Chronic toxicity and environmental risk assessment of antivirals in *Ceriodaphnia dubia* and *Raphidocelis subcapitata*

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**ABSTRACT**

Antiviral drugs are a class of medications used for treating viral infections. Due to their widespread use, especially in cases of pandemics and limited human metabolism, antivirals have been detected in multiple environmental matrices. This study aims to evaluate the chronic effects of acyclovir, efavirenz, lamivudine and zidovudine using *Ceriodaphnia dubia* and *Raphidocelis subcapitata*. The results with *R. subcapitata* showed the following toxicities: zidovudine (IC\(_{50}\) = 5.442 mg L\(^{-1}\)) < acyclovir (IC\(_{50}\) = 3.612 mg L\(^{-1}\)) < lamivudine (IC\(_{50}\) = 3.013 mg L\(^{-1}\)) < efavirenz (IC\(_{50}\) = 0.034 mg L\(^{-1}\)). The results of the chronic bioassay with *C. dubia* demonstrated that zidovudine is the least toxic (EC\(_{50}\) = 5.671 mg L\(^{-1}\)) followed by acyclovir (EC\(_{50}\) = 3.062 mg L\(^{-1}\)), lamivudine (EC\(_{50}\) = 1.345 mg L\(^{-1}\)) and efavirenz (EC\(_{50}\) = 0.026 mg L\(^{-1}\)). Both species have been shown to be sensitive to efavirenz. A risk quotient (RQ) was calculated, and efavirenz had an RQ greater than 1 for both species, and lamivudine had an RQ greater than 1 for *C. dubia*, representing a high ecological risk for these organisms. Antivirals pose a significant environmental risk to aquatic organisms and should be taken into consideration in future monitoring of water sources.

**Key words:** antivirals, *Ceriodaphnia dubia*, chronic bioassay, ecotoxicity, environmental risk assessment, *Raphidocelis subcapitata*

**HIGHLIGHT**

- Very few studies have been published about antivirals toxicity in aquatic organisms. This study is important not only to increase the availability of ecotoxicological data, but also improves the relevance of environmental risks. Results found that Efavirenz was the most toxic for species, suggesting that environmental concentrations can affect the exposed biota. We declare that this manuscript is original.

**INTRODUCTION**

Antiviral drugs have only recently been recognized as emerging contaminants, and conventional wastewater treatment plants (WWTPs) are not designed to remove them efficiently ([Prasse et al. 2010; Ngumba et al. 2016; Nannou et al. 2020]). Therefore, WWTPs play an important role as pollution source of antivirals in the environment. The physicochemical characteristics of antivirals (water solubility, molecular weight, chemical structure, volatility, and polarity) vary widely; therefore, there is a possibility that they would show recalcitrance in wastewater treatment ([Jain et al. 2013; Evgenidou et al. 2015; Schoeman et al. 2017]). Despite advances in water treatment technologies, effluents remain an important source of antiviral load due to low removal efficiencies ([Funke et al. 2016]).

The consumption of antivirals has been increasing due to infections and viral pandemics worldwide. Antiviral drugs are a group of pharmacologically active compounds used for treating viral infections, including herpes, hepatitis, human immunodeficiency virus (HIV) and influenza ([De Clercq 2004]). Despite their importance in treating a variety of diseases, the occurrence and fate of antiviral drugs in the urban water cycle have been less investigated than those of other pharmaceutical products, such as antibiotics, analgesics or antihypertensives ([Evgenidou et al. 2015; Dong et al. 2016]). These are discharged in effluents from production facilities and, after therapeutic use, through human excretion or direct discharge ([Jain et al. 2013]). Consequently, several antivirals have been detected in environmental matrices ([Funke et al. 2016; Abafe et al. 2018; Mosekiemang et al. 2019]); however, they are not always systematically monitored, and ecotoxicological effects remain unknown.

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Acyclovir (ACV) is one of the oldest and most widely used antiviral drugs to treat infections caused by the herpesvirus family, including *herpes simplex* virus (HSV) types 1 and 2, *Varicella zoster* virus (VZ), *Epstein Barr* virus (EBV) and cytomegalovirus (CMV) (De Clercq 2004). It is prescribed for patients with weakened immune systems to control pathologies such as viral conjunctivitis (Bryan-Marrugo et al. 2015). It was found the ACV, of which 45–75% is excreted by patients as unchanged compound (Vergin et al. 1995). Thus, ACV, like other pharmaceuticals, finds its way into the environment through wastewater collection systems (Hernando et al. 2006). Efavirenz (EFV) is a nonnucleoside analogue reverse transcriptase inhibitor (NNRTI) that has been widely used to treat mutant strains of HIV-1 since 1998 and is the third most commonly used antiretroviral by HIV-positive people worldwide (Larru et al. 2014). According to Eckhardt & Gulick (2017), 16–61% of EFV is eliminated in faeces (primarily as parent drug) and 14–54% in urine (primarily as metabolite). Lamivudine (3TC) is a nucleoside analogue reverse transcriptase inhibitor, that interferes with the reverse transcriptase (RT) activity of a virus, causing pro-viral DNA chain termination (De Clercq 2004). Lamivudine is largely excreted in the urine (approximately 70%) as an unchanged drug (FDA 2017). Zidovudine, also known as azidothymidine (AZT), was approved in 1987, making it the first antiretroviral drug approved. AZT is a nucleoside analogue reverse transcriptase inhibitor class that inhibits the activity of reverse transcriptase enzymes, stopping viral replication of HIV (De Clercq 2004). AZT is excreted in urine as a primary metabolite (45%) and as an unchanged drug (29%) (FDA 2008).

Acyclovir is the most investigated drug in many countries (Prasse et al. 2010; Bradley et al. 2014; Azuma et al. 2016). The highest concentration of ACV in surface waters was measured in the United States at 1,410 ng L\(^{-1}\) (Bradley et al. 2014). The concentration levels of EFV indicate that it is a recalcitrant antiviral drug (Schoeman et al. 2017); the drug has a high volume of production and is likely to be persistent and/or bioaccumulative (Howard & Muir 2011). EFV was detected in effluents at concentrations of 17.4 to 34 \(\mu\)g L\(^{-1}\) (Schoeman et al. 2015; Abafe et al. 2018) and in surface waters at a maximum concentration of 2.45 \(\mu\)g L\(^{-1}\) (Mtolo et al. 2019). Some studies have quantified lamivudine in surface waters at concentrations ranging from 11.5 ng L\(^{-1}\) to 167 \(\mu\)g L\(^{-1}\) in Kenya (K’oreje et al. 2016; Ngumba et al. 2016), and in effluent at concentrations ranging from 22 ng L\(^{-1}\) to 31 \(\mu\)g L\(^{-1}\) (K’oreje et al. 2016; Ngumba et al. 2016). Abafe et al. (2018) found concentrations of zidovudine in wastewater influent and effluents to be as high as 53 \(\mu\)g L\(^{-1}\) and 500 ng L\(^{-1}\), respectively. In surface waters, AZT was detected at concentrations up to 17 \(\mu\)g L\(^{-1}\) in Kenya (K’oreje et al. 2016), and Prasse et al. (2010) detected AZT at concentrations up to 170 ng L\(^{-1}\) in Germany.

Some antivirals are bioactive and highly persistent in aquatic environments, and they may negatively affect nontarget organisms (Jain et al. 2013). Antivirals can be toxic and represent one of the most hazardous pharmaceuticals towards algae, daphnia and fish (Sanderson et al. 2004). Antiviral drugs released to the environment are of concern due to potential long-term effects on wildlife and viral resistance development (Nannou et al. 2020). Currently, very few studies have been published on their toxicity in aquatic organisms, requiring further investigation.

*Raphidocelis subcapitata* and *Ceriodaphnia dubia* are important organisms in aquatic ecosystems. Microalgae play an important role in maintaining the balance of water bodies and have been frequently used in ecotoxicological bioassays because they are important for assessing the toxicity of organic and inorganic compounds (Cho et al. 2008; Zhang et al. 2015). Numerous studies have utilized microalgae due to their ecological significance, easy cultivation, rapid response and high sensitivity to various environmental contaminants (Petsas & Vagi 2017). *Ceriodaphnia dubia* belongs to the cladocerans, a group of zooplankton that are broadly distributed in tropical areas, although it is also found in some temperate habitats (Jaikumar et al. 2018). These organisms play an important role in the food chains of freshwater habitats worldwide and can be used in a kind of standardized test (Thaysen et al. 2018).

In the present study, we aimed to perform an environmental risk assessment and assess the chronic effects of four antivirals using two species of aquatic organisms, the cladoceran *Ceriodaphnia dubia* and the green alga *Raphidocelis subcapitata*. The selected compounds, acyclovir, efavirenz, lamivudine and zidovudine, are representative of the class of antivirals and were chosen based on their increasing consumption worldwide and lack of ecotoxicological data. Finally, after assessing the environmental risk based on EMA (2006), the risk quotient (RQ) of each drug was estimated, which will contribute to the knowledge of the environmental risks associated with these antivirals.
METHODS

Chemicals

Acyclovir and lamivudine were purchased from Sigma Aldrich (purity: 94.9 and 96%, respectively), and efavirenz and zidovudine were purchased from the National Institute for Quality Control in Health – INCQS, Fiocruz (purity: 99.6% and 99.4%, respectively). Table 1 presents the physicochemical properties, and predominant species at pH 7 of the antiviral drugs.

Stock solutions of each drug were prepared separately in culture media specific to each organism and stirred for 30 minutes after preparation for complete solubilization of the compounds. Concentrations of 20 mg L⁻¹ acyclovir, lamivudine and zidovudine and 1 mg L⁻¹ efavirenz were used. Efavirenz stock solution (1 mg L⁻¹) was prepared by dissolving the compound in 0.1% methanol, v/v. This procedure was necessary due to the low solubility of this drug in water.

Raphidocelis subcapitata cultures

The green alga strain *Raphidocelis subcapitata* was obtained from the Culture Collection of Sanitary Engineering Laboratory of the University of the State of Río de Janeiro. The algae were cultured in 1,000 mL flasks with approximately 500 mL of medium prepared according to NBR 12,648 (ABNT 2018). The cultures were maintained in the laboratory by reinoculation in sterilized flasks with freshly prepared medium once a week.

Chronic bioassay with *Raphidocelis subcapitata*

Algal growth inhibition tests were performed in accordance with the procedure described by NBR 12,648 (ABNT 2018) using the freshwater alga *Raphidocelis subcapitata* in the exponential growth phase. Erlenmeyer flasks with a capacity of 150 mL

Table 1 | Antivirals, molecular formula, molecular weight, CAS number, predominant species at pH 7, pKa, log Kow and water solubility

| Antivirals   | Molecular formula | Molecular weight | CAS number | Predominant species at pH 7 | pKa | Log Kow | Water solubility (mg mL⁻¹) |
|--------------|-------------------|------------------|------------|-----------------------------|-----|---------|---------------------------|
| Acyclovir    | C₈H₁₁N₅O₃        | 225.20           | 59277-89-3 | 3.02; 11.98                  |     |         | 9.08                      |
| Lamivudine   | C₈H₁₁N₃O₃S      | 229.26           | 134678-17-4| -0.16; 14.29                 |     | -1.1    | 2.76                      |
| Zidovudine   | C₁₀H₁₃N₅O₄      | 267.2            | 30516-87-1 | -3; 9.96                    |     | -0.3    | 16.3                      |

aData from ChemAxon.
bData from VCCLAB-ALOGPS 2.1.
filled with 50 mL of test solution or contaminant-free culture medium (control) were used. In the case of efavirenz, it was necessary to add a solvent control (culture medium + methanol) with a nominal concentration of 0.1% methanol (v/v). After 96 hours of cultivation, the cell density was determined by counting cells in an improved Neubauer haemocytometer using an optical microscope (Eclipse E200, Nikon). After exposure and counting the number of cells in the Neubauer chamber, the percent inhibition of the growth rate was calculated by comparing each treatment group with the control using Equation (1):

\[
\% I_r = \frac{\mu_t - \mu_c}{\mu_c} \times 100
\]

where:

- \( % I_r \): percent inhibition of the average specific growth rate
- \( \mu_c \): mean value for the average specific growth rate (\( \mu \)) in the control group
- \( \mu_t \): average specific growth rate for the treatment replicate.

Initially, a preliminary test was performed with the drugs to determine the concentration range that causes sublethal effects. For each drug, three independent trials were performed, all in triplicate with exposure of the microalgae to five concentrations of acyclovir (1.25, 2.5, 5, 10 and 20 mg L\(^{-1}\)), six concentrations of efavirenz (0.008, 0.016, 0.031, 0.062, 0.125 and 0.25 mg L\(^{-1}\)), five concentrations of lamivudine (1.25, 2.5, 5, 10 and 20 mg L\(^{-1}\)) and five concentrations of zidovudine (1.25, 2.5, 5, 10 and 20 mg L\(^{-1}\)). A negative control maintained with LC oligo culture medium and a positive control prepared with potassium chloride (KCl) were used at concentrations of 20, 10, 5, and 2.5 g L\(^{-1}\). The algal growth inhibition at each test concentration was calculated from the average percentage of cell growth inhibition (% \( I_r \)) of each drug compared with the control, which did not contain acyclovir, efavirenz, lamivudine or zidovudine.

All conditions were in accordance with NBR 12,648 (ABNT 2018). To verify that the chronic effects were not methanol-dependent, a solvent control (0.1% v/v) was used only for the \( R. \ subcapitata \) growth inhibition assay. No significant difference between this solvent control and the negative control was found, which excludes the possibility of solvent effects on the toxicity results.

**Ceriodaphnia dubia cultures**

Group cultures of 30 adults were maintained in reconstituted water with a pH of 7.2–7.6 and a hardness between 44 and 47 mg CaCO\(_3\) L\(^{-1}\) in a 1 L glass beaker. Cultures were incubated under controlled temperature (25 ± 2 °C) and photoperiod (16 h L:8 h D) conditions. Total change of the culture water was carried out three times a week, avoiding temperature differences greater than 2 °C. The neonates were removed and counted every day. These microcrustaceans were fed daily with a suspension of the alga \( R. \ subcapitata \) cultured in CHU-12 medium (10\(^5\) cells mL\(^{-1}\)). A food supplement composed of a yeast suspension (0.5%) and fermented fish food (0.5%) was added (1 mL L\(^{-1}\)) as recommended by NBR 13,373 (ABNT 2017).

**Chronic bioassay with Ceriodaphnia dubia**

Chronic bioassays with \( C. \ dubia \) were conducted following general recommendations by NBR 13,373 (ABNT 2017) and lasted for 8 days. Experiments were initiated with neonates aged between 6 and 24 hours of life. Three independent experiments were performed for the studied antivirals. Each test consisted of 5 concentrations, the control group with 10 replicates per concentration and 1 test organism in each replicate. Test organisms were kept in tubes with 10 mL of the test solution or 10 mL of reconstituted water (control). Hardness, pH, and oxygen were monitored at every total water change.

Tests were performed with acyclovir (20, 10, 5, 2.5 and 1.25 mg L\(^{-1}\)), efavirenz (0.5, 0.25, 0.125, 0.062 and 0.031 mg L\(^{-1}\)), lamivudine (10, 5, 2.5, 1.25 and 0.625 mg L\(^{-1}\)) and zidovudine (20, 10, 5, 2.5 and 1.25 mg L\(^{-1}\)). The 30 control solution replicates were prepared with culture water free of drugs. Over the course of 8 days, the organisms were checked daily for mortality, and the offspring were counted and removed from the test tube. The biological parameter evaluated was the number of neonates.

The range of all abiotic parameters measured during chronic exposures is in accordance with NBR 13,375 (ABNT 2017). In experiments performed with efavirenz, there was no significant difference between the results of the blank control and solvent control group. This excludes the possibility of effects of the solvent (0.1% methanol) on the results of toxicity with \( C. \ dubia \).
Environmental risk assessment

Environmental risk assessments of acyclovir, efavirenz, lamivudine and zidovudine were based on the regulatory scope of the European Medicines Agency (EMA 2006). The risk quotients (RQs) for aquatic organisms were calculated from the measured environmental concentration (MEC) in surface waters and the predicted no-effect concentration (PNEC) (obtained from the battery of bioassays performed in the present study). PNEC is calculated by applying an assessment factor (AF) of 10 to the EC₅₀ (half maximal effective concentration) or NOEC (no observed effect concentration) value of aquatic species (Equation (2)). AF is an expression of the degree of uncertainty in the extrapolation of a limited number of species to complex ecosystems in the real environment and is responsible for intra- and interspecies variations in sensitivity and laboratory data for extrapolation of impact in the field (EMA 2006).

\[
PNEC = \frac{\text{NOEC or } EC_{50}}{10} \tag{2}
\]

RQ values for aquatic organisms were established based on the ratio between the measured environmental concentrations (MECs) (in the present study, the MEC was obtained from data in the literature) and PNEC, the values for which were obtained from the battery of bioassays performed in the present study (Equation (3)).

\[
RQ = \frac{\text{MEC}}{\text{PNEC}} \tag{3}
\]

From the resulting RQ, it is possible to assess the risk that each drug presents for each aquatic organism analysed. A risk classification criterion used by Hernando et al. (2006) was applied: RQ < 0.1 indicated minimum risk, 0.1 < RQ < 1 indicated intermediate risk, and RQ > 1 indicated high environmental risk.

Data analysis

All the compounds were tested 3 times (three independent assays). EC₅₀ and IC₅₀ values were determined as the estimated concentrations at which 50% of the reproduction of *C. dubia* was affected and the growth of the algal population was inhibited by 50%, respectively, as determined in GraphPad Prism® software version 5.0 (GraphPad Software, Inc., San Diego California USA, 2007) using nonlinear regression with a 95% confidence interval. All results are expressed as the mean ± standard deviation of three experiments performed alone with 10 replicates per concentration in the case of *C. dubia* and 3 replicates per concentration for *R. subcapitata*. The results were analysed with GraphPad 5.0 software using analysis of variance (ANOVA) and the Dunnett test for comparisons of means in relation to the control. P values equal to or less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Chronic toxicity assay with *Raphidocelis subcapitata*

The IC₅₀ and NOEC values of each antiviral for *R. subcapitata* are shown in Table 2. The values represent the average of three independent experiments. From these IC₅₀ results, it was found that the chronic *R. subcapitata* toxicity of the antivirals decreased in the order of EFV > 3TC > ACV > ZDV. The most toxic antiviral for the algae was efavirenz, with NOEC and IC₅₀ values of 0.008 mg L⁻¹ and 0.034 mg L⁻¹, respectively.

Chronic toxicity tests (96 h) showed that all compounds had concentrations that significantly inhibited the growth rate of *R. subcapitata* except zidovudine, for which a significant increase in the microalga was found at a concentration of 1.25 mg L⁻¹, indicating a hormesis effect (Figure 1(d)). In the microalgae test shown in Figure 1(a), acyclovir significantly inhibited the growth of the alga at concentrations of 2.5 to 20 mg L⁻¹ compared to that in the control group \( (p < 0.05) \). For efavirenz, significant growth inhibition \( (p < 0.05) \) was observed in *R. subcapitata* populations exposed to concentrations of 0.016 to 0.25 mg L⁻¹ (Figure 1(b)), and lamivudine at concentrations of 2.5 at 20 mg L⁻¹ significantly inhibited \( (p < 0.05) \) microalgal growth (Figure 1(c)). The dose-response curves generated in the growth inhibition assay from the experimental results are shown in Figure 1.
Chronic toxicity assay with *Ceriodaphnia dubia*

The results of short-term chronic exposure tests for *Ceriodaphnia dubia* presented in Table 3 show variation in toxicity among the antivirals, with the following order based on the EC50: zidovudine, acyclovir, lamivudine, efavirenz. The most toxic antiviral for *C. dubia* was efavirenz, with NOEC and EC50 values of 0.016 mg L⁻¹ and 0.026 mg L⁻¹, respectively.

### Table 2 | Chronic toxicity of antivirals to *R. subcapitata*

| Antivirals   | IC50 (mg L⁻¹) | NOEC (mg L⁻¹) | LOEC (mg L⁻¹) |
|--------------|---------------|----------------|---------------|
| Acyclovir    | 3.612 (3.249–4.016) | 1.25           | 2.5           |
| Efavirenz    | 0.034 (0.031–0.038)   | 0.008          | 0.016         |
| Lamivudine   | 3.013 (2.753–3.297)   | 1.25           | 2.5           |
| Zidovudine   | 5.442 (4.969–5.962)   | Nd             | Nd            |

IC50, 50% inhibitory concentration; NOEC, no observed effect concentration; LOEC, lowest observed effect concentration; 95% confidence intervals (in parentheses); Nd, not determined.

### Figure 1 | Growth inhibition of *R. subcapitata* algae after exposure to the drugs acyclovir, efavirenz, lamivudine and zidovudine. Data represent mean ± 95% confidence intervals, presented as log concentration response curves of growth rate inhibition after 96 h exposure. The significant differences compared to the control are marked with a symbol * (p < 0.05).

### Chronic toxicity assay with *Ceriodaphnia dubia*

The results of short-term chronic exposure tests for *Ceriodaphnia dubia* presented in Table 3 show variation in toxicity among the antivirals, with the following order based on the EC50: zidovudine < acyclovir < lamivudine < efavirenz. The most toxic antiviral for *C. dubia* was efavirenz, with NOEC and EC50 values of 0.016 mg L⁻¹ and 0.026 mg L⁻¹, respectively.
The results of the chronic toxicity tests showed that the tested concentrations of acyclovir, efavirenz and lamivudine caused a reduction in the fertility of females. However, synergistic effects at concentrations of 1.25 and 2.5 mg L\(^{-1}\) AZT were also observed (Figure 2(d)). Acyclovir caused a significant fertility decrease in females of *C. dubia* exposed to concentrations of 2.5 to 20 mg L\(^{-1}\) when compared to the control group according to the Dunnett test (*p*, 0.05) (Figure 2(a)). A significant decrease in fertility (*p*, 0.05) was observed in the test organisms exposed to efavirenz concentrations of 0.031 to 0.25 mg L\(^{-1}\), which proved to have a high level of toxicity (Figure 2(b)). For lamivudine, a significant reduction in neonates (*p*, 0.05) was observed at concentrations from 1.25 to 10 mg L\(^{-1}\) (Figure 2(c)).

Ecotoxicological tests performed using *R. subcapitata* and *C. dubia* for the antivirals ACY, AZT and 3TC yielded EC\(_{50}\) values between 1 and 10 mg L\(^{-1}\), indicating that these antivirals can be classified as 'toxic' based on the 93/67/EEC directive on risk assessment (EC 1996). EFV showed an EC\(_{50}\) < 1 mg L\(^{-1}\) and was thus classified as 'very toxic'. Based on ecotoxicity values obtained by chronic exposure assays, the compounds were classified as established by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

### Table 3 | Chronic toxicity of antivirals to *C. dubia*

| Antivirals   | EC\(_{50}\) (mg L\(^{-1}\)) | NOEC (mg L\(^{-1}\)) | LOEC (mg L\(^{-1}\)) |
|-------------|-----------------------------|-----------------------|-----------------------|
| Acyclovir   | 3.062 (2.529–3.707)         | 1.25                  | 2.5                   |
| Efavirenz   | 0.026 (0.024–0.027)         | 0.016                 | 0.031                 |
| Lamivudine  | 1.345 (1.242–1.456)         | 0.625                 | 1.25                  |
| Zidovudine  | 5.671 (5.370–5.989)         | Nd                    | Nd                    |

**EC\(_{50}\)**, 50% effective concentration; **NOEC**, no observed effect concentration; **LOEC**, lowest observed effect concentration; 95% confidence intervals (in parentheses); **Nd**, not determined.

Figure 2 | Average number of neonates produced per *C. dubia* after being exposed to acyclovir, efavirenz, lamivudine and zidovudine for a period of 8 days. Error bars correspond to the standard error of the mean of 30 replicates. Asterisks denote significant differences (*p* < 0.05) between the different concentrations and the negative control.
System of Classification and Labelling of Chemicals (GHS) (UN 2015) as follows: ACY, AZT and 3TC were toxic and EFV was highly toxic for both species.

Chronic values (ChVs) of acyclovir predicted by ECOSAR were estimated at 3.89 mg L\(^{-1}\) for fish, 2.04 mg L\(^{-1}\) for water fleas and 3.62 mg L\(^{-1}\) for microalgae. According to Mayo-Bean et al. (2011), the ChV is defined as the geometric average of the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). The chronic value found in this study for the ACV test for C. dubia and R. subcapitata was 1.76 mg L\(^{-1}\), showing similarity with the data presented in ECOSAR.

The ECOSAR program predicts the acute toxicity of acyclovir, and the LC\(_{50}\) for fish and Daphnia was estimated to be 1.76 \(\times 10^3\) and 69.2 mg L\(^{-1}\), respectively. These values are higher than the threshold for chronic toxicity because the tests are acute, generally exposing the organism during a short period of its life cycle, and criteria such as lethality or immobility are observed. The environmental relevance of laboratory chronic toxicity data is important to consider when interpreting the potential for antivirals to negatively affect aquatic ecosystems. Based on the experimental results presented in this study, the concentrations tested were high, analogous to aquatic environments with a high load of antivirals, such as those observed in densely populated regions with many HIV-positive individuals, poor sanitation systems and drinking water treatment, and low rates of precipitation, which would otherwise dilute the antivirals when entering water bodies (Nannou et al. 2020).

The molecular structure of compounds and their dissociated forms at different pH values can influence their bioavailability in the absorption process and toxicity to organisms (National Research Council 2014). Water pH is an important environmental factor that limits species distributions in aquatic habitats (Schwanke 2013). The ionization state is controlled by the pH of the solution and by the acid-dissociation constants (i.e., pKa values) (Babić et al. 2007). Based on the pKa values of acyclovir (2.27 and 9.25), zidovudine (9.8), lamivudine (4.4) and efavirenz (10.2 and 12.52), at a pH of approximately 7, the ACV, EFV and 3TC antivirals are mostly (above 99%) in their neutral form, while AZT is predominantly in its cationic form (Figure 1). These different chemical species (cationic, neutral or anionic) generally have very different properties, such as water solubility, volatility, UV absorption and reactivity with chemical oxidants (Babić et al. 2007).

In this study, the increase in the number of neonates per female of C. dubia and the hormesis effect on microalgae exposed to low concentrations may have been caused by the presence of nitrogen in the zidovudine molecule. The toxicity test was carried out at pH values ranging from 7.2 to 7.6. Under this condition, the predominant species of the AZT molecule is represented in Figure 1 with positive and negative charges on the chain nitrogen of the chain, facilitating nitrogen fixation by algae and some animals that use it as an energy source (Boyd 2015). According to Babić et al. (2007), the ionized form is generally more soluble in water, while the neutral form is more lipophilic and has greater membrane permeability.

The data obtained in the chronic toxicity tests in this study with C. dubia showed that ZDV causes a reduction in fertility in females by 10 and 20 mg L\(^{-1}\) and high growth inhibition in R. subcapitata at the high concentrations tested (5, 10 and 20 mg L\(^{-1}\)). However, stimulating effects (hormesis) of ARV have also been observed. In this study, the hormesis effect was verified in the test with microalgae at a concentration of 1.25 mg L\(^{-1}\), which represents 33.15% stimulation in relation to the control. This unusual but apparently beneficial effect observed at low doses has been previously reported for some bioindicators, such as microcrustaceans (Stanley et al. 2013), plants and algae (Cedergreen et al. 2007), in the presence of organic nitrogen compounds. Russo et al. (2018) examined the ecotoxicity of treated and untreated solutions of zidovudine, and Raphidocelis subcapitata showed a hormetic effect for stavudine and zidovudine treated with solutions at various UV245 wavelengths after dilution from 1:10 to 1:100. In addition to having a hormesis effect, AZT is known to be a cytostatic drug and can interfere with important physiological functions in nontarget organisms, causing molecular damage. The mechanism of action occurs through the (i) inhibition of nucleotides or DNA synthesis (Garcia-Canton et al. 2013) and (ii) induction of mutations, micronuclei and genotoxicity (Von Tungeln et al. 2002). The genotoxicity of AZT was demonstrated by Onwuamah et al. (2014) using Allium cepa roots, in which the authors identified effects such as inhibition of root growth at high concentrations, changes in mitosis and the induction of chromosomal aberrations.

ECOSAR software estimated chronic values of EFV of 0.13 mg L\(^{-1}\) for fish, 0.14 mg L\(^{-1}\) for water fleas and 0.68 mg L\(^{-1}\) for microalgae. The chronic value calculated for the data collected in this research was 0.022 mg L\(^{-1}\) for C. dubia and 0.01 mg L\(^{-1}\) for the microalgae R. subcapitata. The results obtained in this study for C. dubia (EC\(_{50}\): 0.026 mg L\(^{-1}\)) and for R. subcapitata (IC\(_{50}\): 0.034 mg L\(^{-1}\)) using efavirenz show that this antiviral was the most toxic for both species, suggesting that environmental concentrations can affect the exposed biota. The high growth inhibition of R. subcapitata and the toxic effect on the reproduction of C. dubia by the drug EFV are worrying since they can affect the life cycle of these species and aquatic life at higher trophic levels. Increased resistance or toxicity in aquatic organisms presumably depends on the
mode of action of the compounds (Dietrich et al. 2010). Studies have shown that EFV has mitotoxic action, meaning that it can interfere with the mitochondria of nontarget organisms, such as C. dubia and R. subcapitata. The drug triggers mitochondrial dysfunction characterized by direct inhibition of complex I of the electron transport chain, a decrease in the consumption of O₂, an increase in the production of reactive oxygen species (ROS) and a decrease in the potential of the mitochondrial membrane (Funes et al. 2014; Apostolova et al. 2017). In addition to its mitotoxicity, studies have shown that EFV exposure can cause liver damage. Robson et al. (2017) investigated the exposure (96 h) of the fish Oreochromis mossambicus to an EFV concentration of 20.6 ng L⁻¹ and found liver damage and a general decline in the health of this species, indicating the importance of monitoring efavirenz. These results reinforce the need for more studies on the mechanisms of action of EFV to obtain more sensitive responses.

Unfortunately, no studies have revealed how lamivudine acts on the biochemical and physiological functions of cladocerans and microalgae. Nevertheless, some information about how 3TC could act in other organisms is available. Studies on somatic cells of Drosophila melanogaster have shown that 3TC induces mutagenic and recombinogenic effects, causing genomic instability and loss of heterozygosity. These genetic changes play a primary role in carcinogenesis and are involved in secondary and subsequent stages of carcinogenesis, by which recessive oncogenic mutations are revealed (Franchi et al. 2009). Bayram & Topaktas (2008) classified lamivudine as a weak inducer of exchange between sister chromatids, chromosomal aberrations and the presence of micronuclei in peripheral human lymphocytes in vitro.

The sensitivity was different among the test organisms for the investigated compounds: C. dubia was the most sensitive species to 3 antivirals (ACV, EFV and 3TC). R. subcapitata was more sensitive to AZT. This observation suggests that a battery of bioassays using test organisms belonging to various taxonomic groups and representative of various levels of the food chain is necessary to assess the ecotoxicity of substances (Baran & Tarnawski 2013).

Environmental risk assessment (ERA)
Table 4 presents the worst-case scenario by using maximum MECs in the calculation of RQs for antivirals. Using the RQ classification in Hernando et al. (2006), the value for efavirenz in the Msunduzi River in South Africa was >1, indicating that it poses a potential risk to both aquatic organisms tested in this study. These high RQ values are the result of high concentrations of EFV found in South African rivers and high toxicity of the drug. Risk quotients are calculated to help define the severity of the problem in future risk assessment efforts, determining which environmental components are potentially exposed to toxic concentrations of antivirals. According to this study, it is evident that R. subcapitata and C. dubia are very susceptible to environmental exposure to efavirenz.

The predicted RQ for lamivudine in the Ngong River in Kenya for C. dubia was 2.69 (Table 4), indicating a high risk for this species, which is an important cladoceran and biological indicator of freshwater, and a medium risk for the microalga R. subcapitata. The risk assessment for acyclovir and zidovudine yielded RQ values <1, indicating a low risk for the species studied.

A chronic ecotoxicity experiment performed using the antiviral pharmaceuticals ganciclovir (GCV) and valganciclovir (VGCV) with Raphidocelis subcapitata and Daphnia magna as test organisms did not reveal persistence or bioaccumulation (Straub 2017). In the same study, a comparison between predicted environmental concentrations (PECs) and predicted no-effect concentrations (PNECs) showed no significant risks for treated wastewater, surface water, groundwater or sediment; in addition, potential risks to (semi)aquatic top predators or to human consumers of water and fish were exceedingly low.

**Environmental Risk Assessment (ERA)**

| Antiviral  | MEC Surface Water (ng L⁻¹) | PNEC R. subcapitata | RQ R. subcapitata | PNEC C. dubia | RQ C. dubia |
|------------|----------------------------|--------------------|------------------|---------------|-------------|
| Acyclovir  | 1,410[^a]                  | 0.125              | 0.011            | 0.125         | 0.011       |
| Efavirenz  | 2,450[^b]                  | 0.00078            | 3.14             | 0.0015        | 1.63        |
| Lamivudine | 167,100[^c]                | 0.125              | 1.53             | 0.062         | 2.69        |
| Zidovudine | 17,410[^c]                 | 0.544              | 0.03             | 0.567         | 0.03        |

[^a]: Bradley et al. (2014).
[^b]: Mtolo et al. (2019).
[^c]: Koreje et al. (2016).
Water scarcity conditions have particularly affected highly populated areas in recent years (Fernández-López et al. 2021). Therefore, increasingly reused water as a supplementary source of water to rivers should be considered. Risk assessment of emerging contaminants primarily focuses on their toxicity to aquatic organisms such as fish, algae and daphnia. It is urgent to assess the risks to human health associated with the various pharmaceutical products detected in reused water (Lin et al. 2020). Studies show that these compounds can be toxic, and the risk to humans cannot be considered negligible (Prosser and Sibley, 2015).

CONCLUSIONS

The results of the present study confirmed that one of the most commonly used antiviral drugs, efavirenz, is highly toxic to two aquatic organisms, with an EC50 value of 26 μg L⁻¹ for C. dubia and an IC50 value of 54 μg L⁻¹ for R. subcapitata, which are environmentally relevant concentrations. The RQs of efavirenz for both organisms and lamivudine for C. dubia exceeded 1, indicating a high ecological risk, whereas ACV, 3TC and ZDV could not have direct significant impacts on algae and cladocerans due to their lower toxicity. According to EU Directive 93/67/EEC, ACV, 3TC and AZT were classified as toxic, whereas EFV was a very toxic substance. Based on the results, we suggest monitoring efavirenz, as it poses a significant risk to aquatic organisms, and emphasize the need for testing with other aquatic species, soils and sediments, in order to expand the ecotoxicology spectrum.

DECLARATIONS OF INTEREST

None.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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