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Research Article

Evaluation of the Ecotoxicological Effects of Selected Human Antibiotics; A Case Study of the Guppy Fish (*Poecilia reticulata*)

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Abstract

This study investigated the relative acute single action toxicity and mixture toxicity of four commonly used human antibiotics against *Poecilia reticulata* (Guppy) using laboratory bioassays. The sublethal toxic effects of the antibiotics were also assessed using biochemical endpoints. The antibiotics assessed in this study are amoxicillin, ampicillin, tetracycline and oxytetracycline. The assessment of mixture toxicity carried out with the binary mixtures of the antibiotics (1:1 v/v) showed potential mixture toxicity. The 96 hr LC_{50} values of Amoxicillin+Ampicillin and Tetracycline+Oxytetracycline were 236.129 mg/L and 33.286 mg/L respectively. The pattern of interaction of the antibiotics in binary mixtures assessed using the concentration addition model revealed a synergistic pattern of interaction. Results of the sublethal toxic effects using biochemical endpoints showed that there was a significant ($p<0.05$) decrease in antioxidant enzymes activities in group of fish exposed to the binary combinations of Amoxicillin+Ampicillin and Tetracycline+Oxytetracycline respectively, compared to control groups. A significant ($p<0.05$) increase in Malondialdehyde (MDA) activity was observed in group exposed to both binary mixture combinations compared to the control. Assessment of liver function enzymes revealed a significant increase ($p<0.05$) in enzymes activity in both groups compared to control. The results from this study has shown that the presence of these antibiotics in mixtures in aquatic environment can have potential toxicological effects on non-target aquatic organisms. Regulating the discharge of antibiotics into the environment is therefore necessary to protect aquatic life and ecosystems.

Keywords: Pharmaceuticals, Pollution control, Liver function, Oxidative stress, Synergistic

INTRODUCTION

According to United Nations Report, water pollution is widespread in many developing countries. Wastewater discharged into the environment often drains into nearby open drainage systems (Adeyemo OK, 2003). Much of this untreated water is produced by hospitals, industries, and other water consuming built sources (Altenburger R et al., 2004). Pollution of water bodies hence jeopardize the supply of clean drinking water, hence having negative impacts on marine and freshwater organisms (Ambili TR et al., 2013). Adeyemo et al., noted that the major contributors to aquatic pollution include wastewater, metals and metalloids, industrial effluents, contaminated sediments, nutrients, polycyclic aromatic hydrocarbons, flame retardants, persistent organic pollutants, and pharmaceuticals (Arnold KE et al., 2014). Pharmaceuticals such as antibiotics, painkillers, cardiovascular drugs, blood lipid regulators, and antidepressants, are frequently detected in surface and ground waters around the world (Azanu D et al., 2018). The presence of these active pharmaceutical products in natural environments is however problematic because of their capacity to induce a range of sublethal effects in exposed organisms (Begum G, 2004). Pharmaceuticals can disrupt fundamental behavioral processes, such as reproductive behavior, aggression,

boldness, activity levels, and feeding rates (Betrosian AP et al., 2009). Although pharmaceuticals are used for treatment or prevention of diseases in humans and animals as well as to maintain and improve the quality of daily life, they have been recently identified as a group of emerging environmental contaminants and have been detected in various environmental matrices such as freshwater, marine water, wastewater, soil sediment and fish (Bhagat C et al., 2020). These emerging contaminants are extensively and increasingly used in human and animal medicine, leading to their ubiquitous presence in the environment (Bojarski B et al., 2020).

However, because the majority of pharmaceutical products have low volatility, they disperse easily in water as aqueous solutions or suspensions. Some of these substances may also adhere to soil particles and enter the food chain (Brain RA et al., 2005). The presence of pharmaceuticals in surface waters, groundwater, and aquatic ecosystems have been confirmed, and they range from µg/L to ng/L levels but certain point sources, such as pharmaceutical production and manufacturing facilities, can result in concentrations as high as mg/l in receiving surface waters. As at 2010, Hernández et al., noted that over 4000 pharmaceuticals are in the market and released into the environmental water bodies through different mechanisms and there are no legal maximum allowable limits of these compounds in different environmental matrices. This becomes a threat to countries that lack resources for the proper monitoring and removal of these compounds. Adeyemo reported that freshwater resources in Nigeria are among the resources that are most impacted by environmental stress as they contain a variety of pharmaceuticals. Since the majority of these medications are made to act swiftly and then be expelled from the body without deteriorating, they frequently enter freshwater systems while still being active medications. Sub-lethal effects have been observed in aquatic animals at environmentally relevant quantities, despite the fact that recorded concentrations of these medications in surface waters are often far lower than established levels of toxicity. Most of them are pseudo-persistent; they are not persistent but their constant release into the environment makes them ubiquitous. Qiu, et al. noted that although the environmental concentrations of antibiotics may not be excessive, but the growth and development of aquatic organisms may be disturbed by long-term exposure to the mixtures of antibiotics in their natural habitats. These emerging pollutants, therefore, present a new global water quality challenge with potentially serious implications to human health and ecosystems. Toxicity tests have been performed on fish to evaluate the effect of toxicants under laboratory conditions and their occurrence of pharmaceuticals in their environment has been widely studied, most of which have provided increasing numbers of reports of their occurrence in portable water, river water, sea water and waste water. Fish are good bioindicator species that play important roles in monitoring water pollution because they respond with great sensitivity to changes in the aquatic environment. Guppy fish, *Poecilia reticulata* commonly called guppy, is now one of the most widespread ornamental fish species in the world. They are quite tolerant of a variety of water conditions and have been widely utilized as an experimental model by many researchers for toxicity studies since they are easy to handle, has low cost and does not require large spaces for maintenance and reproduction. Unfortunately, very little is known about the effects of the combinations of various antibiotics in fish, and only the effects of single exposure have been considered. As a matter of fact, only few studies have been carried out in Africa, particularly Nigeria, to investigate the single action of these emerging pollutants in aquatic environment. Evaluation of the acute and chronic ecotoxicity of the selected human antibiotics will therefore provide information about the type of interaction and potential effects that these antibiotics may pose on fish in aquatic environments.

MATERIALS AND METHODS

The experimental study was carried out at the Ecotoxicology unit laboratory of the Department of Zoology, University of Lagos, Nigeria with the coordinates 6°31'06.6"N 3°24'00.0"E (Figure 1).

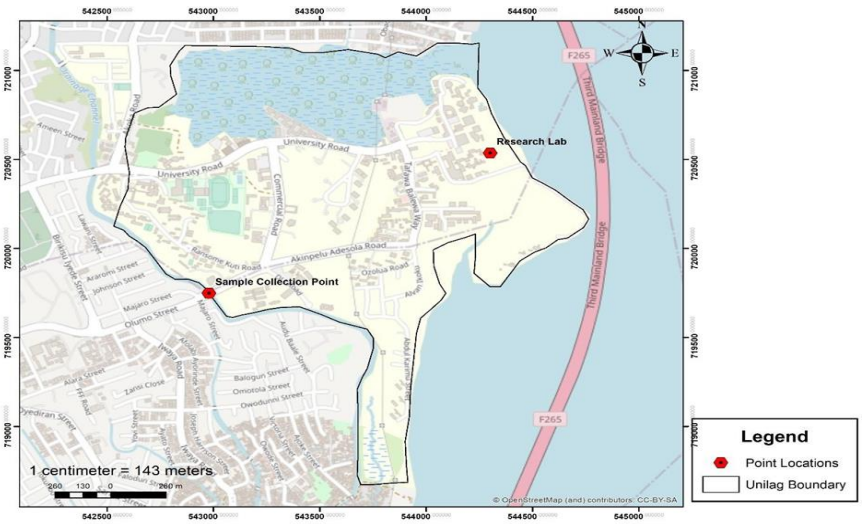


Figure 1. Unilag map showing research lab and sample collection point.

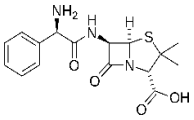
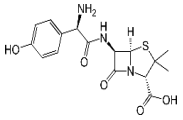
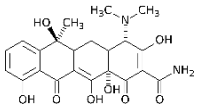
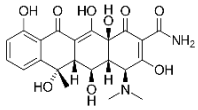
Apparatus

Apparatus used included stock tanks, syringes (10, 20, 50 and 100 ml), micropipette (Eppendorf), pH meter (HI 9813-6N), dissolve oxygen meter (HI 9142), holding tanks (28 x 51 x 29 cm), chemically inert glass tanks (8 x 14 x 9 cm) and measuring cylinder (1000 ml).

Test compound

Two classes of antibiotics were used for this study: Penicillins (Amoxillin (AMX) - Amoxil 500 and Ampicillin-(AMP) - Ampicillin Capsules BP 250 mg) and Tetracyclines (Tetracycline- (TET) - Sumycin and Oxytetracycline (OXY)-Terramycin) (Table 1).

Table 1. Major characteristics and occurrence of the selected antibiotics in aquatic environment.

	Ampicillin	Amoxicillin	Tetracycline	Oxytetracycline
Molecular formula				
CAS-no	69-53-4	26787-78-0	60-54-8	79-57-2
Therapeutic class	Penicillin	Penicillin (β-lactam)	Tetracycline	Tetracycline
Molecular Formula	$C_{16}H_{19}N_3O_4S$	$C_{16}H_{19}N_3O_5S$	$C_{22}H_{24}N_2O_8$	$C_{22}H_{24}N_2O_9$
Molecular weight (g/mol)	349.4	365.4	444.4	460.4
Solubility in H_2O	Slightly soluble	Very slightly soluble	Very slightly soluble	Very slightly soluble
Pharmakocinetical excretion rate in %	≈ 65	80-90	80-90	>80
BCFe (estimated)	-	3	-	0.12
Occurrence in surface water in Nigeria (µg/L)	Not detected	272.2 µg/L	0.05	0.01 µg/L
Maximum Environmental Concentration (MEC) in surface water (µg/L)	13.7 µg/L -India	272.2 µg/L-Nigeria	25.5377 µg/L -China	361.1 µg/L -China
Maximum Environmental Concentration (MEC) in waste water	70600 µg/L- Egypt	6.940 µg/L-India	1.42 µg/L -China	6.44 µg/L-China
Active ingredient	Ampicillin Trihydrate BP 250 mg	Amoxicillin Trihydrate 500 mg	Tetracycline Hydrochloride BP 250 mg	Oxytetracycline Hydrochloride BP 250 mg
Brand name	Vitacillin 250	Amoxil 500 mg	Tetravin Capsules	Cympashn
Manufacturer	Sagar Vitaceuticals Nig Ltd.	Glaxo Smith Kline	Yandzhou Norier Pharmaceutical Co. Ltd.	Jiangzi Xier Kangtal Pharmaceutical Co. Ltd

Test organism

Poecilia reticulata (Guppy) (wet weight: 0.11 ± 0.04 g; length: 2.36 ± 0.08 cm; n=300) were used for this study. Guppy is a widely used for model for ecotoxicity testing since it is easy to handle, has low cost and does not require large spaces for maintenance and reproduction. Juveniles of guppy fish were obtained from a freshwater river situated at the second gate of the University of Lagos, Lagos Nigeria. They were collected and transported early in the morning (7:30 am) to the ecotoxicology unit laboratory, University of Lagos, in plastic tank (28 x 51 x 29 cm). They were stocked in a holding tank (28 x 51 x 29 cm) that was three-quarter filled with dechlorinated water (test medium).

The test organisms were acclimatized for fourteen (14) days. During the period of acclimatization, test organism was fed with coppens fish feed containing 0.7-1.3% fresh weight protein. They were fed daily (evening) until 48 hours before exposure according to OECD. Water was changed every 48h to reduce the accumulation of metabolic waste. Feeding was discontinued 24 h before commencement of definitive experiment according to Mustapha and Bawa-Allah. During the acclimatization period, no mortalities were recorded.

Test media

Dechlorinated tap water was used as dilution water in this study and was aerated prior to exposure to allow the concentration of dissolved oxygen reach saturation according to OECD. Physicochemical parameters including Dissolved Oxygen (DO), pH, and Temperature were measured using a pH meter (Hanna HI 9813-6) and Dissolved oxygen meter (H I9142). The mean physicochemical parameters recorded were 7.1 ± 0.2 mg/L, 24.9 ± 0.6°C and 6.9 ± 0.05 for Dissolved Oxygen (DO), Temperature, and pH respectively. The physicochemical parameters were within the recommended range prescribed by OECD except for temperature which was slightly above the range (21-25). However, the test organisms did not show signs of stress using this test media with the observed physicochemical parameters.

Preparation of test media

Stock solution used in this study were prepared by taking calculated amount of the selected antibiotics and made up to the required volume of water to achieve 1 g/L stock solution. Computed volume of each stock solution were pipetted out using a micro pipette and made up to 1L to serve as exposure medium for the juvenile fish (*Poecilia reticulata*).

General bioassay techniques

Limit test: A 4-day (96 hr) limit test in a static condition was performed during this study to assess the potential acute toxicity of the selected antibiotics. According to OECD toxicants with a LC_{50}>100 mg/l are considered harmless and have no acute toxicity potential. There were no replicates for the limit test as established by OECD guidelines.

For the single action limit test, test organisms (n=7) were exposed to 100 mg/L of the selected antibiotics (AMX, AMP, TET, OXY respectively) and untreated control.

For the joint action limit test, test organisms (n=7) were exposed to 200 mg/L of antibiotic binary mixtures representing equal ratio (1:1) combinations. The antibiotics were combined as binary mixtures as follows: AMX+AMP, TET+OXY, AMX+OXY, TET+AMP. A negative control was also included in the test series.

Visible abnormalities and sub-lethal clinical signs were observed and recorded. Mortality was assessed once every 24 hrs. for 4 days (96 hrs). The physicochemical parameters of the test solution (Dissolved Oxygen-DO, pH, and temperature) were recorded before and after exposure. The recommended maximum loading of 0.8 g/l wet weight of test organism was also achieved according to OECD.

Acute toxicity studies: Based on results from limit tests described above, definitive acute toxicity tests were carried out for binary mixtures of antibiotics that have acute toxicity potential against the test species (*P. reticulata*). The definitive test was carried out according to OECD 203 guideline for fish acute toxicity testing. Definitive acute toxicity tests were carried out for binary mixtures of AMX+AMP and TET+OXY. The antibiotics were combined in ratio 1:1 (v/v). Test organisms were randomly exposed in two replicates of eight organisms each (n=16) to 9 test concentrations (1, 2, 5, 10, 20, 40, 80, 160, 320 mg/L) and untreated control. Mortality was assessed once every 24hrs for 4 days (96 hrs). The physicochemical parameters of the test solution (Dissolved Oxygen-DO, pH, and temperature) were recorded before and after exposure. For the acute test, the recommended maximum loading of 0.8 g/l wet weight of test organism was achieved according to OECD 203 guideline.

$$ECx_{mix}=\left(\sum_i^n \frac{pi}{ECx_i}\right)^{-1}$$

Assessment of quantal response (Mortality): Test organisms were confirmed dead when there was no observable movement when gently prodded with a glass rod. Mortality was monitored in all media at a 24 hours interval over each exposure period. The number of dead organisms were recorded and their carcass removed immediately to avoid contamination of test medium due to decomposition. The test was considered invalid if more than 10% of exposure organisms in the control containers died.

Assessment of pattern of join interaction of binary mixtures toxicity: Pattern of toxic interaction of the antibiotics in mixture toxicity studies were assessed using the Concentration Addition (CA) model. The model assumes that similarly acting toxicants mixed in any proportion, will add together to give the observed response.

In evaluating the pattern of toxic interaction, the predicted ECx_{mix} values derived from the equation above are compared to the observed ECx_{mix} values of the mixture to obtain the Relative Toxicity Unit (RTU) as described by Anderson and Weber. The RTU is used to describe the pattern of interaction between the mixtures.

Relative Toxicity Unit (RTU) is estimated as:

$$RTU=\text{Predict } ECx_{mix}/\text{Experimentally derived } ECx_{mix}$$

Where;

RTU=1 indicates an additive action

RTU<1 indicates antagonism and

RTU>1 indicates synergism.

Ecological risk assessment: To assess ecological risk to aquatic ecosystems, the Risk Quotient (RQ) for the antibiotics and mixtures were calculated as

$$PQ=MEC/PNEC$$

Where,

MEC=Highest measured environmental concentration in surface waters and wastewaters sourced from literature

PNEC=The predicted no effect concentration which was calculated by dividing the 96 hr LC_{50} values by a safety factor of 1000.

The RQ was categorized as described by Verlicchi et al., and Rodriguez-Mozaz et al:

RQ ≤ 0.1: Low environmental risk

0.1<RQ ≤ 1: Moderate environmental risk

RQ>1: High environmental risk

Sublethal toxicity studies: The semi-static renewal bioassay was employed for the sublethal toxicity studies according to OECD 203 guideline. Test organisms (n=8) were randomly selected from the stock tank and exposed to two (2) sub-lethal concentrations (10% 96 h LC_{50} values derived from acute toxicity studies, one environmentally relevant concentration 200 µg/L) and untreated control for 28 days. Test organisms were exposed to binary mixtures of AMX+AMP and TET+OXY respectively. During the sublethal test, test organisms were fed with same feed used during acclimatization.

A semi-static bioassay test procedure was adopted in which the test media was changed once every 2 days to fresh media of the same concentration and untreated control

according to Mustapha and Bawa-Allah. After 28 days, two live organisms per exposure medium and untreated control were randomly selected and placed in transparent sterile specimen tubes (in triplicates A, B, C). The organisms were killed by freezing and stored at 4°C to avoid bio-degradation before they were transported to the laboratory for further analysis.

Biochemical analysis of liver function enzymes (Aspartate Transaminase-AST, Alkaline Phosphatase-ALP, Alanine Aminotransferase-ALT) and antioxidant enzymes (Glutathione-GSH, Glutathione S-Transferases-GST, Superoxide Dismutases-SOD, Catalase-CAT, Malondialdehyde- MDA) were employed as endpoints of the sublethal toxicity studies.

Homogenization of samples: Homogenization of the samples was done with 0.1 M phosphate buffer (pH 7.2) in a glass mortar; containing acid washed sand and blended to form a homogenate. The resulting homogenate was centrifuged at a speed of 2500 rmp for 15 mins and stored at 20°C until analysis.

Liver function enzymes activities: The method of Wright et al., was employed for this assay to determine the Alkaline Phosphatase (ALP) activity. The principle of Wrights method is predicated on the fact that P-Nitrophenyl Phosphate (PNPP) will become hydrolysed to p-nitrophenol and phosphoric acid at a pH of 10.1 by alkaline phosphatase. The p-nitrophenol will then confer a yellowish color on the reaction mixture and its intensity can be monitored by a spectrophotometer at 405 nm which in turn provides a measure of the enzyme activity. Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) activities was determined based on the principle described by Reitman and Frankel.

Antioxidant enzymes activities: Superoxide Dismutase activity was determined based on its ability to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 480 nm as described by Sun and Zigma. The reaction mixture (3 ml) contained 2.95 ml 0.05 M sodium carbonate buffer pH 10.2, 0.02 ml of liver homogenate and 0.03 ml of epinephrine in 0.005 N HCL was used to initiate the reaction. The reference cuvette contained 2.95 ml buffer, 0.03 ml of substrate (epinephrine) and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 min.

Catalase activity was determined in consonance with the methodology of Sinha, et al. It was assayed colorimetrically at 620 nm and expressed as µmoles of H_2O_2 consumed/min/mg protein at 25°C. The reaction mixture (1.5 ml) contained 1.0ml of 0.01M phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 2M H_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

Glutathione-S-transferase activity was determined in consonance with the method described by Habig et al. This principle is predicated on the fact that all known glutathione-S-transferase demonstrate a relatively high activity with 1-Chloro-2,4-Dintrobenzene (CDNB) as the second substrate. Consequently, the conventional assay for glutathione-S-transferase activity utilizes 1-Chloro-2,4-dintrobenzene as substrate. When this substrate is conjugated with reduced Glutathione (GSH), its absorption maximum shifts to a longer wavelength. The absorption increase at the new wavelength of 340 nm which provides a direct measurement of the enzymatic reaction.

Substrate of oxidative stress: Malondialdehyde (MDA) which is an index of lipid peroxidation was determined using the method described by Buege and Aust. To achieve this, 1.0 ml of the supernatant was added to 2 ml of equal ratio (1:1:1) of TCA-TBA-HCl reagent (thiobarbituric acid 0.37%, 0.24N HCl and 15% TCA) (Tricarboxylic acid-Thiobarbituric acid-Hydrochloric acid reagent) and boiled at 100°C for 15 min, which was then allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 min. The supernatant was removed and the absorbance read at 532 nm against a blank.

Statistical analysis: The 96 hr LC_{50} value of acute toxicity was determined by Probit regression analysis including regression line equation as described by Finney using statistical package for the social sciences 26.0 for windows (SPSS 26.0). Data obtained for sub-lethal toxicity tests were subjected to one-way analysis of variance test to test for significance between the treatment means.

The same statistical package (SPSS 26.0) was used to compare the mean of results obtained from biochemical analysis using the one-way Analysis of Variance (ANOVA). The Duncan test was used to detect the source of difference when a significant difference ($p<0.05$) was obtained.

RESULTS

Definitive acute toxicity tests

The selected antibiotics were combined in binary mixtures in equitoxic ratios. Results showed that the binary mixture of TET and OXY were found to be more toxic to *P. reticulata* when compared with the binary mixtures of AMX+AMP with 96 hr LC_{50} values of 33.286 mg/L and 236.129 mg/L respectively (Table 2). The probit transformed response showed a straight line graph which indicated that the mortality was concentration-dependent for the selected antibiotics (Figures 2 and 3).

Table 2. Mixture toxicity of selected antibiotics against *Poecilia reticulata* (Guppy).

Antibiotics	LC_{50} 95% C.L (mg/l)	Slope ± S.E	D.F	Probit line equation	TF
AMX+AMP (1:1)	236.129 (92.439-1806.267)	0.683 ± 0.161	7	Y=-1.4+0.55x	7.09
TET+OXY (1:1)	33.286 (13.448-108.608)	1.183 ± 0.179	7	Y=-1.36+0.64x	1
Note: TF: Toxicity Factor; DF: Degree of Freedom; SE: Standard Error; EC: Effective Concentration; CL: Confidence Limits; AMP: Ampicillin; AMX: Amoxicillin; TET: Tetracycline, OXY: Oxytetracycline					

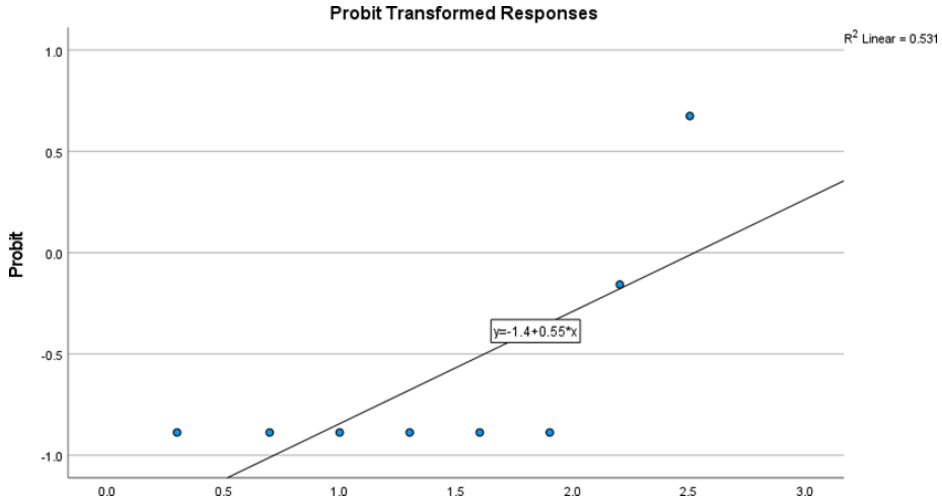


Figure 2. Probit log-dose relationship of joint acute toxicity (AMX+AMP) against *Poecilia reticulata* (Guppy).

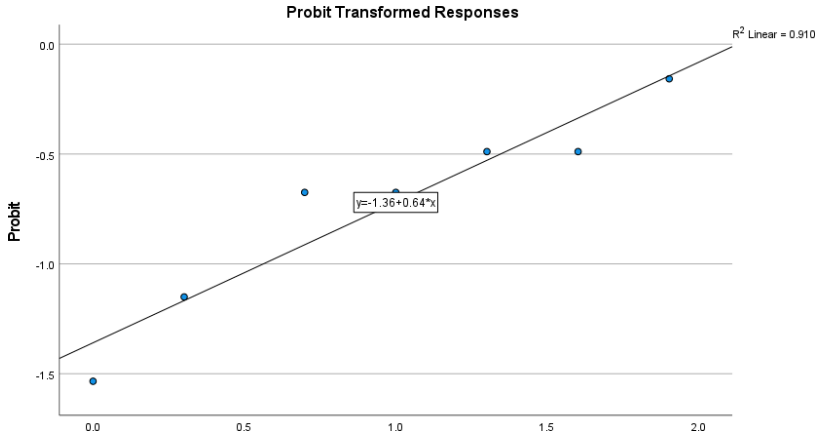


Figure 3. Probit log-dose relationship of joint acute toxicity (TET+OXY) against *Poecilia reticulata* (Guppy).

Pattern of toxic interaction of binary mixtures of antibiotics

The Concentration Addition (CA) model was employed to calculate the predicted LC_{50} of the selected binary mixtures and the RTU in order to determine the pattern of toxic interaction among the selected antibiotics. The values for the predicted 96 h LC_{50} for the binary mixture of AMX+AMP was calculated as 472.258 mg/l and the values observed during the study (experimentally derived) was 236.129 mg/l. The RTU was calculated as 2.0. The values for the predicted 96 h LC_{50} for the binary mixture of TET+OXY was calculated as 66.572 mg/l and the values observed during the study (experimentally derived) was 33.286 mg/l. The RTU was also calculated as 2.0 (Table 3).

Table 3. Pattern of toxic interaction of the binary mixtures of selected antibiotics.

Antibiotics	Ratio	Predicted LC_{50} (mg/l) values	Experimentally derived LC_{50} (mg/l) values	RTU	Pattern of interaction
AMX+AMP	1:1	472.258	236.129	2 (>1)	Synergistic
TET+OXY	1:1	66.572	33.286	2 (>1)	Synergistic
Note: RTU: Relative Toxicity Unit					

Ecological risk assessment of selected antibiotics

To determine the ecological risk of the selected antibiotics to *P. reticulata* (Guppy), the Risk Quotient (RQ) was calculated. RQ was calculated based on the maximum environmental concentrations derived from published literatures and the calculated PNECs. The RQ of AMX+AMP in surface water (0.056) was <0.1 The RQ of AMX+AMP (299.005) in wastewater was >1. The RQ of TET+OXY in surface water was >1 while that of waste water was greater than 0.1 but <1 (Table 4).

Table 4. Risk Quotient (RQs) of selected of selected antibiotics in surface waters and wastewaters.

Antibiotics	LC_{50} (µg/l)	PNEC (µg/l)	MEC surface water (µg/l)	MEC wastewater (µg/l)	RQ surface	RQ wastewater
AMX+AMP	236129	236.129	13.313	70603.8	0.056	299.005
TET+OXY	33286	33.286	81.63	30.566	2.452	0.918
Note: PNEC: Predicted No Effect Concentration; MEC: Maximum Environmental Concentration; RQ: Risk Quotient						

Oxidative stress enzymes and substrate of oxidative stress

The biomarkers of oxidative stress (SOD, CAT, and GST) as well as the levels of GSH and MDA (substrate of oxidative stress) were assessed in fish exposed to sublethal concentrations of the selected antibiotics (AMX+AMP-200 µg/l, 23.6 mg/l and control; TET+OXY-200 µg/l, 3.3 mg/l and control). The result for AMX+AMP showed that the activities of the enzymes exposed to these concentrations were all significantly ($p<0.05$) lower than that of the control with the exception of MDA which showed a higher significant value (6.37 ± 2.61) than that of control (3.81 ± 1.51). Similar activities of these enzymes were recorded for TET+OXY ($p<0.05$) with the exception of MDA which showed a higher significant value (7.93 ± 1.64) than that of control (5.64 ± 0.41) (Tables 5 and 6).

Table 5. Activities of antioxidant enzymes, non-enzymatic antioxidant and substrate of oxidative stress ((U/mg protein) in fish exposed to sublethal concentrations of selected antibiotics (AMX+AMP).

AMX+AMP (mg/l)	SOD (M ± SD)	CAT (M ± SD)	GST (M ± SD)	GSH (M ± SD)	MDA (M ± SD)
0	24.28 ±10.34	34.40 ± 4.89^{a}	170.41 ± 57.76	133.08 ± 45.86	3.81 ± 1.51
0.2	15.65 ± 4.31	30.23 ± 3.28^{b}	131.71 ± 25.50	115.04 ± 27.95	6.37 ± 2.61
23.6	14.64 ± 2.74	20.21 ± 2.15^{c}	143.86 ± 27.42	8.29 ± 67.74	5.91 ± 1.13
Note: M: Mean; SD: Standard Deviation; SOD: Superoxide Dismutase; CAT: Catalase; GST: Glutathione-S-Transferase; GSH: Glutathione; MDA: Malondialdehyde. Values in columns with superscripts of the same or no alphabet do not differ based on Duncan's multiple range test.					

Table 6. Activities of antioxidant enzymes, non-enzymatic antioxidant and substrate of oxidative stress (U/mg protein) in fish exposed to sublethal concentrations of selected antibiotics (TET+OXY).

TET+OXY (mg/l)	SOD (M ± SD)	CAT (M ± SD)	GST (M ± SD)	GSH (M ± SD)	MDA (M ± SD)
0	24.92 ± 11.13	23.68 ± 2.84^{a}	256.35 ± 114.41	176.67 ± 24.50	5.64 ± 0.41
0.2	14.91 ± 3.22	24.07 ± 4.01^{b}	122.97 ± 41.95	47.11 ± 9.29	7.93 ± 1.64
23.6	8.20 ± 1.80	22.78 ± 2.12^{c}	73.70 ± 12.70	47.32 ± 9.84	5.03 ± 0.78
Note: M: Mean; SD: Standard Deviation; SOD: Superoxide Dismutase; CAT: Catalase; GST: Glutathione-S-Transferase; GSH: Glutathione; MDA: Malondialdehyde. Values in columns with superscripts of the same alphabet do not differ based on Duncan's multiple range test.					

Liver function enzyme activity of *P. reticulata* (guppy) exposed to selected antibiotics

The activities of liver enzymes (ALP, AST, and ALT) in fish exposed to sublethal concentrations of the selected antibiotics AMX+AMP revealed that they were significantly higher ($p<0.05$) compared to that of the control with the exception of ALT which showed a higher value in the control (6.03 ± 1.32) than the activities of the enzymes exposed to the other concentrations (2.67 ± 1.44 and 3.88 ± 2.34 respectively). Similar observations were recorded for TET+OXY with the exception of ALT, which showed that fishes exposed to the other concentrations were significantly lower (2.67 ± 1.44, 3.88 ± 2.34) at ($p<0.05$) Tables 7 and 8.

Table 7. Activities of liver function enzymes (U/mg protein) in fish exposed to sublethal concentrations of selected antibiotics (AMX+AMP).

AMX+AMP (mg/l)	ALP (M ± SD)	AST (M ± SD)	ALT (M ± SD)
0	35.53 ± 7.38[a]	0.57 ± 0.29	6.03 ± 1.32[c]
0.2	42.63 ± 24.75[b]	0.97 ± 0.40	2.67 ± 1.44[a]
23.6	179.33 ± 86.70[c]	0.77 ± 0.35	3.88 ± 2.34[b]
Note: M: Mean; SD: Standard Deviation; ALP: Alkaline Phosphatase; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase. Values in columns with superscripts of the same or no alphabet do not differ based on Duncan's multiple range test.			

Table 8. Activities of liver function enzymes (U/mg protein) in fish exposed to sublethal concentrations of selected antibiotics (TET+OXY).

TET+OXY (mg/l)	ALP (M ± SD)	AST (M ± SD)	ALT (M ± SD)
0	64.63 ± 31.74	0.73 ± 0.25	4.70 ± 3.81[c]
0.2	54.97 ± 21.96	1.30 ± 0.89	1.83 ± 1.07[a]
23.6	108.90 ± 63.69	2.33 ± 0.30	2.63 ± 1.99[b]
Note: M: Mean; SD: Standard Deviation; ALP: Alkaline Phosphatase; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase. Values in columns with superscripts of the same or no alphabet do not differ based on Duncan's multiple range test			

DISCUSSION

Limit toxicity tests carried out in this study established that all the 4 selected antibiotics are not acutely toxic to *P. reticulata* when acting singly. Ecotoxicological studies agree that these compounds (pharmaceuticals) generally do not cause acute toxicity to aquatic organisms when acting singly, as their environmental concentrations are typically too low to cause any environmental risk, although potential mixture effects is considered of special importance. Results from the present study agrees with the study of Isidori et al., where they observed that 6 types of antibiotics (Oxytetracycline, Erythromycin, Sulfamethoxazole, Ofloxacin, Lincomycin and Clarithromycin) had no toxic effect on Zebrafish (*Danio rerio*) up to 1000 mg/L suggesting the limited acute ecotoxicity of the antibiotics. In addition, Zhang et al., also observed 96-hr LC_{50} values of 322.8 mg/l, 406.0 mg/l and 759.71- Zhang et al., for tetracycline acting singly (500 mg/L) in zebrafish embryos suggesting low acute toxicity.

Kemper noted that penicillin's and tetracycline's are not usually expected to be found in aquatic environment due to the poor stability of the beta-lactam-ring of penicillin's and the strong adsorption of tetracycline's to organic matter. However, if present in the aquatic environment, it has the potential to exhibit its toxic effect to more sensitive aquatic organism like microalgae, diatoms and crustaceans especially when acting in combination with other antibiotics.

Behavioural responses such as loss of schooling, abnormal surface behaviour, abnormal bottom behaviour, hypoactivity and corkscrew swimming were observed in the test organism upon exposure to the selected antibiotics. Similar observations in this study like hyperactivity, erratic swimming, respiratory difficulty, abnormal surface motion behaviour, slow movement and little or no reaction to stimulus were recorded in the study of Li et al., when they exposed a verapamil to juvenile rainbow trout, Oncorhynchus mykiss. The possible reason for the fish behavioural alterations may be attributed to xenobiotically-induced inhibition of acetylcholinesterase activity leading to accumulation of acetylcholine in cholinergic synapses ending up with hyperstimulation.

The result of the acute toxicity tests indicated that binary mixture of tetracycline and oxytetracycline (broad spectrum antibiotic) was more toxic compared with the binary mixture of Amoxicillin and Ampicillin (β-lactam antibiotics) against *P. reticulata*. Given the lack of toxicity data for the joint action of these mixtures against *P. reticulata*, a few studies that have employed more sensitive aquatic organisms like microalgae, *D. magna* and zebrafish embryos have been highlighted. Eguchi et al., revealed that beta-lactam antibiotics- ampicillin and amoxicillin were less toxic (>1000 mg/L) to *S. capricornutum* and *C. vulgaris*. Also, Zhang et al., showed that fluoroquinolone had an inhibiting effect on the toxicity of Tetracycline when they exposed *D. magna* to a mixture of tetracycline and fluoroquinolone (48 hr LC_{50}-75 mg/L) suggesting a higher toxic effect potential of tetracycline.

The pattern of toxic interaction of the binary mixtures of selected antibiotics showed a synergistic relationship for both AMX+AMP and TET+OXY. Most times, synergistic effects come from the binary mixtures of the similarly acting substances. Magdaleno et al., showed that all binary mixtures of ampicillin, amoxicillin, cephalothin ciprofloxacin, gentamycin, and vancomycin exerted synergistic effect to algae growth. Similarly, Omoya and Ajayi observed a synergistic effect between a range of antibiotics such as Tetracycline+Amoxicillin, Tetracycline+Augmentin, Ciprofloxacin+Amoxicillin, Ofloxacin+Amoxicillin among others. In addition, the joint toxicity of spiramycin and amoxicillin to *M. aeruginosa* was found to be a synergistic interaction as well as the binary antibiotic interactions in the study of Gonzalez-Pleiter et al.

The RQ calculated for the binary mixture of AMX+AMP in surface waters showed a low environmental risk to the fish compared to the RQ in wastewater which revealed a high environmental risk. In contrast, RQ calculated for the binary

mixture of TET+OXY showed a high and moderate environmental risk for surface water and wastewater respectively. This suggest that the joint action of AMX+AMP in wastewater can pose a high level of threat to sensitive aquatic organism compared to its presence in surface water. However, the joint action of TET+OXY will pose a higher threat to organism in surface water. The risk of antibiotics usually increases when antibiotics are present in binary mixtures. Brain et al., reported that tetracycline has been regarded as one of the most threatening antibiotic groups in the ecosystem. In a subsequent study, Brain et al., reported that the mixture of four tetracyclines posed a high environmental risk to selected aquatic macrophytes. Gregorio and Chevre, pointed out that the risk levels associated with the mixed antibiotics greatly exceed the critical aquatic thresholds, and therefore, represent a major challenge in term of risk managements.

In this present study, the activities of antioxidant enzymes in fish exposed to all concentrations for the binary mixture of AMX+AMP and TET+OXY were all lower than that of the control. Zhang et al., noted that oxidative stress will occur if the activities of the antioxidant defence systems decreases or there is an increase in production of ROS (Reactive Oxygen Species). Excessive ROS production induces oxidative damage when fish are exposed to toxic substances. The inhibition of these activities may be due to excess free radical generation as a result of the toxicants which in turn reduced the ability of the enzymes in the organism to protect against oxidative damage. Different studies have also shown that chronic exposure of fish to low, environmentally realistic concentrations of antibiotics may result in physiological disturbances such as haematological alterations, oxidative stress, histopathological lesions, immunosuppression, metabolic disorders, genotoxic damage, general stress response, and reproductive impairment.

The levels of MDA which a product of lipid peroxidation, increased significantly ($p<0.05$) in fish exposed to the binary mixtures of the selected antibiotics. MDA is normally used as a lipid peroxidation marker. Lipid peroxidation occurs when free radicals or ROS oxidatively degrades lipids in cell membranes thereby causing cellular damage. The high levels of MDA in the exposed fish can be attributed to an increase in the generation of free radicals and a reduction in oxidative stress enzyme activities in the fish resulting from the prolonged exposure of the fish to the antibiotics.

Generally, the activities of liver enzymes (with the exception of ALT) in fish exposed to sublethal concentrations of the selected antibiotics were significantly higher ($p<0.05$) compared to the control. The increased activities of phosphatases and aminotransferases in the fish could be a response to an increased energy demand. Increased ALP and AST activities in fish may also indicate cumulative toxicity or a possible mechanism of the fish to meet the increased energy demand under sustained and prolonged toxic stress. The observed decrease in ALT activities in the fish indicates that the detoxification mechanisms may not be sufficiently effective to prevent the action of the antibiotics on the system or that damaged cells may not be able to synthesize AST protein.

CONCLUSION

Results from this study highlights the potential environmental risk posed by antibiotic mixtures, specifically tetracycline and oxytetracycline, to sensitive aquatic organisms. This can lead to alterations in the ecosystem structure of aquatic environments. The study shows that the co- presence of different antibiotics in aquatic environments can lead to synergistic toxicity, indicating an increased potential for ecological harm. This study hence establishes the necessity for in-depth examination of the impacts of antibiotic combinations (binary, tertiary, etc.) and a more comprehensive evaluation of the ecological risk posed by other antibiotics, even at extremely low levels. The implementation of antibiotic pollution control policies and technologies, as well as proper disposal of pharmaceuticals, is necessary especially in developing countries in order to safeguard non-target aquatic organisms hence protecting ecosystem structure.

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