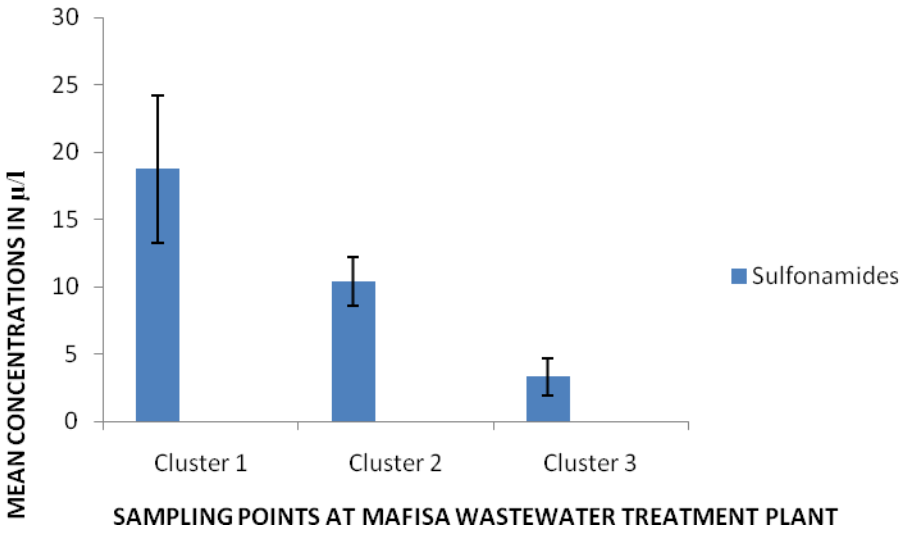

Figure 10: Mean concentrations of sulfonamides at Mafisa Wastewater Treatment ponds.

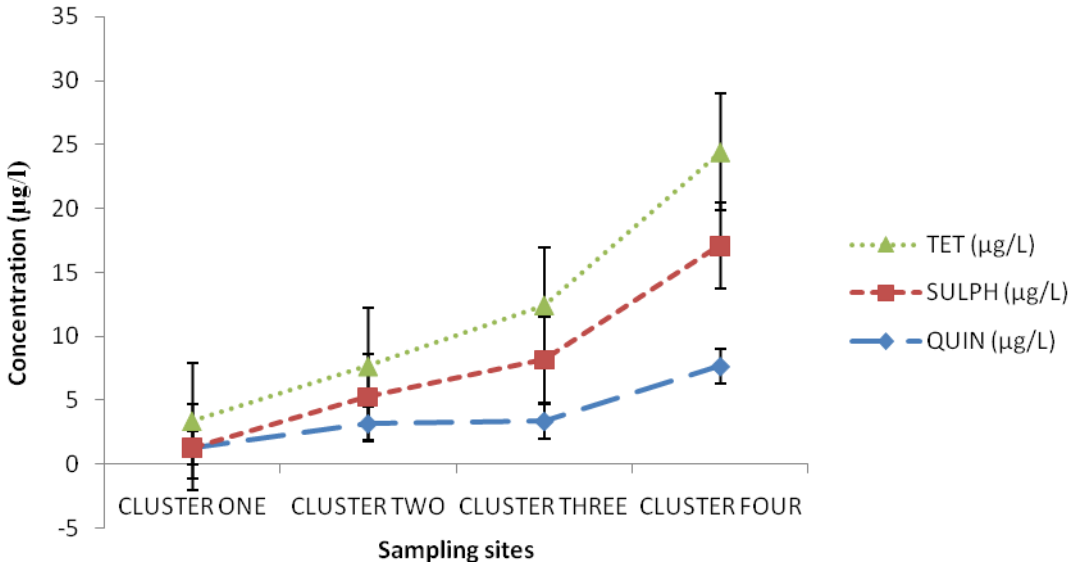

Figure 11: Mean concentrations of antibiotics in different clusters of rivers

DISCUSSION

There were detectable levels of selected antibiotics in rivers, Mindu Dam and Mafisa Wastewater Treatment plant although at low concentrations level. At Mafisa Wastewater Treatment Plant mean concentrations of antibiotics detected were high compared to rivers and Mindu Dam, the difference was statistically significant, $P \leq 0.05$, Figures 7, 8, 9, 10 11. This could be attributed to continuous increased rate of use of antibiotics in both humans and livestock without prescriptions and excreted antibiotics in urine and faeces finally reach wastewater treatment plant as a final place before released into natural environment. It could also be due to low efficiency of the wastewater treatment plant to degrade and remove the antibiotics. However, the removal efficiency of micropollutants including antibiotics in wastewater treatment plants depends on the type of treatment plant set up, hence not all micropollutants are removed (Kenda *et al.*, 2019). Areas close to increased human activities along the rivers showed increased mean concentrations of detectable antibiotics but it was not statistically significant, indicating the impact of anthropogenic source of contamination (Figure 11). At Mafisa

Wastewater Treatment Plant, there was reduction of antibiotics from influent to effluent, but the reduction was not statistically significant (Figures 7, 8 and 9), this indicates that removal efficiency of Mafia's wastewater is not 100 percent.

The detected levels of antibiotics in this study were in line with other studies done in different parts of the world although in some studies higher concentrations were reported. Concentrations of tetracycline in Northern China wastewater treatment plants as high as 32.0±6mg/l in effluent and 5,481.1±123.0mg/kg in dewatered sludge (Jie *et al*., 2015). Also concentrations of tetracycline in River Naerincheon in Korea were 2.30±46µg/l in June and 38.60±254.82µg/l in September, while the concentration of sulfonamide was 4.01±10.57µg/l (Yasser *et al*., 2014). Studies in United Kingdom rivers found that the concentrations of tetracycline, sulfonamide and quinolone were 32µg/l, 4.130µg/l and 0.405µg/l, respectively (Boxall *et al*., 2005). Investigation of pharmaceuticals in water and sediment of Msunduzi river in Kwazulu Natal, South Africa found that antipyretic ibuprofen was in highest concentrations of up to 117 µg/l, 84.60 µg/l and 659 ng/g in wastewater, surface water and sediment, respectively. The detection of antibiotics was low in concentrations of less than 10 µg/l in surface water samples and up to 34.5 µ/g in wastewater. Also, there was no complete removal during wastewater treatment. The percentage removal efficiency for antibiotics was only 48.80% (Solomon *et al.*, 2015).

Use of antibiotics in human and animals can cause selection for antibiotic resistant bacteria in feaces. Thus, manure or other organic material that contains human or animal wastes used as soil amendments, as practiced worldwide, have the potential to disseminate residues. The fate of these antimicrobial-resistant bacteria, ARGs and antimicrobial residues following application of soil amendments will vary with environmental conditions: For example, the selective properties of the antimicrobial residues can last for weeks to months, and possibly more than a single growing season in humid-temperate regions (Marti *et al.,* 2014; Chen *et al.,* 2018).

CONCLUSION

Environmental contamination by antibiotics has become an increasingly serious concern worldwide. In this study antibiotics have been detected in all sampled water bodies with much lower concentration in drinking water source example Mindu Dam, probably due to minimal human activities in and around the dam. Further studies are recommended, more sampling sites be included, use of high-performance liquid chromatography technique and sampling at different seasons also recommended. Best management practices should be adhered to with respect to the use of material of human (sewage sludge; biosolids) or animal origin (manures) in primary food production environments. Also, greater understanding of the persistence dynamics of antibiotic-resistant bacteria, antimicrobial residues and the potential for exchange of antibiotic resistance genes in human and animal wastes and wastewater, and how these factors vary with treatment, will allow for the more precise assessment of risks associated with environmental sources of antibiotic residues

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CONFLICT OF INTERESTS

I declare that there is no conflict of interest between this work and funding organization

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