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The use of *Bacillus subtilis* bacteria as a tool to assess the toxicity of pharmaceuticals in the environment

Williams Kwari Joshua[1*] and Yaduma Gaiuson Wandiahyel[2]

[1]Faculty of Pure and Applied Sciences, Federal University Wukari, Taraba State, Nigeria. E-mail: kwariwills@gmail.com
[2]Department of Chemistry, Adamawa State College of Education, Hong, Adamawa State, Nigeria. E-mail: wandiayaduma@gmail.com

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Abstract

Pharmaceuticals and personal care products are now known as micro-contaminants due to their effects on bacterial resistance and effect on non-targeted organisms. Most recently, these substances have been found in surface water, sewage, hospital and care home wastewaters and landfill. Pharmaceuticals have been known to pose acute and chronic effects especially when exposed at higher concentrations and for longer durations. This study adopted the spectrophotometric method to assess the acute and chronic effects of seven pharmaceuticals on *Bacillus subtilis* bacteria. The effects were observed in terms of the conversion of 3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; (MTS) to a colored formazan product. The effect of pharmaceuticals was measured as a reduction of tetrazolium solution and expressed as percentage. The results indicate that both acute and chronic effects exist with Bezafibrate, Diclofenac, Diatrizoate, Ibuprofen and Atenolol inhibiting bacterial growth at 50 µg/ml. Consequently, at 500 µg/ml, all the pharmaceuticals inhibited growth thereby posing acute effects. In addition, all the eight pharmaceutical substances tested inhibited bacterial growth at 50 µg/ml and 500 µg/ml when exposed to pharmaceuticals for more than 24 h. The implication is that wastewater from hospitals can likely inhibit biological process of breaking waste in the wastewater treatment plants.

Keywords: *Pharmaceuticals, Toxicity, Wastewater, Bacteria, Bacillus subtilis*

1. Introduction

In the past, the composition of substances that contaminate the environment were centered around heavy metals and emissions from factories as a result of industrialization (Cioca and Munteanu, 2019; Veras *et al.*, 2019; White *et al.*, 2019). However, in recent times, the attention of contaminants is gradually changing from the previously known contaminants to micro contaminants (Cabral *et al.*, 2019; Fick *et al.*, 2009). These micro contaminants include organic chemicals, pesticides, pharmaceuticals and plastics have been found in the environment in several samples at different places and concentrations (Kümmerer *et al.*, 2019; Pal *et al.*, 2010). These toxic substances have been known to enter the environment via hospitals, care homes, production industries, household waste and wastewater (Bonnefille *et al.*, 2018; Hu *et al.*, 2018; Verlicchi *et al.*, 2010). Although their toxic effects have widely been studied in recent years, they continue to persist in the environment at concentrations that can likely cause severe impacts.

* *Corresponding author: Williams Kwari Joshua, Faculty of Pure and Applied Sciences, Federal University Wukari, Taraba State, Nigeria. E-mail: kwariwills@gmail.com*

Pharmaceuticals and personal care products have emerged as one of the micro contaminants due to their persistent presence and adverse effect on human and environmental health (Behera *et al*., 2011). Pharmaceuticals are now increasingly used globally both in humans and animals to treat and prevent diseases. This therefore has increased the production, use and consumption of pharmaceuticals and consequently their input in the environment (Verlicchi *et al*., 2010). Several studies have shown wastewaters from hospitals, veterinary clinics, households and even pharmaceutical manufacturing facilities containing pharmaceutical active compounds (Behera *et al*., 2011). This eventually reaches the Wastewater Treatment Plants (WWTPs) and since the removal of pharmaceuticals from the conventional wastewater treatment has been reported to be inefficient (Agunbiade and Moodley 2014; Casas *et al*., 2015; Santos *et al*., 2013; Santos *et al*., 2010), effluents from WWTPs will likely contain pharmaceuticals compounds which can potentially affect the environment. Onsite wastewater treatment or pre-treatment to remove pharmaceuticals must be incorporated in hospitals, veterinary clinics and every source of pharmaceuticals input to the environment before discharging effluents to WWTPs for further treatment.

These useful compounds yet harmful can be toxic to the environment especially when found in concentrations that have the potential to target and adversely affect humans and animals. Pharmaceuticals are complex molecules with different physiochemical and biological properties, which makes them ideal to be used for the specific activity they perform in the body (Jelic *et al*., 2011; Lagesson *et al*., 2019; Pereira *et al*., 2015). There have been over 3,000 various active pharmaceutical substances with more than 200 compounds already identified as contaminants (Deblonde *et al*., 2011; Mackulak *et al*., 2019). These substances identified include antibiotics, anti-inflammatory, anti-epileptics, anti-diabetics, antihistamines, cardiovascular drugs, hormones and nonsteroidal substances (Binh *et al*., 2018; Fekadu *et al*., 2019; Lagesson *et al*., 2019; Mackulak *et al*., 2017; Terechovs *et al*., 2019). These substances when in contact with other chemical substances in the environment have the potential to cause synergistic acute and chronic toxic effects (Cui *et al*., 2019; Frédéric and Yves, 2014; Kümmerer *et al*., 2019). In addition to acute and chronic effects of pharmaceuticals, their effect on non-targeted organisms and pathogen and bacterial resistance is of great concern especially to water and environmental scholars (Taylor and Senac 2014; Wu *et al*., 2016; Zhu *et al*., 2013).

Understanding the impacts of pharmaceuticals especially on the biological activity in the WWTPs is vital in ensuring a limit to these harmful substances from effluents are strictly adhered. To understand this vital impact, this study aims to use bacteria as a tool to assess the toxicity of single pharmaceuticals substances both acute and chronic.

2. Material and methods

The experiment adopted the spectrophotometric method as described by Capka *et al*. (2015); El-Didamony and Ali (2013a); El-Didamony and Ali (2013b) and El-Didamony *et al*. (2015). The procedure is based on exposure of bacteria (*Bacillus subtilis)* to individual pharmaceutical substances at a concentration of 50 µg/ml and 500 µg/ml and computing the activity (%) by measuring the spectrophotometric absorbance at 490 nm and 660 nm. The apparatus and reagents used include: the Iso-sentitest broth, *Bacillus subtilis* (BGA) spore suspension, Cell Titre 96® Aqueous One Solution cell proliferation Assay, methanol, distilled water, spectrophotometer, test tubes, 10 ml and 100 ml volumetric flask, beakers, pipettes and cuvettes.

2.1. Pharmaceutical stock preparation

The pharmaceuticals substances assessed were sulfamethoxazole, lidocaine, erythromycin, bezafibrate, diclofenac, ibuprofen and atenolol. First off, 0.1 g of each of the pharmaceuticals was prepared separately by suspending it in 10 ml methanol solution. Thereafter, 8 ml of the drug-methanol solution was transferred into separate capped containers for storage. One ml of the stock solution was mixed with 99 ml of distilled water to provide a nominal concentration of 100 µg/ml. A second concentration of the pharmaceuticals was prepared using 1 ml of methanol stock solution and mixed with 9 ml of distilled water to provide a nominal concentration of 1000 µg/ml. In addition, 23.4 g of Iso-sentitest broth was taken and mixed to 1 l of distilled water before being autoclaved alongside cuvettes, test tips and other apparatus at 121^0 for 15 min (sterilization).

2.2. Acute toxicity test of pharmaceuticals at 50 µg/ml and 500 µg/ml concentration

Using a sterile test tip, 1 ml of *Bacillus subtilis* spores was added to the sterile broth and shaken for even distribution. 1 ml of each pharmaceutical substance was measured into 3 uvettes and to another set of 3 cuvettes, 1 ml of methanol-water solution (1 ml water and 99 ml distilled water) was measured. To each

cuvette, 1 ml of inoculated broth was added and incubated at 28^0 for 24 h. 20 μl of cell titer was added to the samples and incubated at 28^0 for 4 h in the dark before placing in the spectrophotometer to read the absorbance at a wavelength of 490 nm and 660 nm, respectively. Toxicity was tested at 50 μg/ml and 500 μg/ml and the activity (%) was computed using the expression:

$$Activity(\%) = \left\{ \frac{(Ab_{490}Pharm - Ab_{660}Pharm) - (Ab_{490}Meth - Ab_{660}Meth)}{(Ab_{490}Meth - Ab_{660}Meth)} \right\} \times 100$$

where

$Ab_{490}Pharm$ and $Ab_{660}Pharm$ are both the absorbance of pharmaceuticals at 490 nm and 660 nm respectively,

And $Ab_{490}Meth$ and $Ab_{660}Meth$ are absorbance of methanol at 490 nm and 660 nm, respectively.

2.3. Chronic toxicity test of pharmaceuticals at 50 µg/ml and 500 µg/ml concentration

To a set of 9 cuvettes, 1 ml of each pharmaceutical was added. To another set of 9 cuvettes, methanol-water solution was added and finally to another set of 9 cuvettes, water was added. To each of the cuvettes, 1 ml of diluted broth solution was added and incubated at 28^0 for 24 h. 3 cuvettes from each of the 9 cuvettes was taken out and 20 μl of cell titer was added and reincubated at 28^0 for 4 h in the dark before reading the absorbance at 490 nm and 660 nm using the spectrophotometer. Same procedure was repeated after 48 h and after 72 h and the reading computed and tabulated. The procedure was repeated with a concentration of 500 μg/ml.

3. Statistical analysis

The data obtained was analyzed descriptively using excel package 2010 version to plot the graphical representation of the data and to infer acute and chronic effects of pharmaceuticals on bacteria. The activity was computed, and the result tabulated with results greater than the water solution signifying growth stimulation while results lower showing growth inhibition.

4. Results and discussion

Acute (24 h) and chronic (72 h) effects were observed at concentrations of 50 μg/ml and 500 μg/ml, respectively. The effects were observed in terms of the conversion of 3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; (MTS) to a colored formazan product. The activities were computed, tabulated and discussed as follows:

4.1. Acute testing of pharmaceuticals at 50 µg/ml and 500 µg/ml

The methanol-water solution was used as a baseline against which the stimulatory or inhibitory effects of pharmaceuticals were measured. Therefore, any activity higher than the water solution signifies a stimulation of growth whereas, activities lower indicates growth inhibition. The results indicate both stimulatory and inhibitory effects (Figure 1) of pharmaceuticals at 50 μg/ml with Bezafibrate, Diclofenac, Diatrizoate, and

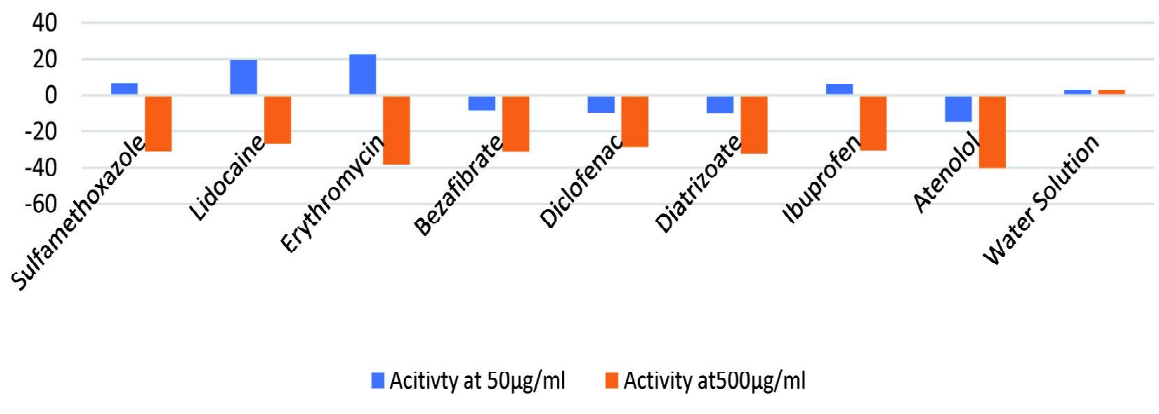

Figure 1: Acute growth stimulation and inhibition of *Bacillus subtilis* at 50 µg/ml and 500 µg/ml

Atenolol showing acute effects at both concentrations. Studies have found these compounds and many others to be present in surface waters and pose serious concerns to aquatic organisms (Deblonde *et al.*, 2011; Wu *et al.*, 2016). Consequently, with a 10 times dosage (500 µg/ml), all the pharmaceuticals showed growth inhibition implying acute effects (Figure 1) of compounds on bacteria.

4.2. Chronic testing of pharmaceuticals at 50 µg/ml & 500 µg/ml

Chronic effects were analyzed by exposing the bacteria to pharmaceutical compounds for 72 h. The activities were recorded after 24 h, 48 h and 72 h, respectively. The study results found out that at 24 h exposure, the bacterial growth was stimulated by Sulfamethoxazole, Lidocaine, Erythromycin and Ibuprofen (Figure 2). However, Bezafibrate, Diclofenac, Diatrizoate, and Atenolol inhibited bacterial growth. Consequently, all pharmaceuticals inhibited growth activities after 48 h and 72 h except for Lidocaine which still stimulated growth at 48 h but when further exposed to 72 h, growth was inhibited. This indicates effects when pharmaceuticals are exposed for longer period.

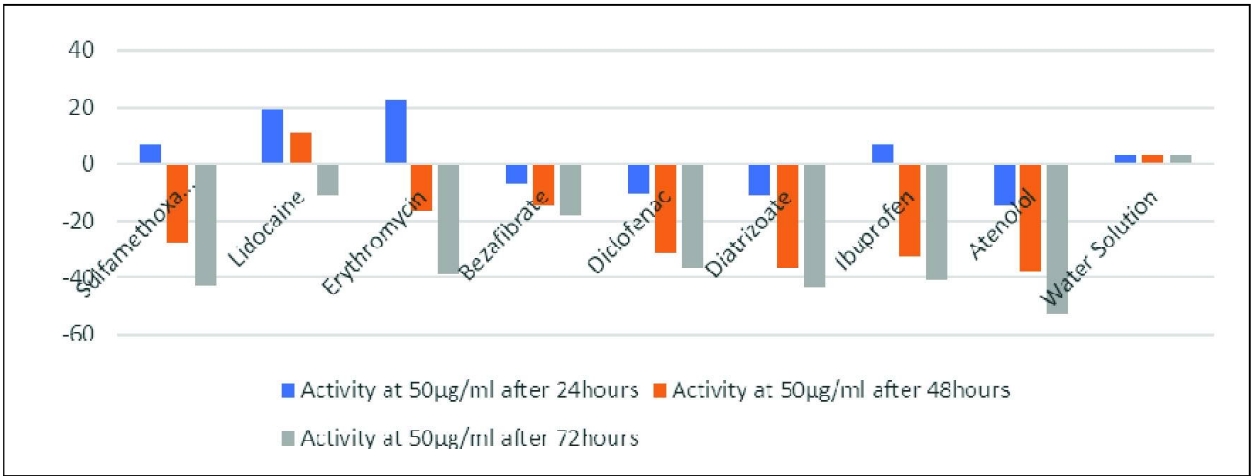

Figure 2: Chronic growth stimulation and inhibition of *Bacillus subtilis* at 50 µg/ml

In addition, at 500 µg/ml, all pharmaceutical substances inhibited the growth of *Bacillus subtilis*, indicating impact at higher dosages even after 24 h exposure (Figure 3). The indication of a chronic effect implies that wastewater from hospitals, veterinary clinics and other sources of pharmaceuticals in the environment can severely affect aquatic and other terrestrial organism when exposed at intolerable concentrations.

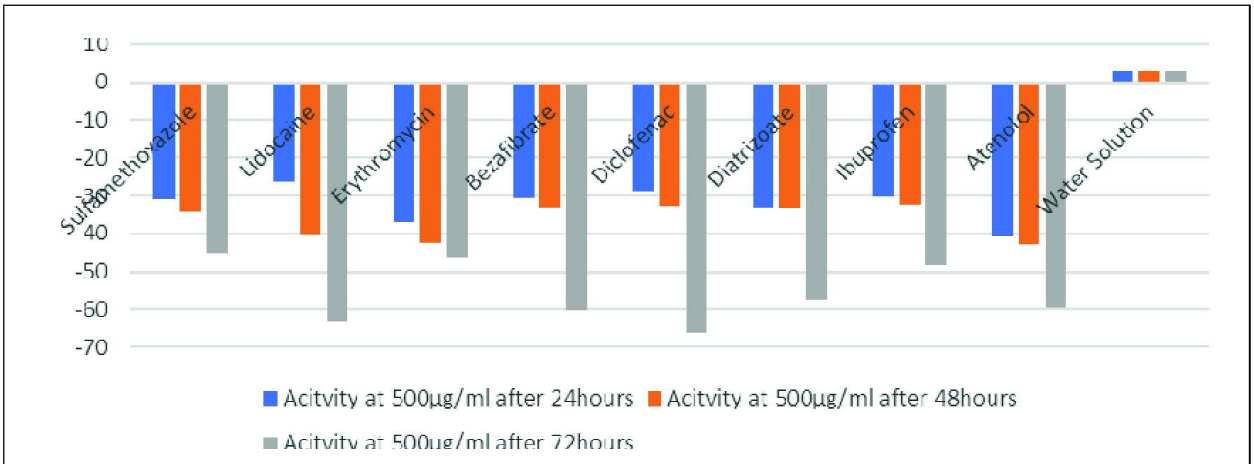

Figure 3: Chronic growth stimulation and inhibition of *Bacillus subtilis* at 500 µg/ml

4.3. Ecotoxicology of pharmaceutical substances

Pharmaceuticals have been detected and their effects in the environment widely investigated. For some of these pharmaceuticals, their effects on aquatic organisms have been studied in acute toxicity assays. However, their chronic toxicity is only marginally known and is becoming an area of interest to scientist. Only little is widely

known about the long-term effects of pharmaceuticals and other personal care product to aquatic organisms, with respect to biological targets (Fent *et al*., 2006). From the literature assessed, acute effects to aquatic organisms are unlikely, however, reports from Nambirajan *et al*. (2018); Oaks *et al*. (2004); Prakash *et al*. (2012), indicated an unusually high death rate (>95%) among three species of vultures (*Gyps bengalensis*, *Gyps indicus* and *Gyps tenuirostris*) in India and Pakistan: believed to be caused by diclofenac, a widely used analgesic and anti-inflammatory drug. Thus, pharmaceutical toxicity is off great concern to scholars and scientist especially in developing countries where restrictions on the use, disposal and purchase of drugs are not effectively monitored.

Long-term exposure of drugs in the environment can subtly give rise to bacterial resistance and severe effects on non-targeted organisms. The study results indicate a likelihood of severe long-term effects on bacterial activities and since the WWTPs sometimes rely on biological treatment to breakdown waste, hospital wastewater containing drugs will likely inhibit the efficiency of the organisms used in biological treatment of waste. In addition, pharmaceuticals from hospital waste water rarely exist as single compounds but rather come in a mixture with other drugs, creating a likelihood of a more toxic substance (Fent *et al*., 2006; Frédéric and Yves, 2014). Mixture effects of pharmaceuticals have shown to be dangerous and pose both acute and long-term impact on aquatic life, leading to high mortality in fish and other aquatic organisms.

Pharmaceutical substances have been designed to be biologically active and therefore may have effects on non-target organisms even at trace concentrations, therefore, continuous consumption of drugs even at sub-therapeutic concentrations may likely lead to potential threat to public health. Studies show that many non-target organisms (which possess human- and animal-alike metabolic pathways, similar receptors or biomolecules) are therefore inadvertently exposed to active substances released into the environment (Fent *et al*., 2006). Thus, the need for monitoring of pharmaceuticals in the environment.

The study results infer that both acute and chronic effects exist, and that any or both effects can likely occur depending on the concentration of pharmaceuticals. Since removal of pharmaceuticals from conventional treatment is unlikely in some cases (Cui *et al*., 2019; Frédéric and Yves 2014), pre-treatment of hospital and care home wastes including drug companies must be ensured to reduce drug load in WWTPs. In addition, developing countries with little or no restrictions on drug use and disposals must enact regulations that will seek to reduce the input of drugs in the environment.

5. Conclusion

Pharmaceuticals have emerged to become contaminants with a likelihood of devastating consequences for both humans and animals. Effects of pharmaceuticals on non-targeted organism and bacterial and pathogen resistance have shown to pose a severe risk to both humans and animals. The study found out that both acute and chronic effects on bacteria exist and therefore imply that wastewater from hospitals and other sources of pharmaceuticals in the environment can potentially inhibit the biological process of degrading waste in most conventional wastewater treatment. Drugs can likely pose severe risk as single compounds but their mixture effects in the environment will most likely pose a greater risk to both aquatic organisms, humans and other terrestrial animals. There is therefore the need to incorporate a pre-treatment of hospital waste before discharging to the wastewater treatment facility for further treatment. In addition, government and scientist must ensure a strict compliance to the use and disposal of drugs and unwanted/expired drugs. There should also be a constant monitoring, assessment and evaluation of pharmaceuticals and other micro-contaminants in the environment.

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