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Comparative Study of the Ecotoxicological and Histopathological Impacts of Effluent, Sludge Water, and Commonly Used Inorganic Fertilizers on Juvenile *Oreochromis niloticus* (Linnaeus, 1758)

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Wastewater treatment systems, designed to treat domestic wastes, produce effluents and sludge that are high in organic matter and nutrient content. These effluents and sludge are now being used as organic fertilizers because such nutrients and organic matter are vital to plant growth. However, without proper treatment, these substances may eventually find their way into bodies of water through run off and/or infiltration with potentially dangerous consequences. This study, therefore, investigated the potential toxic effects of effluents and sludge produced from wastewater treatment facilities against commonly used inorganic fertilizers to an aquatic species. Toxicity tests (expressed as mean 96-hr LC_{50} in mg/L) and histopathological examinations of the liver were conducted using juvenile *Oreochromis niloticus* (Linnaeus, 1758) exposed to varying concentrations of effluent, sludge, and inorganic fertilizers (*i.e.*, urea and complete fertilizer) to assess both acute and sublethal effects. The results of the acute toxicity tests show concentrations (expressed as mean 96-hr LC_{50} in mg/L or ppm) arranged in decreasing order of toxicity to tilapia: complete fertilizer 14-14-14 (1,396 ppm) > urea (16,152 ppm) > sludge (145,900 ppm) > effluent (465,000 ppm). Histopathological examinations of liver tissues showed that exposure to the two inorganic fertilizers resulted to blood congestion and degeneration in comparison to those exposed to the sludge. Furthermore, results for fishes exposed to the lowest concentrations of the effluent also showed alterations in the liver tissue. These results demonstrate that the sludge and effluent are less toxic by several orders of magnitude than the inorganic fertilizers. It is suggested that further chronic toxicity and histopathological studies be done to determine their long-term impacts to receiving aquatic organisms to establish their potential for agricultural applications.

Keywords: ecotoxicology, effluent, histopathology, inorganic fertilizers, *Oreochromis niloticus* L., sludge water

INTRODUCTION

Waste management continues to be one of the environmental challenges that many countries face today. Wastes from households, industries, and agricultural runoff are discharged into bodies of water resulting in a significant level of pollution that cause potential risks to human health and the environment (World Bank 2003). In response, domestic wastewater treatment systems (DWWTS) have been devised to lessen the impact of domestic wastewater on the environment while at the same time allowing for the byproducts, effluent, and sludge to be utilized by humans (Boer and Blaga 2016).

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Studies have shown that effluents and sludge can provide essential nutrients for plant growth and improve soil characteristics and, thus, may act as cost-efficient alternatives to organic fertilizers (Mohapatra *et al*. 2016, Toze 2006). In Cyprus, for example, it was reported that there was a subsequent increase in the yield of crops irrigated with the wastewater particularly because of the presence of nitrogen and phosphorus (Al-Nakshabandi *et al*. 1997). Other countries also utilize biosolids for agricultural use such as Japan and India (Kumazawa 1997, Saha *et al.* 2017). However, biosolids are known to contain significant amounts of heavy metals such as Cu, Cr, Ni, Zn, Cd, and Hg that can run off to surface water – hence potentially harming aquatic organisms and, ultimately, human health (Marguí *et al*. 2015).

Although organic fertilizers are widely used in the Philippines, inorganic fertilizers such as urea and complete fertilizers are still more commonly used in many farms in the country (FPA 2015). However, there have been very few studies that investigated the safety of both inorganic fertilizers, as well as that of organic fertilizers such as sludge and effluents for agricultural applications and their eventual impact on aquatic environments as agricultural runoffs. It is essential then that the potential impacts of sludge and effluent, when used as alternatives to inorganic fertilizers, be assessed by comparing the toxicity of these byproducts with those of commonly used inorganic fertilizers to verify whether these are truly sustainable and environmentally safe.

This study aimed to determine and compare the acute and sublethal effects of wastewater treatment effluent, sludge water, urea, and complete fertilizer on juvenile *O. niloticus* through toxicological and histopathological tests to determine their impact on aquatic ecosystems.

METHODS

Ninety-six (96) -hour acute toxicity tests and histopathological examinations of the liver tissues of juvenile tilapia exposed to various toxicants were conducted. The procedures are described as follows.

Acquisition, Handling, and Acclimation of Test Organism

Juvenile tilapia, 2–3 cm long and less than 3 g in weight, were acquired from a local hatchery. The organisms were placed inside polyethylene bags filled with oxygenated water and transported to the laboratory. Upon arrival, the bags were submerged for one hour in a 200-L acclimation tank containing filtered and dechlorinated water to allow for transitional adaptation before releasing the test organisms. The fish were fed daily throughout the five-day acclimation period, except for the first 24 hours and provided with standardized controlled aeration supply in an air-conditioned laboratory to maintain constant temperature. Water quality parameters such as dissolved oxygen and temperature were monitored daily using the Ohaus Dissolved Oxygen Starter 300D (SN B229127874) and Horiba Water Checker U-10 (SN 308006). Water was replaced up to 70% of the volume every other day to ensure optimal conditions (Espiritu *et al.* 2011).

Toxicants Tested

Five toxicants were used in this study – wastewater treatment effluent; wastewater treatment sludge water; urea [$CO(NH_2)_2$, Ramgo brand]; complete fertilizer (*i.e.*, 14% nitrogen, 14% phosphorous, and 14% potassium content, Ramgo brand); and copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$, HIMEDIA brand, reagent grade) as the reference toxicant. Both effluents and sludge samples were obtained from a domestic wastewater treatment facility and transported to the laboratory following US EPA guidelines (EPA 2004). Specifically, the effluent samples were obtained from the holding tank prior to chlorination and discharge to a receiving body of water while the liquid sludge samples were drawn from the drums used to store sludge water drippings from the facility's dewatering unit. Copper sulfate pentahydrate, urea, and complete fertilizer were obtained from local commercial chemical suppliers.

Stock solutions were prepared by dissolving the required amount of the test substance in distilled water. From these stock solutions, various concentrations of test solutions were prepared through a series of dilution. Control solutions were prepared using filtered and aerated tap water.

Acute Toxicity Tests (Range-finding and Definitive Tests)

After the acclimation period, the test organisms were transferred to 20-L holding tanks for an hour to expose them to an environment similar to the actual experimentation setup. Static renewal toxicity tests were performed in 2-L glass aquaria using five varying concentrations of the test substance and a control – all prepared in three replicates per concentration. Ten (10) fishes were then randomly transferred to each experimental tank using dip nets progressing from the control to the highest test concentration to prevent contamination (Espiritu *et al*. 2011). The test organisms were exposed to the toxicant for a period of 96 hours without feeding. During this period, the test solutions were replaced every other day up to approximately 70%

of the volume. In addition, water quality parameters such as dissolved oxygen, temperature, salinity, electrical conductivity, and pH were monitored every other day. The behavior of the fish was also observed and dead fishes were removed and counted from each tank daily. Mortality was interpreted as the absence of any movement (immobility) and/or reaction to mechanical stimulus. These procedures were followed for both range-finding and definitive tests.

Based on the results of the range-finding tests, definitive tests were conducted using five varying concentrations of the toxicants including a control – as shown in Table 1. The test results were considered valid if (a) the control mortality for each replicate was below 10% and (b) the resulting 96-hr LC_{50} for the reference toxicant was within the same order of magnitude as previously reported (Espiritu *et al*. 2011).

Table 1. Range of toxicant concentrations (ppm) used in in the 96-hour acute toxicity tests with tilapia.

Concentrations of Toxicants per Trial (mg/L, ul/L, or ppm)				
Urea ($x10^3$)	Complete Fertilizer ($x10^3$)	Effluent ($x10^4$)	Sludge water ($x10^4$)	Copper Sulfate Pentahydrate*
10	0.7	10	10	10
30	1.0	30	30	30
50	3.0	50	50	50
70	5.0	70	70	70
100	7.0	Pure effluent	Pure sludge water	100
	10			

*Reference toxicant

Histopathological Assessment

At the end of the 96-h acute toxicity tests with urea, complete fertilizer, sludge water, and effluent, the remaining live test organisms were taken from each concentration to determine possible cellular morphological changes that may be indicative of toxicity. Samples of the liver were preserved in glass vials containing 10% formalin. These were processed into 5 µm thick sections, stained with hematoxylin-eosin, and mounted on a glass slide and analyzed under a light microscope at low power objective (LPO) magnification.

Data Treatment

The toxicity tests with each toxicant were performed thrice to ensure statistical validity. Mortality results were expressed as 96-hr LC_{50} (ppm) using the Trimmed Spearman-Karber Method (Hamilton and Thurston 1977). Daily mortality results were added by the end of the 96-hour exposure period and expressed in cumulative percent mortality. One-way ANOVA was used to determine significant differences in mortality results among replicates and between trials**.** One-way ANOVA was also used to determine significant difference in daily LC_{50} values among toxicants. Statistical tests were done in Microsoft Excel 2016 and R version 3.4.3.

RESULTS

The results of the toxicity tests (expressed as cumulative percent mortality over time) showed a normal dose-response relationship, wherein mortality increased as the concentration of the toxicant increased (Figures 1–4). The results of the definitive tests showed 100% mortality within the first 24 hours in all three trials for the following test concentrations: sludge water (pure), sludge water (70 x 10^4 ppm), sludge water (50 x 10^4 ppm), sludge water (10 x 10^4 ppm), urea (70 x 10^3 ppm), urea (50 x 10^3 ppm), urea (30 x 10^3 ppm), urea (10 x 10^3 ppm), and complete fertilizer (7.0 x 10^3 ppm). In contrast, the test organisms exposed to the

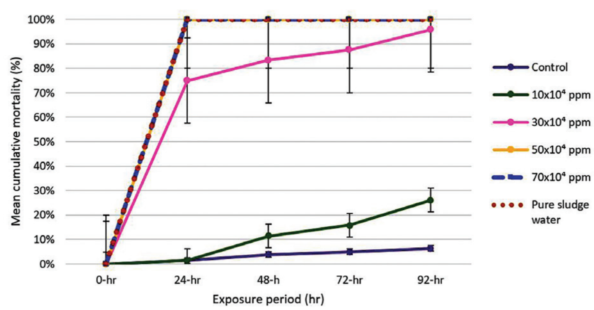

Figure 1. Mean cumulative mortality (in %) of *O. niloticus* (Juvenile Tilapia) exposed to sludge water in function of exposure period (hr) and toxicant concentration (in uL/L, ppm). Note that the results for the 50 x 10^4 ppm, 70 x 10^4 ppm, and pure sludge exhibited similar patterns. Data expressed as mean ± SD.

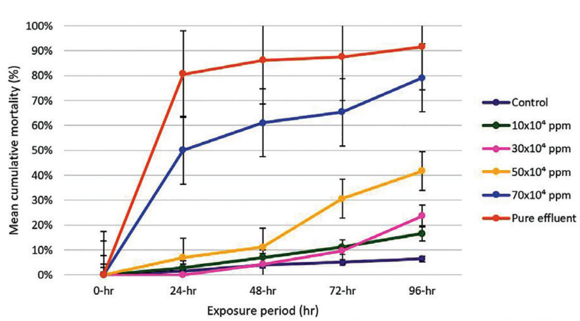

Figure 2. Mean cumulative mortality (in %) of *O. niloticus* (Juvenile Tilapia) exposed to effluent in function of exposure period (hr) and toxicant concentration (in uL/L, ppm). Data expressed as mean ± SD.

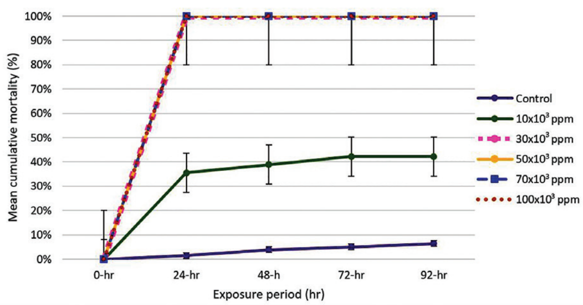

Figure 3. Mean cumulative mortality (in %) of *O. niloticus* (Juvenile Tilapia) exposed to urea in function of exposure period (hr) and toxicant concentration (in mg/L, ppm). Note that the results for the 30 x 10^3 ppm, 50 x 10^3 ppm, 70 x 10^3 ppm, and 100 x 10^3 ppm exhibited similar patterns. Data expressed as mean ± SD.

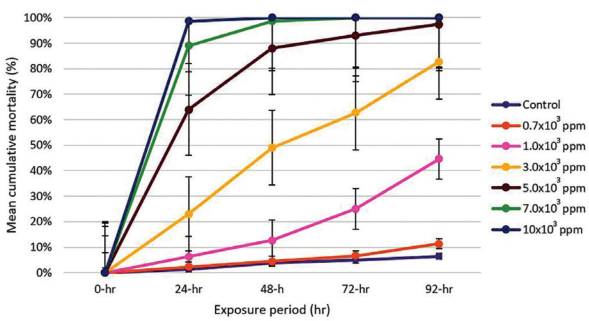

Figure 4. Mean cumulative mortality (in %) *O. niloticus* (Juvenile Tilapia) exposed to complete fertilizer in function of exposure period (hr) and toxicant concentration (in mg/L, ppm). Data expressed as mean ± SD.

higher concentrations of the effluent reached almost 100% mortality only towards the end of the 96-hour test. Results of the one-way ANOVA showed no significant differences in percent mortality after 96 h among the replicates in each trial and between the trials for all toxicants ($p > 0.05$). No significant differences were also observed among the concentrations in each trial for all toxicants except for effluents, which had *p*-values less than 0.05 in the 70% and 50% concentrations. Using Tukey's HSD, significant differences among the trials were found for the 70% effluent concentration during the first trial and for the 50% effluent concentration during the second trial. This difference may be attributed to variations in the design of septic tanks (*e.g.*, number of chambers, height, porosity, connections, *etc.*) that may have affected the septage and effluent quality.

A comparison of the results of the definitive toxicity tests for the four toxicants is shown in Figure 5 in decreasing order of toxicity: complete fertilizer (96-hr LC_{50} = 1,396 ppm) > urea (96-hr LC_{50} = 16,152 ppm) > sludge water (96-hr LC_{50} = 145,900 ppm) > effluent (96-hr LC_{50} = of 465,000 ppm). The resulting 96-hr LC_{50} values differed by one order of magnitude among the toxicants. The effluent

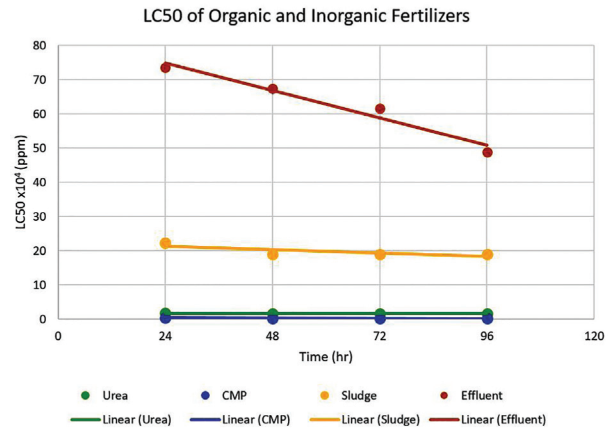

Figure 5. Trends in the 96-hr LC_{50} values in function of time for *O. niloticus* exposed to urea, complete fertilizer, effluent, and sludge. Complete fertilizer is the most toxic of the four and effluent as the least toxic.

and sludge water were far less toxic than the commonly used inorganic fertilizers. A comparison of daily LC_{50} values showed significant differences between toxicants ($p < 0.05$). Tukey's HSD test revealed that among the five toxicants investigated, only the values for complete fertilizer and urea had no significant difference ($p > 0.05$). Figure 5 shows a clear manifestation of this trend.

Figures 6 and 7 show the results of the histopathological examinations between the control and the exposed organisms. Examinations of the liver samples of the surviving test organisms showed that blood congestion and necrosis had already occurred in the fish that were exposed to the lowest concentration of the toxicants (*i.e.*, 0.7 x 10^3 ppm for complete fertilizer, 10x 10^3 ppm for urea, and 10 x 10^4 ppm for both the effluent and sludge water) in comparison with the control. This is indicative of the occurrence of sublethal effects on the test organisms prior to the onset of mortality.

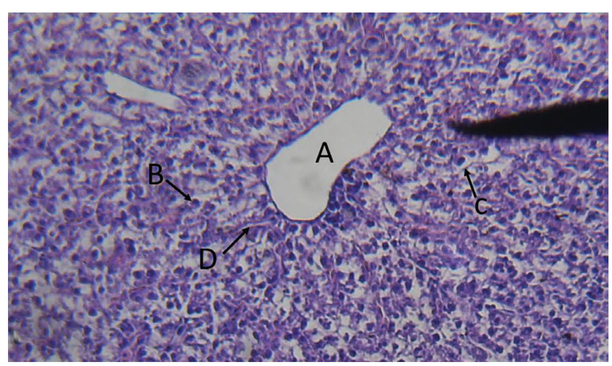

Figure 6. Liver specimen of *O. niloticus* obtained from the control showing no alterations at LPO magnification: (A) central vein; (B) round, central nucleus; (C) hepatocyte with granular cytoplasm; and (D) Sinusoid.

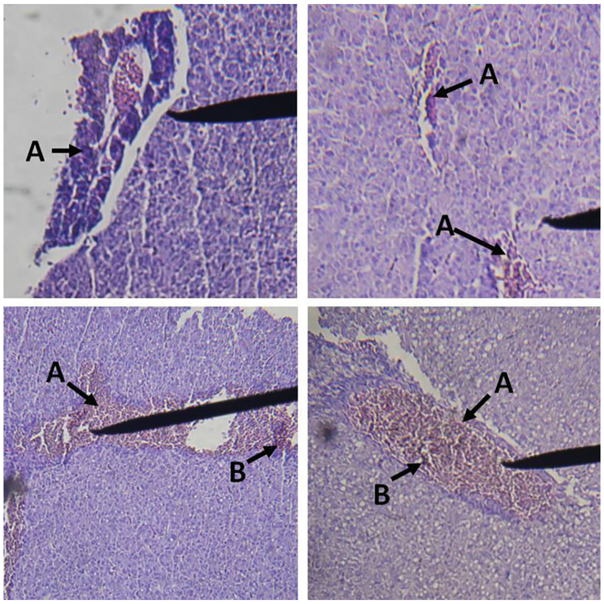

Figure 7. Liver samples obtained from *O. niloticus* exposed to 100,000 ppm effluent (top left); 100,000 ppm sludge water (top right); 10,000 ppm urea (bottom left); and 700 ppm complete fertilizer (bottom right) viewed under a light microscope at LPO magnification. Samples showed signs of sinusoidal congestion (A) and necrosis (B).

DISCUSSION

There have been numerous studies done in other countries on the use of treated wastewater for agriculture – particularly for crop irrigation to increase crop yields (Al-Nakshabandi *et al.* 1997, Oron *et al.* 1999, Kivaisi 2001, Toze 2006) – and for aquaculture to fertilize fishponds (Mara 2004). However, the use of treated wastewater can pose some risks to the health and safety of both humans and the environment in the form of bacteria, protozoa, viruses, and helminths that can still be present even after treatment. Helminths, in particular, are difficult to detect using conventional microbiological monitoring techniques and are not easily removed through conventional treatment processes (Akin *et al*. 1989). Such is the case in many wastewater treatment facilities in the Philippines (ADB 2016). Only 10% of the country's population are connected to piped sewage systems (DPWH 2013) and only a fraction of the country's total wastewater undergoes treatment. Moreover, in many cases, the resulting effluents do not even meet prescribed standards. Most of these are discharged into rivers and other bodies of water along with the remaining untreated wastewater, causing detrimental effects to the quality of the receiving water body (World Bank 2003, 2007).

Moreover, sludge can also be used for agricultural purposes as fertilizer for crops (EPA 2015). However, a review by Clark and Smith (2010) showed the alarming levels of perfluorinated compounds (PFCs) and polychlorinated alkanes (PCAs) found in sludge. In addition, sludge may also contain nitrogen at levels beyond what the soil needs, thus increasing the risk of nitrogen emissions to the environment (Rigby *et al.* 2017).

In this study, all four toxicants exhibited the typical dose-response of an increase in toxicity with increasing concentrations and duration of exposure to the toxicant – the common trend in toxicity studies (Asuquo and Essienibok 2014). The results are also consistent with previous studies wherein inorganic fertilizers were found to have a much higher toxicity compared to sludge and effluent. For example, a toxicity study by Uhuo *et al.* (2013) with *O. niloticus* exposed to a 20-10-10 grade inorganic NPK fertilizer revealed a 96-h LC_{50} of 820 ppm. Other nitrogen-based inorganic fertilizers tested on other fish species reported values that were more toxic than sludge and have closer toxicities with other NPK-grade fertilizers (Capkin *et al*. 2010, Uhuo *et al.* 2013).

The nitrogen component in fertilizers presents itself as organic-N and inorganic-N, with the inorganic form mainly as ammonium (Clarkson *et al*. 1986). Studies have shown that high concentrations of ammonium can have adverse effects on aquatic life including inorganic fertilizers from agricultural runoff (Capkin *et al*. 2010). It can thus be inferred that this ammonium component contributes to the significant disparity of the toxicity between the inorganic fertilizers and sludge.

The liver is an important organ in histopathological studies for its role in storage, metabolism, and biochemical transformation of pollutants from the environment (Channa and Mir 2009). However, its ability to perform such functions can be impaired by the accumulation of toxicants resulting in structural damage to the organ (Camargo and Martínez 2006). Exposures to contaminants are generally reflected as lesions and histopathological alterations in the organ (Velkova-Jordanoska and Kostoski 2005, Cengiz 2006). In this study, the results of the histopathological examinations generally showed blood congestion and necrosis in the liver tissues of juvenile tilapia exposed to the lowest concentration of the toxicants (*i.e.*, 700 ppm for complete fertilizer; 10,000 ppm for urea; and 100,000 pm for both the effluent and sludge water). These lesions and alterations in the organ may be attributed to direct toxic effects on hepatocytes and the inability of the liver to detoxify pollutants in the water (Soufy *et al.* 2007). Moreover, these are early signs of liver dysfunction that could have led to the mortality of fish exposed to higher concentrations of the toxicants (Laith *et al.* 2017). Congestion and necrosis of liver cells were also observed in other studies involving fish exposed to polluted waters (Figueiredo-Fernandes *et al.* 2007, Velmurugan *et al*. 2009).

These histopathological effects thus imply that relatively low concentrations of the toxicants may still lead to damaging effects to aquatic ecosystems in the long term. Signs of liver impairment, which appear to have been the cause of the mortality of the tilapia samples, were evident in the remaining survivors – showing the possibility that further exposure to the toxicants might eventually lead to their deaths. Further studies should be conducted to determine the long-term impacts of prolonged exposure to these substances on aquatic organisms.

CONCLUSION

The study has demonstrated that effluent and sludge are relatively less toxic compared to commonly used ammonium-based inorganic fertilizers (urea and complete fertilizers) based on their respective 96-hr LC_{50} (ppm) values. However, as the results of the histopathological tests had shown, even low concentrations of the effluent – which has been found to be the least toxic among the test substances – have acute sublethal effects to the test organisms. Given this, further chronic toxicity and histopathological studies should be conducted to investigate the environmental safety and sustainability of these substances prior to their use for agricultural applications. With the current practice of using sludge and effluents in agriculture, there is a need to review the existing guidelines on wastewater reuse in agri- and aquaculture – as provided in the Department of Agriculture Administrative Order 26 series of 2007 to include toxicity testing in determining the suitability of the effluents and sludge for these types of applications.

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