ECOTOXICOLOGICAL EFFECTS OF IBUPROFEN (NSAID) ON FRESHWATER SHRIMP, *Gammarus pulex* COLLECTED FROM BRAMHAM ESTATE, LEEDS, WEST YORKSHIRE UNITED KINGDOM.

[*1]Ogunbanwo, O. M. A., [1]P. Kay, L. Brown, [2]T. A. Okeowo
[1]Faculty of Environment, University of Leeds, UK
[2]School of Agriculture, Lagos State Polytechnic, Ikorodu
Corresponding author: tayogunbanwo@yahoo.com & ogunbanwo.o@mylaspotech.edu.ng
Mobile: +2348100244181; WhatsApp: +447753312067

ABSTRACT
An increasing number of pharmaceuticals have been found in the aquatic environment and the issue has become of human and environmental health concern. Many pharmaceuticals are not fully degraded in wastewater treatment plants (WWTPs) and are continuously released into the aquatic environment resulting in low range concentrations (?gL^{-1}) in the receiving waters. Ibuprofen is a widely used non-steroidal anti-inflammatory drug (NSAID) and is persistent in the aquatic environment. This pharmaceutical has been frequently reported in wastewater effluents, surface waters, groundwater and even drinking water. In this study, the ecotoxicological effects of ibuprofen on the growth, feeding, and mortality of freshwater shrimp, Gammarus pulex was investigated in a laboratory experiment. Seventy-five (75) male G. pulex were assigned at random among five experimental groups (three treatments: 420.8 ngL^{-1}, 2629.6 ngL^{-1} and 4838.4 ngL^{-1} and two controls: negative and solvent controls) in a static-renewal bioassay for four weeks, with 100% renewal of water and the respective treatments weekly. The results showed that exposure of G. pulex to environmentally relevant concentrations of ibuprofen had no significant effects on feed intake (p=0.36), growth rate (p=0.09), and mortality (p=0.53). However, further investigations on the multigeneration effects of ibuprofen on G. pulex may be needed to confirm that, ibuprofen effects on G. pulex is non-consequential.

Keywords: Ecotoxicology; Ibuprofen, *Gammarus pulex*, Pharmaceuticals, Emerging Contaminants

INTRODUCTION

Pharmaceutical drugs are extensively used in aquaculture, veterinary, and human medicines to save a life. In recent times, the presence of these active ingredients and their metabolites have been detected in treated and untreated sewage effluents, groundwater, surface water, drinking water, and estuaries at concentrations in the range of µgL^{-1} to ngL^{-1} (Fent *et al*., 2006; Gomez *et al.,* 2006; Metcalfe *et al.,* 2003). It has been established that the main source of these drugs and their metabolites into the aquatic environment is through sewage treatment plants (Ferrari *et al*., 2005; Heberer, 2002). The continuous input of pharmaceuticals into the aquatic environment may influence the non-target organisms including macro-invertebrates (Fent *et al*., 2006; Li *et al.,* 2014).

Ibuprofen is a non-steroidal anti-inflammatory drug used worldwide as an analgesic and antipyretic and found in the environmental matrix as a result of a high volume of usage and incomplete removal during wastewater treatment (Nakada *et al*., 2006; Tauxe-Wuersch *et al*., 2005; Tixier *et al.,* 2003). It has high over the counter sales and it is poorly metabolised in humans, with 70-80 % of the parent compound and metabolites being excreted. In addition to this, ibuprofen is not readily degradable and has the potential to bio-accumulate (log K is 3.5), hence it is not surprising that it is found in effluents and rivers at relatively high concentrations (Ashton *et al.,* 2010; Thomas & Hilton, 2004).

Ibuprofen has been found at a concentration of 22µgL^{-1} in effluents and up to 84 µgL^{-1} in sewage treatment plants (STPs) (Gömez *et al.,* 2006). However, the concentration may vary from country to country because of usage level. For instance, 0.1 µgL^{-1} concentration of ibuprofen was detected in surface water of South Wales, UK (Kasprzyk-Hordern *et al.,* 2008), 0.03 µgL^{-1} in major rivers of Korea (Kim and Carlson., 2007), 27.3 µgL^{-1} in the UK (Roberts and Thomas, 2006), 6.7 µgL^{-1} in Canada (Buser *et al.,* 2008), 7.1 µgL^{-1} in Sweden (Bendz *et al.,* 2005), 0.15 and 4.8 mgL^{-1} in European STPs (Santos *et al.,* 2016). In developing nations of Africa, the information on the occurrences and effects of pharmaceuticals in the environment is very sparse (Agunbiade and Moodley, 2014; Hughes et al., 2013; K'oreje et al., 2012; Ngumba et al., 2016; Ogunbanwo, 2018; Wood et al., 2015) but none on ibuprofen.

Ibuprofen is considered to be a dangerous compound because of its low affinity to water (Christensen *et al.,* 2006), and persistence in the environment because of its inherent properties (Bendz *et al.,* 2005; Boxall et al., 2003) and has 32 days half-life (Tixier *et al.,* 2003). It affects the reproductive behaviours of invertebrate and vertebrate animals e.g. it has been shown to affect the spawning habits of Medaka (*Oryzias latipes)* and *Daphnia magna* (Han *et al.,* 2010; Flippin *et al.,* 2007) Ibuprofen stimulates the growth of aquatic organisms. For example, the growth of cyanobacteria was stimulated after 5days of

exposure at a concentration of 1 ?gL^{-1} and reduced the growth of duckweed *Lemna. minor* after 7days (Pomati *et al.*, 2014).

After six (6) weeks of exposing female Japanese medaka to various concentrations of ibuprofen, a sharp rise in liver weight and an increase in egg production was observed which also led to a reduction in weekly spawning events (Flippin *et al.*, 2007). The population growth of *D. magna* exposed to ibuprofen was reduced significantly within concentrations of 10-80 mgL^{-1} and reproduction was inhibited at all concentrations (Heckmann *et al.*, 2012). When freshwater amphipods were exposed to 1-10 ngl^{-1} concentrations of ibuprofen, a decrease in all activities of *G. pulex* was observed (De-Lange *et al.*, 2006). Brown *et al.*, (2006) carried out a study on the concentrations of several non-steroidal anti-inflammatory drugs-diclofenac, ketoprofen, ibuprofen, and naproxen on Rainbow trout at three sewage treatment plants. *Oncorhynchus mykiss* was used for the study. It was found out that the plasma concentrations were highest for ibuprofen compared to other Non-steroidal anti-inflammatory drugs (NSAIDs). The finding revealed that the bio-concentration factors for the compounds varied between sites, and ibuprofen had the highest value. Similarly, Fick *et al.*, (2009) detected tramadol, ibuprofen, diclofenac, naproxen, and ketoprofen in *Oncorhynchus mykiss* plasma from the sites of three sewage treatment plants. Ibuprofen had the highest concentrations of all the identified NSAIDs.

In a study by Cleuvers, (2004) involving the mixture of pharmaceuticals and non-steroidal anti-inflammatory drugs (e.g. naproxen, diclofenac, and ibuprofen), Ibuprofen was detected with the highest concentrations of all the NSAIDs.

Macro-invertebrates are a sundry group of aquatic life that are important in the ecology of aquatic habitats (Ogunbanwo, 2018, 2018a). For Example, *Gammarus pulex*, which is generally classified as an omnivorous shredder, dwells in the water column and is considered _pollution sensitive_ (Ogunbanwo, 2018). They serve as food to many fishes and play a vital role in particulate organic matter decomposition in the aquatic ecosystem. Due to their widespread distribution, significance in the food web, and sensitivity to a wide range of pollutants, they serve as a bio-indicator of the aquatic environment. They have been successfully used in various toxicity testing (Taylor *et al*., 1991; Williams et al., 2013). Hence, they are standard test species in ecotoxicity testing in many parts of the world (Ogunbanwo, 2018, 2018a).

This investigation aimed to improve the understanding of the ecotoxicological effects of prolong low-level exposure of *G. pulex* to ibuprofen at environmentally relevant concentrations. Response variables assessed include feeding, growth, and mortality.

MATERIALS AND METHODS

Materials

Ibuprofen (IB) (CAS number: 15687-27-1, purity ? 98%), molecular weight 206.29gmol^{-1}, and molecular formula $C_{13}H_{18}O_2$, the structure of the compound is shown in Fig.1, was purchased from Sigma-Aldrich, (Dorset, UK). High-performance liquid chromatography (HPLC) grade methanol (CAS no.67-56-1, purity ?99.9%) was purchased from Fisher Scientific (Loughborough, UK). Ultra-pure water was obtained from Sartorius Purite Select HP160/BP/IT water purification system with a specific resistance of 18.2 M?cm. The chemical stock solution was prepared in methanol on a weight basis in 100ml of 100% methanol and stored at ?20 °C, and the working solutions were diluted aliquots of the stock solutions. Glassware and vessels were disinfected then pre-rinsed with 100% methanol and ultra-pure water twice and left to dry in the fume cupboard before the experiments.

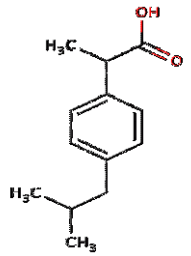

Figure:1 Chemical structure of ibuprofen

Preparation of Solutions

Environmentally relevant concentrations of IBU were used in this experiment (UK mean measured environmental concentration [LT] and UK maximum measured environmental concentration [HT] and medium concentration [MT] was the average of the LT and HT). These treatment concentrations were chosen as likely exposure concentrations based on published mean for UK Rivers and a measure of worst-case exposure scenario based on maximum concentrations in UK Rivers.

One hundred mgL^{-1} of the test solution was prepared by dissolving 10mg of IBU in 100 ml of HPLC grade methanol (100mgL^{-1} = 10mg/100ml). The desired experimental concentrations were achieved through serial dilutions. All solutions were stored at ?20°C and in the dark for optimum stability and to avoid photo-degradation.

Test Animals

Two hundred and fifty (250) *Gammarus pulex* were collected from ponds at Bramham Estate, Leeds, West Yorkshire, the United Kingdom using a net at 1.5 to 4 m depth. They were brought to the laboratory in cool boxes. Amphipods of

approximately the same size averaging (22.47 ± 3.16mg) were sorted out sexed and kept in incubators at 12°C with a diurnal light rhythm of 16 h: 8 h (day-night) and allowed to acclimatize in aerated pond water before the experiments started. They were fed on conditioned alder leaves (*Alnus glutinosa*), the leaves also acted as a shelter for the animals. The leaves were conditioned in an aerated bucket at room temperature where they were inoculated with bacteria and fungi. Sexing was achieved by placing the pre-copular pairs on a dry filter paper and allowed them to detangle from each other. The test animals were chosen because they play important role in the food chain, eliminating them will disrupt the balance in the ecosystem. They are also bioindicators of the stream_s health, generally abundant, easy to sample, and most likely to be affected by pollution because they have little mobility.

Exposure Media

Water from Bramham Park where the animals were sourced was used for this experiment. The pH, DO, water temperature and electrical conductivity were measured weekly with HACH HQ40d multi. The instruments were rinsed with ultra-pure water. (Sartorius, model: Arium Comfort) before every reading was taken.

Methods

The experiment was carried out in a glass clear SS jar (500mL) with face lined cap kept in incubators at a temperature of 12°C and 16:8h light: dark regime each containing 300mL of pond water. Each glass jar contained one *G. pulex* which was assigned using a random integer generator to avoid the subjectivity of the experimenter, weighed individually at the start of the experiment and subsequently every week using Sartorius (Model: Quintex 224-1s) balance. Exposure glass jars were arranged randomly and temperature data loggers were placed in the incubator, to monitor the temperature at every compartment of the incubator. Water samples for chemical analyses of the compounds were collected every week. They were fed with 0.1gm of standardised alder leaves (*Alnus glutinosa*). There were three treatments: low treatment (LT), medium treatment (MT), high treatment (HT), and two controls: negative control (NCTR) and solvent control (SCTR) with 15 replicates of each treatment and 15 replicates of each control. The solvent control (HPLC grade methanol) used in the experiment was tested for different responses of the physiological measurements compared to the negative control (pond water from Braham Estate where the test animals were sourced and exposed to in the laboratory). No statistical difference was found between control treatments with and without solvent.

Test concentrations were selected to mimic environmental detection levels reported for UK Rivers in the literature. The low treatment (LT) was UK mean measured environmental concentration of 420.8 ngL^{-1} (LT), 2,629.6 ngL^{-1} medium treatment (MT) and 4,838.4 ngL^{-1} high treatment (HT) for ibuprofen respectively (Hughes *et al*., 2013, Bound and Voulvoulis, 2006). The negative control contained no treatment and the solvent control contained 0.1 mL L^{-1} of methanol. The working solutions of LT, MT, and HT were poured on transparent silica glass beads and allowed to evaporate to dryness in the fume cupboard in other to avoid methanol toxicity, then the dried extracts were reconstituted/re-suspended with 10 mL of pond water and washed into the beakers before *G. pulex* were introduced. Before the transparent silica glass beads were re-used, they were washed with ultra-clean water, ashed in the oven at 400° C, and allow to cool in the fume cupboard to prevent toxicity in any form to the test animals.

Seventy-five (75) male *G. pulex* were assigned at random among the five experimental groups. Exposures were in a static-renewal bioassay; with 100% renewal of water and the respective treatments weekly. The collected water samples were stored at -20°C and filtered, extracted with Solid-phase extraction (SPE) units into 4 ml amber glass vial and analysed. The experiment was run for 4 weeks. Response variables, growth, mortality, and physicochemical parameters of the water were measured weekly, and feed materials loss was measured at the end of the experiment.

Data analysis

Data were organised using Excel (Microsoft, 2013) spreadsheet. Residuals of the data were checked for normal distribution (Kolmogorov and Smirnov method, 2016) and homogeneity of variance (Bartlett method). R (R Development Core Team, 2008) was used to analysed the data and create figures. Tukey post-hoc tests were used to identify the means that differed. Change in *G. pulex* mass, physicochemical parameters, and mass of feed materials (*Alnus glutinosa*) loss from week 1 to week 4 were tested using generalised linear model and Chi-square. Mortality was analysed using one-way ANOVA where assumptions of normality and homogeneity were met followed by a Bonferroni test to compare the treatment means with the respective controls.

RESULTS

Growth

When the experiment was initiated, the cumulative mean mass of *Gammarus pulex* (across treatments and controls) was 22.47 ± 3.16mg and no statistically significant difference (ANOVA: $F_{4, 70} = 0.14$, $p = 0.97$) was recorded between treatment and the control groups. The cumulative mean dissolved

oxygen (DO) was 9.61 ± 0.07mgL^{-1}, pH was 8.69 ± 0.04, water temp was 11.89 ± 0.32°C, and mean electrical conductivity (EC) was 694.28 ± 64.34µS/cm. There were no statistically significant differences in water chemistry.

When the change in mass was analysed, the growth was not significant. Hence, there were no statistically significant differences between the treatment and the control groups (ANOVA: $F_{4, 65}$ = 2.10, p =0.09) (Figure 2).

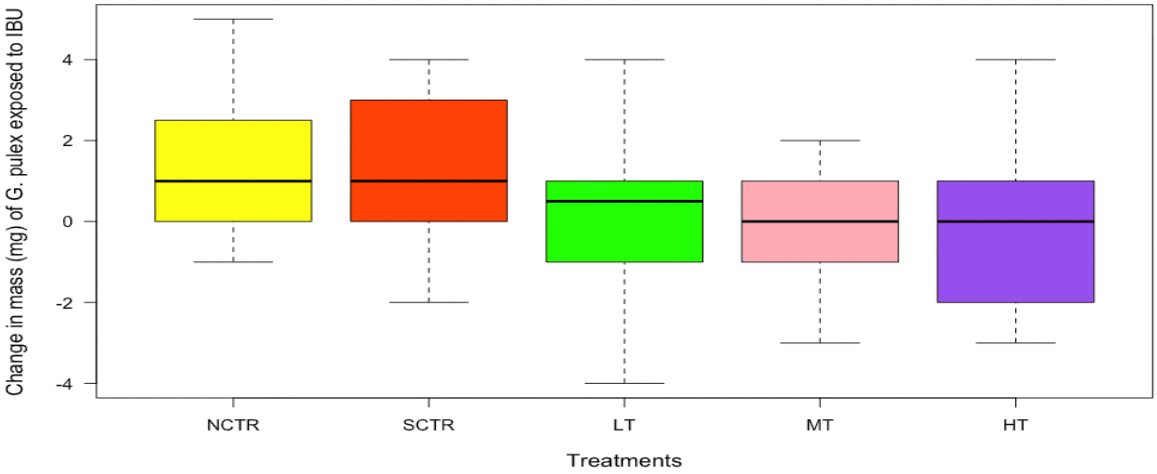

Figure 2: Boxplots displaying change in the mass of *G. pulex* exposed to environmentally relevant concentrations of Ibuprofen after a 4week static renewal experiments. Negative control (NCTR), solvent control (SCTR), low treatment (LT), medium treatment (MT), and high treatment (HT), The dark horizontal line inside the box represents the median (50th percentile), top of the coloured box represents 3rd quartiles (75th percentile), top whisker represents 4th quartiles (90th percentile), the bottom of the coloured box represents the 2nd quartiles (25th percentile) and the vertical lines represent the 1st quartiles (10th percentile). There were no outliers.

Feeding

The change in feed materials (*Alnus glutinosa*) per *G. pulex* per feeding day were NCTR = 0.030gm ± 0.003 SD, SCTR = 0.029gm ± 0.007 SD, LT = 0.028gm ± 0.003 SD, MT = 0.027gm ± 0.004 SD and HT = 0.026gm± 0.008 SD (Figure 3). There were no statistically significant differences in feeding between controls and treatments (GLM: $?^2$ (4) = 0.00013, *p* =0.356).

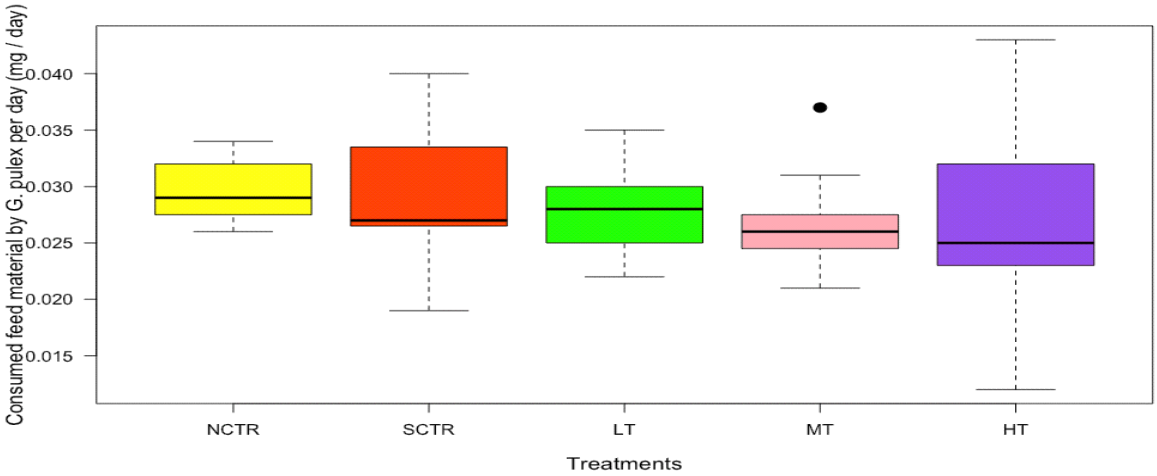

Figure 3: Boxplots displaying change in feed materials of *G. pulex* exposed to environmentally relevant concentrations of Ibuprofen after a 4-week static renewal experiment. Negative control (NCTR), solvent control (SCTR), low treatment (LT), medium treatment (MT), and high treatment (HT). The dark horizontal line inside the box represents the median (50th percentile), the top of the coloured box represents 3rd quartiles (75th percentile), the top whisker represents 4th quartiles (90th percentile), the bottom of the coloured box represents the 2nd quartiles (25th percentile) and the vertical lines represents the 1st quartiles (10th percentile). There was an outlier.

Mortality

In the first week of the experiment, there were no deaths recorded in all the treatments (LT, MT, HT) and the controls (NCTR, SCTR). Mortality commenced in the second and third week for HT with two *G. pulex* recorded for both weeks. While in

the fourth week, one and two mortalities were recorded for LT and MT respectively. No mortality was recorded for the control group (Figure 4). There were no statistically significant differences (GLM: $?^2$ (4) = 211.12, p = 0.53) between treatment and control groups at the end of the study.

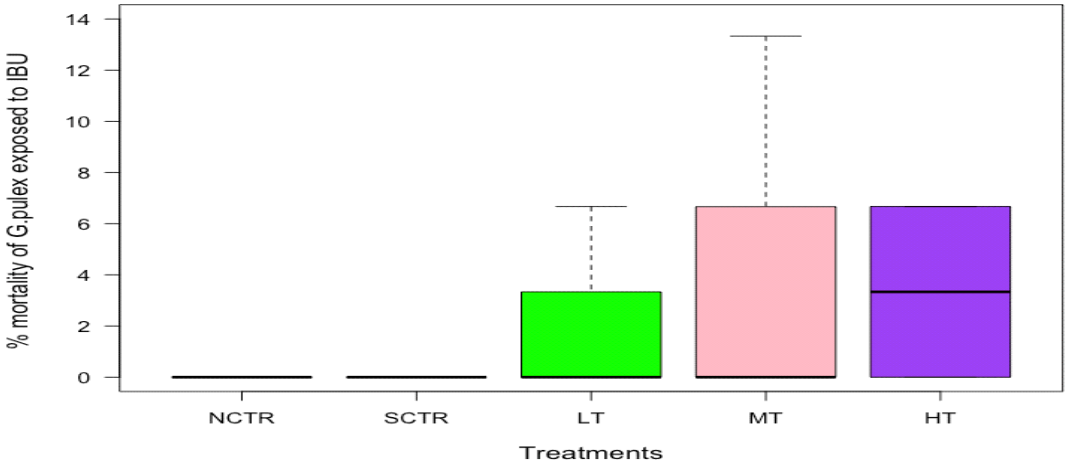

Figure 4 Boxplots displaying % mortality of *G. pulex* exposed to environmentally relevant concentrations of Ibuprofen after a 4-week static renewal experiment. Negative control (NCTR), solvent control (SCTR), low treatment (LT), medium treatment (MT), and high treatment (HT). The dark horizontal line inside the box represents the median (50^{th} percentile), the top of the coloured box represents 3^{rd} quartiles (75^{th} percentile), the top whisker represents 4^{th} quartiles (90^{th} percentile), the bottom of the coloured box represents the 2^{nd} quartiles (25^{th} percentile) and the vertical lines represents the 1^{st} quartiles (10^{th} percentile). There were no outliers.

DISCUSSION

Effects of Ibuprofen on Growth, Feeding, and Mortality of Gammarus pulex

Ibuprofen has shown to significantly affects the growth of some fungi and bacteria species (Ogunbanwo, 2018). However, for invertebrate animals, the reverse is the case although, there has been conflicting reports about the effects of this drug on invertebrate animals (Ogunbanwo, 2018).

In this study, the exposure of *G. pulex* to environmentally realistic concentrations of ibuprofen had no statistically significant effects on feeding, growth, and mortality even though the feeding rate in the control groups were higher than the treatment groups. Hence, an increase in the mass of *G. pulex* in the control groups than the treatment groups was noticed. The results indicate that the exposure of *G. pulex* to environmentally realistic concentrations of ibuprofen was inconsequential i.e. would most likely be of minor importance. However, a definite conclusion might not be reached about the risks of environmentally relevant ibuprofen concentrations before potential effects on multi-generational exposure have been assessed, which was beyond the scope of this investigation. Furthermore, no significant differences were observed in mortality between the treatments and controls, although a minimal increase in the mass of the treated *G. pulex* was noticed, this was not significant. This indicated that the concentration thresholds at which ibuprofen could potentially cause toxicity effects were not reached. Hence, the concentration of ibuprofen in *G. pulex* was not enough to affect a significant change within the 4 weeks of exposure, however, this could have effects later on in offspring of *G. pulex* or a multi-generational experiment. In a similar experiment conducted on *Hydra vulgaris*, a freshwater invertebrate Pascoe *et al.*, (2003), showed no negative effects of pharmaceuticals (ibuprofen, paracetamol, acetylsalicylic acid, amoxicillin, bendroflumethiazide, furosemide, atenolol, diazepam, digoxin, and amlodipine) on the survival, feeding, and bud formation at concentrations up to 1000 μgL^{-1}. Cleuvers (2003) exposed daphnia, chlorophyte, and macrophyte to environmentally relevant concentrations of major pharmaceuticals and concluded that acute effects stemming from single substances in the aquatic environment are very unlikely. Recent findings have also shown that the population effects of ibuprofen in *D. magna* were reversible, consistent with the known action of ibuprofen on eicosanoid synthesis in mammals.

Similarly, when duckweed was exposed to 1 ?g $L^{?1}$ of the ibuprofen this resulted in no negative effect on growth compared to the other treatments. In a behavioural experiment conducted by De-Lange et al., (2006) on *G. pulex* using multispecies freshwater biomonitoring (MFB), the exposure to ibuprofen resulted in decreased activity of *G. pulex* from 65% in the control to 45% at concentrations of 1 and 10 ngL^{-1} but the difference was not significant. In other studies, ibuprofen has been shown to inhibit the growth of *Synechocystis* and *Lemna*, the effect however turned into a growth stimulation after the second day of freshly added ibuprofen (Pomati *et al.*, 2014). Similar findings were reported by Han *et al.*, (2010) who reported that environmentally

relevant concentrations of ibuprofen do not cause detrimental effects on the early life stages of zebrafish if they were exposed via the water only. The same study also reported that no differences were observed in either mortality or incidence of malformations between the treated and control embryos.

Similar works supported these results; a study on killifish (*Oryzias latipes*) revealed that, although reproduction was delayed following a 6-week chronic exposure to μgL^{-1} levels of ibuprofen, there was no statistically significant difference between the treatments (Flippin *et al.,* 2007; Ogunbanwo, 2018). When *D. magna* was exposed to a metabolite of acetylsalicylic acid (o-hydroxyhippuric) at 10 mgL^{-1}, egg abortion and reduced PGR was reported. Although, the parent compound has no effect on *D. magna* at the same concentration (Marques *et al.,* 2004).

The mode of action of ibuprofen in humans is the inhibition of prostaglandin biosynthesis. Prostaglandins are capable of causing contractions or atony of muscles in different organs (Cleuvers, 2003). Some studies have indicated the presence of prostaglandins in some vertebrates and invertebrates such as crustaceans (Bundy, *et al.*, 1985). A possible explanation of the observed reduced activity of *G. pulex* maybe that ibuprofen interfered with the normal pattern of muscle contractions in *G. pulex* (Ogunbanwo, 2018).

Pomati *et al*., (2014), in their studies, suggested that ibuprofen metabolites are nontoxic (*Synechocystis* and *Lemna*); and may also have growth stimulating properties. Hence, *G. pulex* exposed to ibuprofen did not demonstrate significantly increased mortality, feeding, and growth.

CONCLUSION

These results showed that exposure of *G. pulex* to environmentally relevant concentrations of ibuprofen had no significant effect on feeding activity, growth, and mortality; suggesting that, prolonged exposure, use of sensitive points (behavioural signs), and use of susceptible test species (*G. pulex*) are more useful for assessing sub-lethal impacts of contaminants and are sensitive indicators of toxicity in benthic macro-invertebrates (Ogunbanwo, 2018). Hence, these tools are useful in aquatic environmental risk assessment of drugs. However, further investigations on the multi-generation effects of ibuprofen on aquatic organisms may be needed to confirm that, ibuprofen effect on aquatic organisms is non-consequential.

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