RESPONSE OF *Gammarus pulex* TO LOW CONCENTRATIONS OF ERYTHROMYCIN

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ABSTRACT
Pharmaceuticals are manufactured for the benefit of mankind and used for specific biological functions in veterinary and human medicine. However, these substances after use enter the aquatic environment through different sources either as parent compounds, metabolites or a combination of both. There are significant research gaps for thousands of existing pharmaceuticals with regards to their potential ecological consequences on non-target species. Pharmaceutical pollution is an emerging area of research in Africa and no work had been done in Nigeria on effects of pharmaceuticals in river systems. In this study, the biological effects of one of the most frequently detected pharmaceuticals in the aquatic environment, erythromycin was investigated at environmentally relevant concentrations reported for UK Rivers. Seventy-five (75) Gammarus pulex were employed in the laboratory experiments. Exposure resulted in statistically significant decreases in feeding, growth and an increase in mortality. The feeding rate in the control groups were higher than those in the treatment groups and G. pulex in the control groups gain weight. Mortality among the treatment groups were consistent compared to the controls. Although pharmaceuticals are indispensable to human health, but their usage and discharge into the aquatic environment may lead to ecological problems and antibiotic resistance.
Keywords: Aquatic environment, Concentrations, Ecological effects, Erythromycin, *Gammarus pulex*,

INTRODUCTION

Widespread occurrences of pharmaceuticals and personal care products (PPCPs) have been found in the surface waters worldwide including Africa in recent times. This release of pharmaceuticals and their metabolites into the aquatic environment has become a significant issue of increasing importance (Huggett et al. 2003, Fent *et al*. 2006). Wastewater treatment plants are implicated as the main routes of entrance of PPCPs into freshwater systems as a large portion of PPCPs that are ingested are excreted by the human body and ultimately end up in sewage treatment systems. Some of these compounds are not completely eliminated by wastewater treatment plants and they are expelled with the effluent into surface waters (e.g. Heberer, 2002; Blair *et al.,* 2013a, b). There is little known about the effects of a large number of these PPCPs on aquatic organisms that inhabit these areas, specifically at the concentrations they are found in the environment, e.g. the macrolide antibiotic erythromycin (Cleuvers 2008, Fent *et al*. 2012). Overall, there is not enough experimental data to determine which PPCPs may be of most concern (Brooks *et al*., 2010).

Pharmaceuticals are intended to have a biological effect, which makes them potentially harmful xenobiotics. Antibiotics, a class of common pharmaceuticals, have received growing attention on account of their widespread use, ubiquitous environmental distribution, great bioaccumulation potential, and possible toxicity to non-target organisms on physiological and behavioural processes, such as development, reproduction, and nervous-system function. However, in most cases in which effects were detected, the concentrations were environmentally irrelevant (Kümmerer, 2009; Liu *et al.*, 2013; Rocco *et al.*, 2012). Due to the continuous release into the environment because of various human activities, via wastewater effluent discharge, agricultural runoff, or improper disposal of unused drugs, antibiotics were detected at concentrations ranging from ngL^{-1} to several μgL^{-1} in surface waters (Liu and Wong, 2013; Zhu *et al*., 2013; Lu *et al*., 2014).

Erythromycin (ERY, CAS 114-07-8) is a macrolide antibiotic and is widely used to treat upper/lower respiratory tract infections, skin infections, acute pelvic inflammatory disease and erythrasma by binding irreversibly to the subunit 50S of the bacterial ribosome. ERY is a lipophilic pharmaceutical, and its log octanol-water partition coefficient (LogKow) is 3.06 (Jones *et al.*, 2002, Lu *et al*., 2014). Due to its frequent usage, ERY is detected in surface water between levels of several ngL^{-1} and 1.7 μgL^{-1} (Kleywegt *et al.*, 2011; Kolpin *et al.,* 2002; Li *et al.,* 2012a), and its photo degradation rate was below 36.7% under natural light for 45 days (Xiao *et al*., 2008). However, relatively higher concentrations of ERY (6.0 μgL^{-1}) found in the effluent of sewage treatment plants (STPs) (Hirsch *et al*., 1999; Kümmerer, 2009) might be due to its low removal efficiency (generally < 50%) (Gao *et al.,* 2012a). Moreover, ERY has been selected to the Drinking Water Contaminant Candidate List formulated by the U.S. Environmental Protection Agency (US EPA, 2010). Clearly, more information is required to guide decisions about regulations on ERY (Sara Rodriguez-Mozaz, 2010).

Recent studies have indicated unintended biological activity of ERY on non-target organisms. The administration of ERY, even at low concentrations of 6.3 μgL^{-1} (the maximum concentration of STP effluents reported by Hirsch *et al*. (1999), can strongly inhibit the growth of certain cyanobacteria (Ando *et al*., 2007). Jessick (2010) observed that the freshwater species *Daphnia magna* and *Lumbriculus variegatus* could absorb ERY from water and sludge, respectively. After 21 days of chronic exposure, the survival of

Daphnia magna and the number of young per brood was significantly reduced at 100 mgL^{-1} and 33.3 mgL^{-1} of ERY, respectively (Ji *et al.*, 2012). Moreover, ERY showed a more powerful genotoxic effect on zebrafish erythrocytes and hepatocytes at an exposure concentration of 100 mgL^{-1} (Rocco *et al.*, 2012) and exhibited potential bioaccumulation in crucian carp, with a mean BAF (bioaccumulation factor) value of 4492 kgL^{-1} (Gao *et al.*, 2012b). In mammals, the principal elimination pathway for ERY is N-demethylation by hepatic cytochrome P-450 (CYP); only a small proportion of the drug is excreted unchanged in urine and bile (Craig *et al.*, 1993). Yet, biological effects of ERY have not been well studied in aquatic organisms, particularly using *G. pulex*, which are vulnerable to exposure to environmental toxins.

The current work investigated the ecological effects of prolong low-level exposure at environmentally relevant concentrations of ERY on feeding, growth and mortality of freshwater benthic macroinvertebrate animals (*G. pulex)* with the aim of broadening knowledge about the potential risk of such contamination to aquatic ecosystems.

MATERIALS AND METHODS

Materials

ERY (CAS no.114-07-08, purity > 99%), molecular weight 733.93$gmol^{-1}$ and molecular formula $C_{37}H_{67}NO_{13}$, (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.67-56-01, purity ≥99.9%) was purchased from Fischer 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 MΩcm. Chemical stock solutions were 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 prior to the experiments.

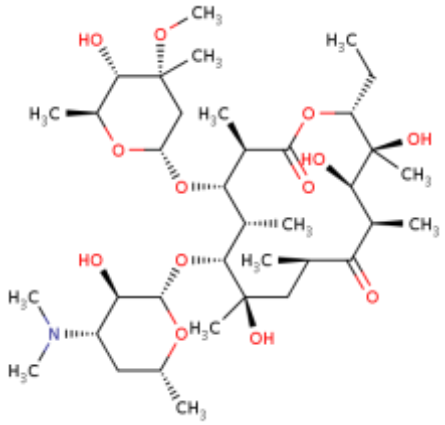

Fig. 1 Chemical structure of erythromycin

Preparation of solutions

Environmentally relevant concentrations of ERY were used in this experiment (UK mean measured environmental concentration [LT - Low Treatment] and UK maximum measured environmental concentration [HT - High Treatment] and medium concentration [MT - Medium Treatment] was the average of the LT and HT). These treatment concentrations were chosen as an indicator of likely exposures based on published mean for UK rivers and an indicator of worst-case exposure scenario based on maximum concentrations in UK rivers. One hundred mgL^{-1} ($100mgL^{-1}$ = 10mg/100ml) of ERY was prepared by dissolving in methanol (HPLC grade). The desired experimental concentrations were achieved through a series of dilutions. All solutions were stored at −20 °C and in the dark for optimum stability and to avoid photodegradation.

Test animals: origin and maintenance

Gammarus pulex were collected in ponds at Bramham Estate, Leeds, West Yorkshire, United Kingdom and were sampled with a net from 1.5 to 4 m depth. They were brought to the laboratory in cool boxes. Amphipods of approximately the same size averaging (21.47 ± 2.45 mg) 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 acclimatise in aerated pond water before the exposure experiments started. They were fed on conditioned alder leaves (*Alnus glutinosa)*, the leaves also act as shelter for the animals. The leaves were conditioned in an aerated bucket at room temperature where they were inoculated with bacteria and fungi. Sexing is 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 serve as fish food, played important role in the food chain, eliminating them will disrupt the balance in the ecosystem. They are also bio indicators of the stream health, generally abundant, easy to sample and most likely to be affected by pollution because they have little mobility.

Exposure media

Water from Bramham Estate 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) for every reading taken.

Methods

The experiment was carried out in glass clear SS jar (500 mL) with face lined cap kept in incubators at a temperature of 12° C and 16:8 h light: dark regime each containing 300mL of pond water. Each glass jar contained one *G. pulex* which was assigned randomly, weighed individually at the start of the experiment and subsequently every week with Sartorius (model: Quintex 224-1s) balance. Exposure glass jars were

arranged randomly to avoid the influence of potential gradients in the incubator, and all the *G. pulex* were assigned in the experimental chambers using random integer generator to avoid subjectivity of the experimenter. Water samples for chemical analyses of the compounds were collected every week and analysed. They were fed with 0.1gm of standardised alder leaves.

There were three treatments (LT, MT and HT), negative and solvent controls with 15 replicates of each treatment and 15 replicates of each control. The solvent control used in the experiment was tested for different responses of the physiological measurements compared to the negative control. 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 159.7 ngL^{-1} (LT), 768.75 ngL^{-1} medium treatment (MT) and 1,377.8 ngL^{-1} high treatment (HT) for erythromycin respectively (Hughes *et al*., 2013, Bound and Voulvoulis, 2006). The negative control contained no treatment and the solvent control contained 0.1mlL^{-1} of methanol. The working solutions of LT, MT and HT were poured on a 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 *G. pulex* were introduced. Before the transparent silica glass beads were reused 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.

In all seventy-five (75) male *G. pulex* were assigned at random among the five experimental groups. Exposures were static-renewal with 100% water replacement every week with fresh concentrations of the pharmaceutical. The collected water samples were stored at -20° C and filtered, extracted with SPE units into 4 ml amber glass vial and analysed. The experiments were run for 4 weeks. Response variables such as growth, mortality and physicochemical parameters were measured weekly and mass of feed materials loss measured at the end of the experiment.

Data analyses

Data were organised using Excel (Microsoft, 2013) spreadsheet. Residuals of the data were checked for normal distribution (Kolmogorov and Smirnov method) and homogeneity of variance (Bartlett method). R (R Development Core Team, 2008) was used to analysed the data and create figures. Tukey's post-hoc tests were used to identify the means that differed. Percentage change in *G. pulex* mass, physicochemical parameters and feed materials (*Alnus glutinosa*) were tested using generalised linear model. Mortality were 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

Initial test conditions

When the experiment was initiated (day 0) the cumulative mean mass of *G. pulex* (across treatments and controls) was 21.47 ± 2.45 mg and no statistically significant difference (ANOVA: $F_{4, 70} = 0.09$, $p = 0.99$) were recorded between treatment groups and the control groups. The cumulative mean dissolved oxygen (DO) was 9.30 ± 0.01 mgL^{-1} and p=0.57, pH was 8.5 ± 0.03 and p=0.43, water temp was 14.10 ± 0.19°C and p=0.18, mean electrical conductivity (EC) was 598.09 ± 4.64 μScm^{-1} and p=0.14. There were no statistically significant differences in the water chemistry.

Feeding

There were statistically significant differences in mass of feed materials between controls and treatments (H^{2} (4) = 21.308, p<0.0005). The % mass loss of *Alnus glutinosa* litter by the control group were higher than those in the treatment group i. e. feeding rate in the controls were higher than the treatments. Even between the treatments group, the feed materials loss was dose dependant and significantly influenced by erythromycin figure 2.

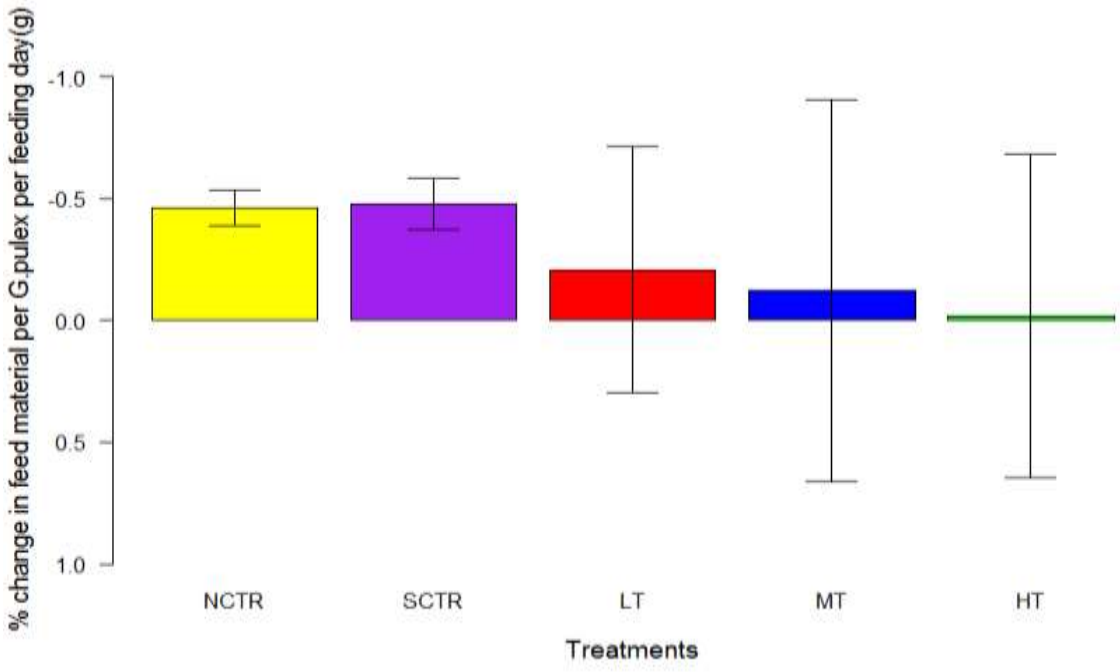

Figure 2. Mean % change in mass of Alder leaves (*Alnus glutinosa)* per *G. pulex* per day following a 4-week static renewal exposure to erythromycin, negative control (NCTR), solvent control (SCTR), low treatment (LT), medium treatment (MT) and high treatment (HT), [n=15, bars represent means and vertical lines represent error bars at ±1SD].

Growth

The growth of *G. pulex* was reduced at 0.16 μgL^{-1}, 0.77 μgL^{-1} and 1.38 μgL^{-1} concentrations of erythromycin over a 4 week period. When the % change in mass was analysed there were statistically significant difference between the treatment groups and the control groups (ANOVA: $F_{4, 37} = 30.49$, $p < 0.001$), figure 3. In the experimental period *G. pulex* increased in mean mass in the control groups (NCTR: 23.35 ± 2.99 mg & SCTR:
23.09 ± 3.96 mg) and there were decreased mass in the treatment groups (LT: 20.29 ± 2.54 mg, MT: 20.03 ± 2.85 mg and HT: 19.97 ± 2.62 mg). However, mean mass decreased, was more pronounced in the high dose treatment than the other treatments. The results showed sign that erythromycin reduces the growth of *G. pulex* and may pose a risk to the environment and possibly the wider health of humans and animals.

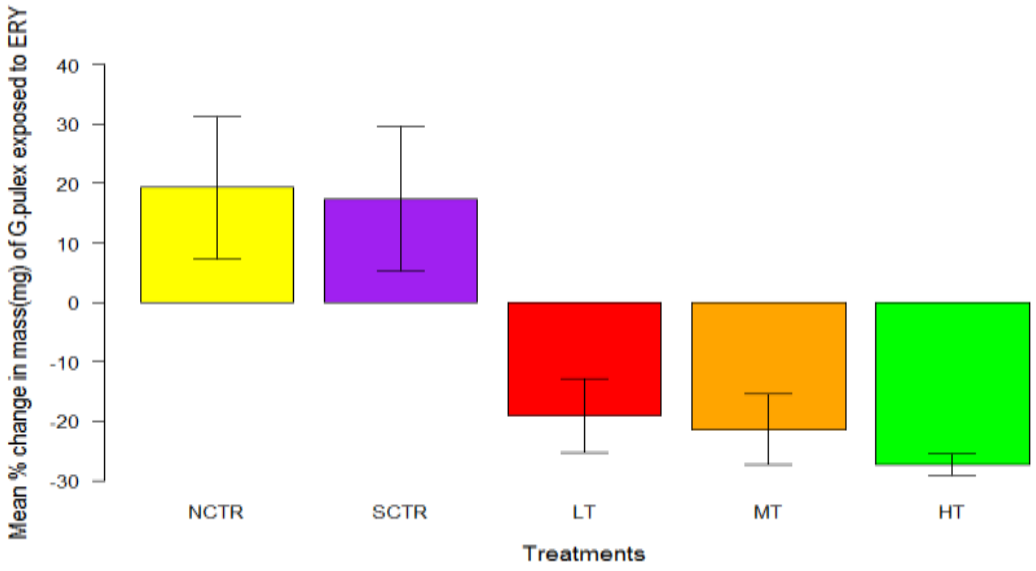

Figure 3. Mean % change in mass of *G. pulex* following a 4-week static renewal exposure to erythromycin, negative control (NCTR), solvent control (SCTR), low treatment (LT), medium treatment (MT) and high treatment (HT), [n=15, bars represent means and vertical lines represent error bars at ±1SD].

Mortality

In the first week of the experiment there were no mortality recorded in all the treatments (LT, MT, HT) and the controls (NCTR, SCTR). Mortality commenced in the second week with 4, 5 and 5 *G. pulex* recorded corresponding to LT, MT, HT respectively and none recorded for the controls (figure 4). For both medium and high treatments, more than 50% of the mortality had taken place before the fourth week.

In the fourth and final week of the exposure 53.33%, 73.33% and 86.67% mortality were recorded for LT, MT, HT respectively. In the control group, total mortality amounted to one individual (6.67%) and this was recorded in the third week by SCTR. Statistically significant differences (GLM: χ^2 (4) = 24.95, $p < 0.001$), were found in mean % cumulative mortality comparing high, medium and low-dose treatments at the end of the study with the control groups.

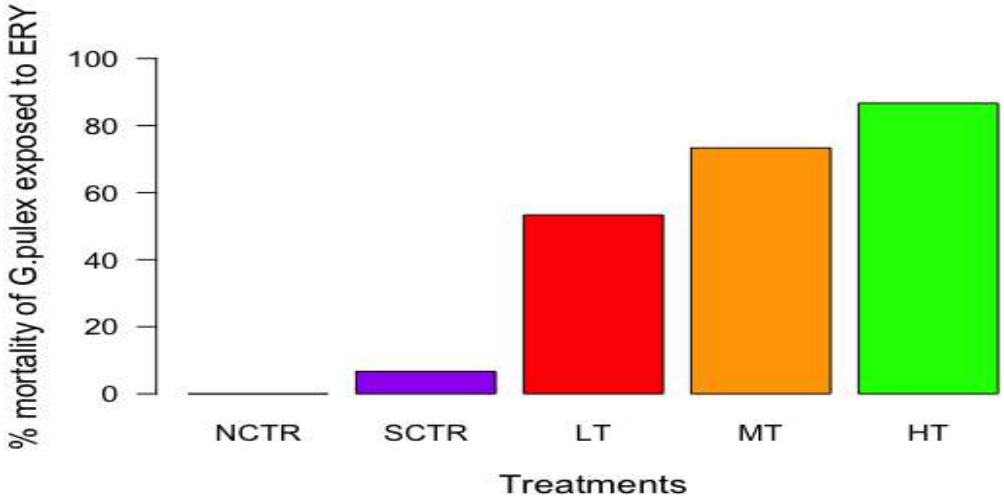

Figure 4. % mortality of *G. pulex* following a 4-week static renewal exposure to erythromycin, negative control (NCTR), solvent control (SCTR), low treatment (LT), medium treatment (MT) and high treatment (HT), [n=4, bars represent means].

DISCUSSION

The objective of this experiment was to assess the ecological effects of prolonged low-level exposure to environmentally realistic concentrations of erythromycin on feeding, growth, and mortality of *G. pulex*. It is a well-known fact that growth and feeding are sensitive indicators of varieties of stressors in freshwater amphipods (Hughes *et al.*, 2013; Naylor, 1989; Maltby & Naylor, 1990). Erythromycin, a macrolide antibiotics that blocks bacterial protein synthesis had negative effects on the feeding, growth and mortality of *G. pulex* at all concentrations tested. At concentrations 0.16 μgL^{-1}, 0.77 μgL^{-1} and 1.38 μgL^{-1} erythromycin seemed to affect the feeding of *G. pulex*. Decreased feeding inevitably led to reduce energy intake, which had far-fetched effects on growth, mortality and other behavioural activities. In the wild this may affect reproduction, population success and may alter predator avoidance behaviour and will disturb the predator–prey balance. However, this may have a short-term advantage for the predator (i.e. increased prey consumption), but long-term effects may be negative when the prey source is overexploited. Overall, substantial increases in mortality, reduced feeding and growth of *G. pulex* were found as a result of erythromycin exposure. The anti-histamine cimetidine has been shown to reduce the growth and biomass of other gammarids (*Gammarus fasciatus*) at environmentally realistic concentrations (Hoppe *et al.*, 2012; Hughes *et al.*, 2013). The exposure of *G. pulex* to low level concentrations of erythromycin for 4 weeks indicated a similar statistically significant trend of decreased feeding, reduced growth and increase mortality. The exposed *G. pulex* were feeding at a reduced rate compared to the control and this was significant. The reduced feeding rate and decreased in growth in this study might had been due to the fact that the animals were standardised by size and gender as suggested by Hughes *et al.*, 2013; Willoughby & Sutcliffe, 1976; Sutcliffe *et al.*, 1981 "that feeding and growth of *G. pulex* may be strongly dependent on size and gender so future studies should seek to standardise by these factors". During the experiments, care was taken to minimise stress and prevent injury to *G. pulex* when separating the precopula pairs and during the weekly measurement. Sublethal effects of pharmaceuticals on the behaviour and activity of other amphipod species have also been established at concentrations similar to those reported here (De Lange *et al.*, 2006; De Lange *et al.*, 2009), albeit with different compound classes (antidepressants and anti-inflammatories).

Clear and consistent increases in mortality were observed for erythromycin starting from 2nd week. This compound caused a doubling in mortality after relatively long exposures in the medium (MT) and high treatment (HT) at environmentally relevant concentrations. Lethal

concentrations for erythromycin ($\leq$1400ngL^{-1}) demonstrated in this study were up to four orders of magnitude below those demonstrated in short-term exposure tests of other aquatic invertebrates (Webb, 2001; Kim *et al.,* 2009). Other freshwater taxa such as green algae appear to be more sensitive than *D. magna* but even these effects manifest at levels 60x the nominal concentration of the lowest exposure reported here. This demonstrates that longer experimental exposures are likely to change the conclusions drawn from short-term experiments substantially. In terms of the ecological impact of therapeutic drugs on aquatic environments, the results presented here may have potential implications for the future assessment of risk associated with freshwater ecosystems and consumption of water.

CONCLUSION

These results show that environmental concentrations of erythromycin reduced the growth, feeding rate and increased mortality of *G. pulex*. These concentrations are much lower than previously reported lowest observed effect concentrations (LOEC) for other organisms, indicating that growth, feeding and mortality are much more sensitive endpoint than other endpoints. Environmental concentrations of those compounds in surface waters are high enough to reduce *G. pulex* activity. The potential negative cascading effects on *G. pulex* population growth and benthic community structure, and the mixture effect of pharmaceuticals present in surface waters should be studied further. This result, however, suggest that even ngL^{-1} concentrations of therapeutic drugs can affect the growth, feeding and survival of aquatic macro-invertebrate animals.

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