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Effects of Mixture Toxicity of Erythromycin, Diclofenac and Ibuprofen on the Freshwater Isopod, *Asellus aquaticus*

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Abstract

Pharmaceuticals are continuously released into the aquatic environment mostly as waste water effluents through sewage treatment plants, run-offs, effluents from pharmaceutical manufacturing companies etc. This results in chronic exposure of aquatic organisms to these substances and their metabolites. Although, the concentrations of pharmaceuticals in the aquatic environment are usually in ngL-1 to □g L-1 range, they are not likely to result in lethal toxicity. Nevertheless, extended and unabated exposure to low concentrations of drugs could lead to sublethal effects or even multigenerational effects. The aim of this study was to seek to improve the understanding of the effects of prolonged low-level exposure of Asellus aquaticus (aquatic macro-invertebrates) to mixtures of erythromycin, diclofenac and ibuprofen. On exposure to the mixture, growth rate decreased, feed intake was reduced but mortality was not significant for A. aquaticus. The effects of these pharmaceuticals on the growth, feeding and mortality of the test animal were as a result of the actions of the drugs and not attributed to a more general stress response. Although pharmaceuticals are indispensable to human health their usage and discharge to the aquatic environment coupled with their ecotoxicity to aquatic life may lead to ecological problems in the near future. Furthermore, this research confirms the suitability of the test species (A. aquaticus) as ecotoxicological test species that is both amenable to laboratory culture and sufficiently sensitive to provide reliable quantification of environmental risk.

Keywords: Pharmaceuticals; Sub-lethal; Asellus aquaticus; Mixture toxicity; EcotoxicologyNAPCC

Introduction

Pharmaceuticals are consumed all over the world including the poorest countries on the planet because it increases life span, sustainability of lives, increases human productivity and mass production of food and livestock to sustain ever-growing human population [1]. As a result, in the last few decades, global manufacturing of pharmaceuticals had increased geometrically [1,2]. However, the presence of these drugs in the aquatic environment may elicit unintended biological response on non-target organisms among other responses, physiological changes, such as feeding, growth, mobility and behavioural changes [3-5] are most vulnerable/important endpoints for assessing the effects of pharmaceuticals on aquatic organisms [6].

Over the years, invertebrates have been found useful as model animals for investigating the toxicity of compounds in the environmental [7-11]. Macro invertebrates have been used regularly in the past for measuring the toxicity of chemicals because they are sensitive to toxic compounds and environmentally significant [12,13]. They are simple to handle, easy to rear, varieties of animal species to choose from and have short life span; hence, they are suitable for toxicity testing of water [11].

The test animal-Asellus aquaticus, a freshwater isopod, was chosen because they play a significant part in freshwater environment; they are leaf shredders and transfer and store metabolic energy within the ecosystems [14,15]. They also serve as food for both fish and invertebrate predators [16-19]. Asellus aquaticus has a life cycle of one year and has been used as a test species in toxicity testing experiments both in the laboratory and the field [16,20-22]. They serve as an indicator of the health of stream, can be found in large number and breed

in captivity and very slow in movement in water. Unlike G. pulex that is a water column dweller *A. aquaticus* are sediment-dwellers and constantly in contact with contaminants both in the water column and sediments [17]. They are seen as a robust organism, tolerant to fluctuations of pH value, dissolved oxygen concentrations and other physico-chemical parameters [10]. They are considered to be relatively tolerant to pollution but can be sensitive to trace metals [20-25]. They play a prominent role in transfer of contaminants in the aquatic food chain [3,26,27]. Their small size and robust nature make them ideally suited for application in toxicity tests and eliminating them will disrupt the balance in the ecosystem [16,19]. Hence, they are of great importance for the sustainability and balancing in the ecosystem. Very few studies have investigated effects of pharmaceuticals on *A. aquaticus* in the aquatic environment; in the past three decades the majority of work done using this model organism focused on metal pollution. For example, mercury, cadmium and copper were found by [28] to be toxic to A. aquaticus. Long-term effects of metals on *A. aquaticus* mortality were investigated by [29] and found that lethal concentrations were lower than nominal and effective concentrations [8]. Investigated the effects of exposure of *A. aquaticus* to selected organic pollutants and found that there were significant effects on the mortality rate. In a similar experiment by [30], the effects of chronic exposure to 5 µg L^{-1} cadmium and copper on *A. aquaticus* were investigated and it was found that the juvenile body growth was stimulated by cadmium and depressed by copper. Other studies in which *A. aquaticus* were exposed to metal toxicity were [19,20,31-36].

The current work investigated the use of A. aquaticus (bottom/sediment dweller) as an indicator to evaluate the potential effects of contaminants and the ecological effects of prolong low-level exposure of *A. aquaticus* to mixtures of erythromycin, diclofenac, ibuprofen at environmentally relevant concentrations on growth, feeding and mortality with the aim of broadening knowledge about the potential risk of such contaminants to aquatic ecosystems [37-39].

Materials and Methods

Study compounds

The study compounds (erythromycin, diclofenac and ibuprofen) were chosen based on their high prescription rates, volumes and availability of a reliable analytical method. They are among the 25 most prescribed drugs in the United Kingdom (UK) and because of their widespread occurrence in rivers worldwide. Calculations of the ratio of predicted environmental concentration (PEC) and predicted no effect concentration (PNEC) has shown that the ratios for these drugs exceeded one. A risk quotient (RQ) ≥1 indicates the potential for impacts on aquatic organisms. Hence, the basis for their selection (Table 1).

Table 1 Physico-chemical properties of the study compounds

Compound	CAS number	Purity (%)	Molecular weight (g/mol)	Molecular formula	Physico-chemical properties and risk quotients
Erythromycin	114-07-08	>99	733.93	$C_{37}H_{67}NO_{13}$	Solubility (mgL-1)=1.44, pKa=8.9, log Kow=2.48, Excretion rate=5% parent, RQmin=0.01, RQmax=1.25
Diclofenac	15307-79-6	>98	296.148	$C_{14}H_{10}Cl_2NNaO_2$	Solubility (mgL-1)=2430, pKa=4.0, log Kow=4.02, Excretion rate=15% parent, <1% conjugate, RQmin=0.01, RQmax=1.13
Ibuprofen	15687-27-1	98	206.29	$C_{13}H_{18}O_2$	Solubility(mgL-1)=21.00, pKa=4.91, log Kow=3.79, Excretion rate=1% parent, RQmin=0.55, RQmax=4.20
pKa=dissociation constant; log Kow=octanol: water partition coefficient; RQ data from: (Jones et al., 2002; Quinn et al., 2014; Sun et al., 2016; Ogunbanwo, 2018)					

Materials

Erythromycin, diclofenac and ibuprofen (Table 1) (Figure 1) were purchased from Sigma-Aldrich, (Dorset, UK). High performance liquid chromatography (HPLC) grade methanol was purchased from Fischer Scientific (Loughborough, UK). Ultra-pure water was obtained from a Sartorius Purite Select HP160/BP/IT water purification system with a specific resistance of 18.2 MΩcm. Chemical stock solutions for each compound were prepared in methanol on a weight basis in 100 ml of 100% methanol and stored at -20°C, and the working solutions were diluted aliquots of the stock solutions (100 mgL^{-1}=10 mg/100 ml). Glassware and vessels were disinfected then pre-rinsed with 100% methanol and ultra-pure water twice and left to dry in the fume cupboard prior to the experiments.

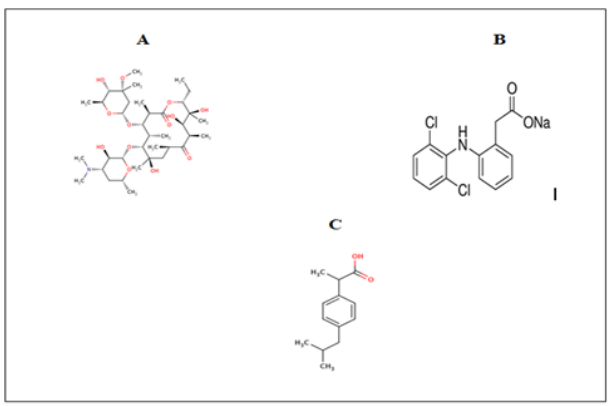

Figure 1 Chemical structures of erythromycin (A), diclofenac (B) and ibuprofen (C) from left to right respectively. Image from (Sigma-Aldrich).

Preparation of solutions

Environmentally relevant concentrations of each of the compounds ERY, DIC, IBU were mixed together and used in these experiments UK mean measured environmental concentration (LT) and UK maximum measured environmental concentration (HT). These treatment concentrations were chosen as an indicator of likely exposures based on published data for UK Rivers and an indicator of worst-case exposure scenario based on maximum concentrations in UK Rivers.

One hundred mgL^{-1} solutions (100 mgL^{-1}=10 mg/100 ml) of each of the compounds (ERY, DIC and IBU) were prepared by dissolving each separately in methanol (HPLC grade) to make the stock solutions. 1 mL was measured from each stock solution and each dissolved in 100 mL of solvent to make the intermediate solution for each compound. For the mixture experiment environmental concentrations of each of the compounds were measured from the intermediate solutions, mixed together and dissolved in 250 mL of solvent to form the working solution. All solutions were stored at -20°C in the dark for optimum stability and to avoid photodegradation.

The working solutions of LT and HT were poured on transparent silica glass beads and allowed to evaporate to dryness in the fume cupboard in order to avoid methanol toxicity, and then the dried extracts were reconstituted/resuspended with 10 mL of pond water and washed into the beakers before *A. aquaticus* were introduced.

Before the transparent silica glass beads were reused, they were washed with ultra clean water, ashed in the furnace at 550°C and allow cooling in the fume cupboard to prevent toxicity in any form to the test animals. Separate beads were used for the different treatments and controls to prevent contamination.

Test animals: origin and maintenance

Asellus aquaticus (Figure 2) used for the experiments were collected in ponds at Bramham estate, Leeds, West Yorkshire, United Kingdom. This site was chosen because it was located upstream of any STP effluent inputs, hence reducing the possibility for pollution by the compounds being investigated. The test animals were sampled with a net from 1.5 to 4 m depth. *A. aquaticus* individuals were hand selected from other organisms and detritus and then brought to the laboratory in cool boxes (5°C). Isopods of approximately the same size averaging 22.29 ± 1.31 mg were used for the experiments. Individuals were sexed by placing pre-copular pairs on a dry filter paper and allowing them to disentangle from each other and kept in incubators at 12°C with a diurnal light rhythm of 16 h: 8 h (day-night) and allowed to acclimatise in aerated pond water before the exposure experiments started.

Figure 2 Asellus aquaticus (pollution tolerant) Source: Ogunbanwo, 2018

Preparation of leaf material for feeding of test animals

Alnus glutinosa (Alder leaves) were collected from Bramham Estate near the ponds and oven dried at 60°C for 24 hrs. The leaves were conditioned in a nutrient medium in an aerated bucket at room temperature for 10 days together with alder leaves previously exposed in the ponds in which the test animals were collected. This was to establish a natural microbial community consisting of fungi and bacteria. This conditioning process increases the nutritive value of leaf material for shredders, such as *A. aquaticus* and simulates the environmentally relevant processes. *A. aquaticus* were fed with 0.1 g of the conditioned/standardised alder leaves (*Alnus glutinosa*).

Exposure media

Water from Bramham Park ponds (where the animals were sourced) was used for this experiment. The physico chemical parameters at the point of collection of the culture media were, dissolved oxygen (DO): 12.3 mgL^{-1}, water temperature: 17.2°C, electrical conductivity (EC): 662 µS cm-1 and pH: 7.5. The pH, DO, water temperature and EC were measured weekly with a HACH HQ40d multimeter and the instruments were rinsed with deionised water before every reading taken.

Experimental design

For the mixture experiments, there were two treatments (LT and HT) and solvent controls (SCTR) with 15 replicates of each treatment and 15 replicates of the control. Test concentrations were selected to mimic environmental detection levels reported for UK rivers in the literature. The low treatments (LT) were UK mean measured environmental concentrations of 159.7 ngL^{-1}(ERY), 202.2 ngL^{-1} (DIC), 420.8 ngL^{-1}(IBU) and the high treatments were 1377.8 ngL^{-1}(ERY), 2990.7 ngL^{-1}(DIC) and 4838.4 ngL^{-1}(IBU) respectively and the solvent control contained 0.1 ml L^{-1} of methanol.

For the mixture experiments, the low and high treatments were

mixtures of ERY, DIC and IBU concentrations. The experiments were carried out in clear glass SS jar (500 mL) kept in incubators (Table 2) (Figure 3) at a temperature of 12°C and 16:8 h light: dark regime. The animals were illuminated with a fluorescent light (with a specification for freshwater invertebrates), to simulate on a small scale the macroinvertebrates' natural climatic condition. The glow mimicked the thermal warmth and daytime illumination obtained from the sun radiation.

Table 2 Concentrations of the test compounds (environmental detection levels reported for UK).

Compound	Low concenteration(ngL^{-1})	High Concentration (ngL^{-1})
Erythromycin	159.7	1377.8
Diclofenac	202.2	2990.7
Ibuprofen	420.8	838.4

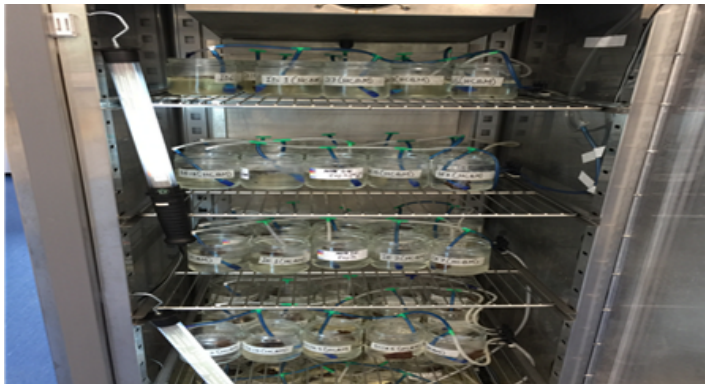

Figure 3 One of the experimental set-ups in the incubators showing the arrangement of the jars with one *A. aquaticus* in each jar exposed to experimental media (Solvent control (STCR), Low treatment (LT) and High treatment (HT)).

Each glass jar contained one *A. aquaticus* with 300 ml of pond water, which was assigned and arranged randomly in the experimental chambers using a random integer generator. Individuals were weighed individually at the start of the experiment and subsequently every week with a Sartorius Quintex 224-1 s balance.

The working solutions of LT 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/resuspended with 10 ml of pond water and washed into the beakers before *A. aquaticus* were introduced.

Before the transparent silica glass beads were reused, they were washed with ultra-clean water, ashed in the furnace at 550°C and allow cooling in the fume cupboard to prevent toxicity in any form to the test animals.

For the mixture experiments, forty-five (45) *A. aquaticus* were used, Exposures were static-renewal with 100% water replacement every week with fresh concentrations of the pharmaceuticals and the experiments were run for 4 (four) weeks. Growth was measured weekly by deducting the initial mass of each *A. aquaticus* from the mass each week. Mortality was determined at the end of the experiments by counting the surviving animals and calculating percentage mortality. Remaining alder leaves (feed material) at the end of the experiments were oven dried, weighed and combusted to determine the feeding rate (ash free dry mass).

Data analysis

Data were organised using Excel (Microsoft, 2013) and residuals of the data were checked for normal distribution using the Shapiro-Wilk normality test and homogeneity of variance using the Bartlett test of homogeneity of variances. R (R Development Core Team, 2008) was used to analyse the data and create figures (Box-and-Whisker). The box-and-whisker plots display a statistical summary of variables: median, quartiles, range and possibly extreme values (outliers). An outlier value is defined as a value that is smaller than the lower quartile (25 percentile) minus 1.5 times the interquartile range, or larger than the upper quartile (75 percentile) plus 1.5 times the interquartile range. Changes in Asellus aquaticus mass, physicochemical parameters and mass of feed materials (*Alnus glutinosa*) 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 Tukey's post-hoc tests to identify and compare the treatment means with the respective controls

Results

Initial test conditions

When the experiment was initiated (day 0) the average mass of *A. aquaticus* was 22.32 mg ± 1.45 SD for control (SCTR), 22.19 mg ± 1.31 SD for low treatment (MIX-LT) and 22.37 mg ± 1.24 SD for high treatment (MIX-HT). There was no statistically significant difference in test organism mass between the treatments and the control (ANOVA: $F_{2, 42}$=0.073, p=0.929).

Growth

When the residuals of the data were analysed for change in mass over the course of the experiment, there were statistically significant differences between the treatments and the control (GLM: χ^2 (2)=10.07, p<0.01), (Figure 4).

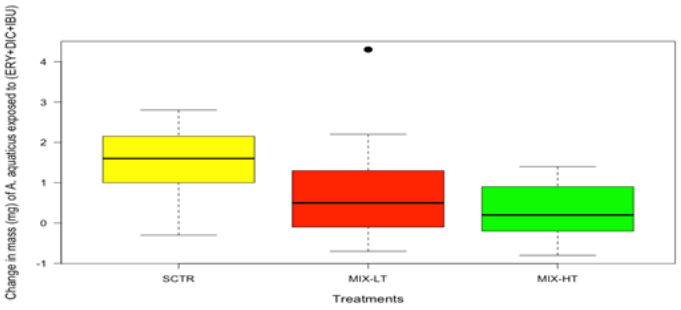

Figure 4 Boxplots displaying change in mass of *A. aquaticus* exposed to mixtures of erythromycin, diclofenac and ibuprofen

after 4 weeks static renewal experiments. Solvent control (SCTR), low treatment (MIX-LT) and high treatment (MIX-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), bottom of the coloured box represents the 2ndquartiles (25th percentile) and the vertical lines represents the 1st quartiles (10th percentile). There was outlier.

Feeding

There were statistically significant differences in the mass of feed materials between control and treatments (ANOVA: $F_{(2,42)}$=6.72, p<0.01). The mass loss of *Alnus glutinosa* litter by the control was higher than those in the treatment groups i. e. feeding rate in the control was higher than the treatments, (Figure 5).

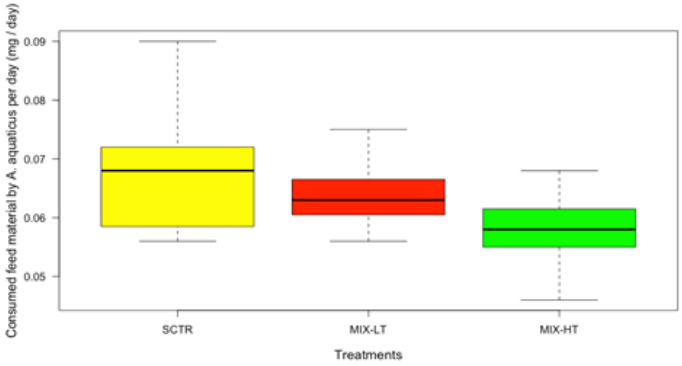

Figure 5 Boxplots displaying consumed feed materials by *A. aquaticus* exposed to environmental relevant concentrations of mixtures of erythromycin, diclofenac and ibuprofen after 4 weeks static renewal experiments. Solvent control (SCTR), low treatment (MIX-LT) and high treatment (MIX-HT). The dark 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), bottom of the coloured box represents the 2nd quartiles (25th percentile) and the vertical lines represents the 1st quartiles (10th percentile). There were no outliers.

Mortality

Mortality did not occur in the control throughout the duration of the experiments. One and two mortalities were recorded in the fourth week of the experiments in the MIX-LT and MIX-HT respectively (Figure 6) but there was no statistically significant difference between the treatments and control (GLM: $\chi 2$ (2)=22.11, p=0.56).

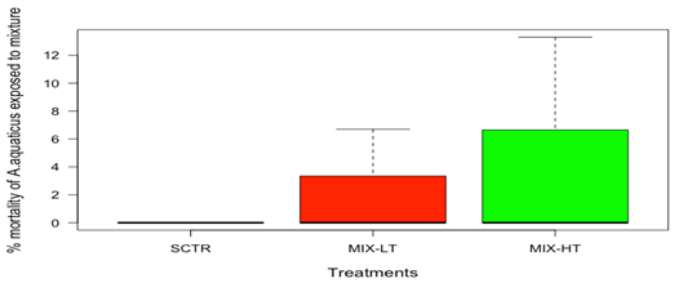

Figure 6 Boxplots displaying % mortality of *A. aquaticus* exposed to environmental relevant concentrations of mixtures of erythromycin, diclofenac and ibuprofen after 4 weeks static renewal experiments. Solvent control (SCTR), low treatment (MIX-LT) and high treatment (MIX-HT). The dark horizontal line 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), bottom of the coloured box represents the 2nd quartiles (25th percentile) and the vertical lines represents the 1st quartiles (10th perc entile). There were no outliers.

Discussion

The aim of this study was to seek to improve the understanding of the effects of prolonged low-level exposure of Asellus aquaticus to mixtures of erythromycin, diclofenac and ibuprofen. There are few data on the use of *A. aquaticus* as a test species in pharmaceutical effect studies but there is substantial information on its use in metal toxicity. This study is one of the few in which *A. aquaticus* is used in pharmaceutical effect studies.

Effects of mixtures of erythromycin, diclofenac and ibuprofen on growth, feeding and mortality on A. aquaticus.

Going forward in the present study, effects of mixtures of erythromycin, diclofenac and ibuprofen at relevant environmental concentrations via direct (waterborne) exposure pathway on Asellus aquaticus in a 4 weeks bioassay was investigated. Sublethal responses such as growth, feeding behaviour and mortality were analysed. It was observed that the mixture negatively affected the growth and feeding activities of the test organism. However, synergism was exhibited in this present study, this maybe as a result of different receptors targeted by the compounds, NSAIDs targeting COX 1 and 2 and ERY targeting prokaryotic cells. In a similar investigation by [40] in which Hydra was exposed to mixtures of pharmaceuticals for 96 h, there was reduction in the ability of the freshwater Hydra to regenerate.

Investigations by [39] also supported the findings in this study, although, Pimephales promelas (Fathead minnow) was used to study the effects of mixtures of seven drugs at concentrations of 1 µg L^{-1} for 3 months. The degree of defects observed in the fathead minnow were small. Investigated binary mixtures of a hormone (17 β-estradiol) with letrozole at environmentally realistic concentration and detected significant decrease in fertility and fecundity after 21 d of exposure [41].

In a similar study to this experiment, even though different compounds and test species exposed Lemna gibba (Fat Duckweed) to different mixture of drugs similar sensitivity was demonstrated by the test species at concentrations 1–300 µg L^{-1}. After 7 d, the test specie showed sign of necrosis.

In a multigenerational mixture experiment in which acetaminophen, diclofenac, ibuprofen and a host of other compounds were used, it was observed that the sex ratio was altered by 17% more males. In a binary combination (diclofenac and ibuprofen) and quaternary (ibuprofen, acetylsalicylic,

naproxen and diclofenac) exposed to D. magna, a very strong additive effect was observed at concentrations of 34-54 mgL^{-1} (Cleuvers, 2004). Very strong additive effects were also observed when D. magna was exposed at concentrations 10-fold lower than the quaternary concentrations. The body size and reproduction were affected (Cleuvers, 2008).

In a study carried out by [38]. Carcinus maenas was shown to have significant changes in haemolymph osmolality and osmoregulatory capacity after being exposed to relevant environmental concentrations of the mixtures (10 ngL^{-1} and 17.5 psu of salinity). The osmoregulatory ability of the mixture was improved, implying a reduction in benefit by organisms and a rise in haemolymph osmolality. The *A. aquaticus* exposed to the mixtures of erythromycin, diclofenac and ibuprofen started losing weight as a result of the exposure while the control animals are gaining weight weekly. Feeding rate was equally affected; the exposed isopod was feeding at reduced rate in the low and high treatments compared to the control. Hence there was alteration in feeding rate of *A. aquaticus* exposed to the mixture. Similar investigation on Hydra attenuata, showed that minimum concentrations of 10 mgL^{-1} and 50 mgL^{-1} was needed to observe a significant reduction in feeding activities when exposed for 96 h to ibuprofen and carbamazepine respectively [42]. This concentration was 1000 times higher than the concentrations employed in this study though, with different study compounds and test animals and duration.

De Lange established the effects of pharmaceuticals on feeding activities and behaviour of other macro-invertebrate animals, using concentrations similar to those used in this study but different pharmaceutical compounds were used.

Considering the feeding rate and growth between the control and treatments, one-way ANOVA/GLM results suggested that there was a significant interaction.

The realistic environmental concentrations of isolated compounds such as diclofenac and erythromycin do cause increase mortality, reduced feeding rate and growth. However, when they are in mixtures, these compounds may present increased (synergistic) or reduced (antagonistic) inherent toxicity. Aside this, diclofenac and ibuprofen have similar mode of action (MoA) and hence they may act (additively) synergistically. Addition of erythromycin to this mixture may cause it to act antagonistically, hence the result obtained in this study.

The low and high treatments did not show any sign of increase mortality as a result of the exposure to the mixture. There were only three mortalities throughout the duration of the study, one in the low and two in high treatments and none in the control.

Generally, many scientists agree that concentration addition (CA) is appropriate for estimating mixture toxicity of substances acting in a similar manner, while independent action (IA) assumes that in a mixture of different chemicals, the effects exerted by individual chemical are not dependent on others. The key limitation of the concentration addition model, as noted, is that differences may be detected for some mixtures containing drugs for which only low effects are detected [43].

Conclusion

Based on this study, it can be suggested that *A. aquaticus* can be recognized as a reference model test animal and good indicator to evaluate the potential effects of contaminants. The results of this study showed that the toxicity of drug mixtures is unpredictable, and complex compared to effects of single pharmaceuticals. However, the mixtures showed concentration addition (CA) effects and one of the weaknesses of this model is that differences are sometimes seen for some mixtures containing drugs for which only little effects are detected.

Causes of Climate Change

The underlying causes of climate change are human actions. The measurements made in the last 50 years show that there has been a rise in air temperature of around 0.1°C per decade. The key reasons for the rise in temperature are industry, trade, power supply.

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