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Research Article

Comprehensive eco-geno-toxicity and environmental risk of common antiviral drugs in aquatic environments post-pandemic

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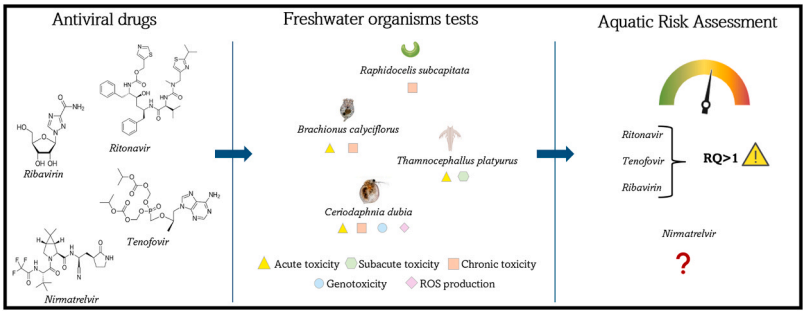
HIGHLIGHTS

- Eco-genotoxicity of ritonavir, ribavirin, tenofovir and nirmatrelvir was evaluated.
- Primary consumers were more sensitive than producers to the antiviral drugs.
- Chronic toxicity and genotoxicity occurred at concentrations of environmental concern.
- Chronic and genotoxic risk quotients were higher than the threshold value of 1.

GRAPHICAL ABSTRACT



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ABSTRACT

The eco-geno-toxicological impacts of the most widely used antiviral drugs against SARS-CoV2 - ribavirin, ritonavir, nirmatrelvir and tenofovir - were investigated in freshwater organisms. Ribavirin and tenofovir exhibited the highest acute toxicity in the rotifer *Brachionus calyciflorus* at concentrations of a few mg/L while ritonavir and nirmatrelvir showed similar effects at tens of mg/L; acute toxicity of ribavirin was also observed in the crustacean *Ceriodaphnia dubia* at similar concentrations. In contrast, the crustacean *Thamnocephalus platyurus* showed the lowest sensitivity to the antiviral drugs tested with no sublethal effects. Chronic toxicity tests revelead that these antivirals induced effects in consumers at concentrations of environmental concern (ng-μg/L). Ribavirin showed the highest toxicity to the alga *Raphidocelis subcapitata*, while ritonavir showed the highest toxicity to *B. calyciflorus* and *C. dubia*. DNA damage and oxidative stress were observed in *C. dubia* at 0.001 μg/L and 0.1 μg/L when exposed to ritonavir and nirmatrelvir respectively, and at 1 μg/L when exposed to ribavirin and tenofovir. Toxic and genotoxic environmental risks were assessed with risk quotients for ritonavir, tenofovir and ribavirin exceeding the threshold of 1, indicating significant environmental concern.

1. Introduction

COVID-19 infection has been mainly treated with antiviral drugs (ATVs) which significantly reduce the severity of the disease in patients at the highest risk of severe disease. In recent years, the unprecedented increase in the consumption of antivirals for the disease control and prevention has led to a continuous increase of such drugs into aquatic systems, resulting in environmental pollution and risks for aquatic

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organisms [1-6]. During the pandemic, antivirals as well as antibiotics and antidepressants, coming from conventional hospital and municipal wastewaters treatment plants (WWTPs), most of the time inefficient in removing drugs, have been detected in surface waters at concentrations in the order of tens/thousands of ng/L, often as pseudo-persistent drugs [7,8,1,9,2,4,10]. Specifically, it was estimated that the concentration of ATVs in effluents has increased by more than 70 % [11].

Among the mentioned antiviral drugs, ritonavir (RTV), ribavirin (RBV), tenofovir (TFV, as its prodrug tenofovir disoproxil fumarate TDF) and nirmatrelvir (NMV) have been extensively used both in pandemic and post-pandemic era [12-15,3,16,17]; (www.covid19treatmentguidelines.nih.gov/; www.nhs.uk/conditions/covid-19/treatments-for-covid-19/).

Ribavirin principally acts on hepatitis C virus [18] and its mechanism of action is focused on the inhibition of RNA synthesis; tenofovir acts as an inhibitor of reverse-transcriptase and is used to treat HIV infection [19] with the simultaneous administration of ritonavir and lopinavir, since RTV inhibits cytochrome P450 3A4 responsible for the biotransformation of lopinavir, increasing its half-life and efficacy [18,20]. Nirmatrelvir is a novel antiviral drug associated to ritonavir which delays its metabolism through the inhibition of cytochrome P450 3A4 enzyme, maintaining higher concentrations of the main drug in circulation [21]. The combination of NMV and RTV (PAXLOVID™, Pfizer trade name) has been extensively adopt to treat adult and paediatric patients (https://www.pfizer.com/).

The concentrations of ATVs has been detected in surface waters all over the world. Notably, during the pandemic, ribavirin has been detected at concentration of 52.2 ng/L and ritonavir in the range of 0.1–4.2 ng/L in Chinese surface waters [1,22,23] while tenofovir was in the order of tenths of μg/L in Brazilian surface waters (0.31 μg/L, [24]). To our knowledge, the concentrations of nirmatrelvir in the aquatic environment are not reported in the recent literature, for its recent use during the COVID-19 pandemic [25]. Furthermore, albeit the publications of numerous studies on the use of therapeutic drugs for COVID-19, data on the ecotoxicity and risk quotients of these drugs in freshwater aquatic organisms are still lacking [26]. Most existing studies depend on predictions generated by Ecological Structure Activity Relationships (ECOSAR) rather than on tests performed by exposing *in vivo* organisms to the drugs [1,27,24,28,26,29,22,23], highlighting the need to assess the overall environmental impact of such novel generation pollutants. To the extent of our understanding, data are lacking in relation to genotoxic damage and oxidative stress observed in aquatic organisms following *in vivo* exposure to the selected drugs.

In light of these considerations, the objectives of the present study were to acquire understanding of the ecotoxicity of ritonavir, ribavirin, tenofovir and nirmatrelvir by conducting acute, sub-acute and chronic toxicity investigations in organisms of freshwater, belonging to two different trophic levels. Specifically, the alga *Raphidocelis subcapitata* was used as a producer, and the rotifer *Brachionus calyciflorus,* the anostracan crustacean *Thamnocephalus platyurus* and the cladoceran *Ceriodaphnia dubia* were used as primary consumers. Additionally, genotoxicity was assessed in the latter organism, by detecting the potential DNA damage and evaluating the ability of the four antivirals to induce reactive oxygen species and quantify oxidative stress. The final objective was to estimate toxic and genotoxic risks of the selected antivirals.

2. Materials and methods

2.1. Test compounds

Ritonavir (CAS: 155213–67-5), ribavirin (CAS: 36791–04-5), tenofovir (CAS: 147127–20-6) and nirmatrelvir (CAS: 2628280–40-8) were purchased from Sigma-Aldrich (Milano, Italy). As regards physical and chemical properties and mode of action, ribavirin ($C_8H_{12}N_4O_5$, molecular weight 244.20 g/mol, logKow = −1.85) is a water-soluble synthetic guanosine with a broad spectrum of action on RNA and DNA viruses; tenofovir ($C_9H_{14}N_5O_4P$, molecular weight 287.21 g/mol, logKow = −1.6) is an acyclic nucleotide analogue of adenosine 5′ monophosphate; ritonavir ($C_{37}H_{48}N_6O_5S_2$, molecular weight = 720.94 g/mol, logKow = 6.27) is an inhibitor of the protease used for the treatment of HIV infection. Nirmatrelvir ($C_{23}H_{32}F_3N_5O_4$, molecular weight 499.535 g/mol, logKow = 1.10), also known with the code PF-07321332, is a second-generation cysteine protease inhibitor. Both Ritonavir and nirmatrelvir inhibit the cytochrome P450 3A4 enzyme.

Ritonavir was dissolved in 100 % dimethyl sulfoxide (DMSO, CAS: 67–68-5) to obtain a stock solution of 25 000 mg/L. Nirmatrelvir was solubilized in 1 % DMSO to obtain a stock solution equal to 500 mg/L. Ribavirin and tenofovir were prepared by dissolving powders in Milli-Q water, resulting in stock solutions of 1300 mg/L and 2500 mg/L, respectively. Each test compound was then diluted starting from the stock solutions using the respective synthetic medium for each organism tested. For each antiviral drug, and for each type of experiment, concentrations were chosen following appropriate range finding tests.

2.2. Toxicity testing

2.2.1. Acute and sub-acute toxicity tests

The rotifer *B. calyciflorus*, the anostracan crustacean *T. platyurus,* and the cladoceran crustacean *C. dubia* were used to detect acute toxicity of the antivirals according to [30,31], and to US EPA-600–4-90 [32], respectively. *T. platyurus* was also used to assess sub-acute toxicity following the same standard guideline of the acute test [31].

Cysts of *B. calyciflorus* (MicroBioTest Inc, Nazareth, Belgium) were hatched in synthetic freshwater (pH 7.5 ± 0.3; hardness: 80–100 mg/L $CaCO_3$) in a Petri dish at 25 ± 1 °C, under continuous light at 3000–4000 lux for 16–18 h. Ritonavir and nirmatrelvir were tested from 100 to 0.3 mg/L, while ribavirin and tenofovir were tested from 10 mg/L to 0.009 mg/L using, for each of the four compounds, a dilution factor (DF) of 3.2. Then, 36-well plates were assembled with 0.3 mL per well of each dilution and five rotifers were transferred to each well, in six replicates. Negative control (NC) was prepared using only the synthetic freshwater. Plates were incubated for 24 h at 25 °C in the dark.

The *C. dubia* organisms were obtained from an initial culture of gravid females purchased from Aquatic Research Organisms, Inc., Hampton, NH, USA) and mantained in synthetic standard freshwater (pH 8.0 ± 0.3; hardness: 250 ± 20 mg/L $CaCO_3$) at 25 °C with 16 h:8 h light:dark cycle (600 lux) and fed with the green alga *R. subcapitata*, yeast, alfalfa and food fish. When the organisms reached the third or fourth/fifth generation, 24-well plates were prepared with 1 mL per well of different concentrations of the antivirals (ribavirin: 200 to 1.56 mg/L; tenofovir: 100 to 6.25 mg/L; ritonavir: 2 to 0.156 mg/L; nirmatrelvir: 2 to 0.6 mg/L, with DF=2) in three replicates, then ten organisms per well were distributed. The plates were incubated for 24 h at 25 °C in the dark. A percentage of DMSO less than 0.01 % was used for the antivirals solubilized in this solvent.

Cysts of *T. platyurus* (MicroBioTest Inc, Nazareth, Belgium) were hatched in the same synthetic medium and light condition of the rotifer for 20–22 h of incubation at 25 °C under 3000–4000 lux. Ritonavir was tested from 0.75 to 0.0071 mg/L, nirmatrelvir was tested from 1.5 to 0.0045 mg/L while ribavirin and tenofovir were tested from 100 to 0.95 mg/L with DF of 3.2. Then, 1 mL/well of each dilution was placed in 24-well plates and ten organisms were added to each well. NC was prepared using synthetic freshwater only. Plates were incubated for 24 h at 25 °C in the dark.

For each test, a solvent control (from 0.003 to 0.4 % DMSO) was prepared, and the endpoint considered was mortality, calculated by comparing the number of dead rotifers/crustaceans in the test dilutions to those in the negative control.

Cysts of *T. platyurus* were let to hatch for a longer time (30–45 h) in the same synthetic medium mentioned above, at the same light and temperature conditions to assess sub-acute toxicity. The increased

incubation time allows the nauplii to molt into II and III stages in which the mouth and digestive tract are fully formed. The hatched larvae were exposed to antiviral drugs solutions for 1 h in darkness. Ritonavir, nirmatrelvir, ribavirin and tenofovir were tested at the highest concentrations used for acute toxicity (0.75, 1.5, 100, and 100 mg/L, respectively). The negative control was prepared with standard freshwater only. Then, red coated polystyrene beads (5 μm diameter-microspheres) were added to samples and control (two replicates per sample). After a 30 min-incubation at 25 °C, Lugol solution was added in each tube to immobilize and fix the test organisms. The uptake of colored particles by the organisms was noted using a stereomicroscope. The stressed (intoxicated) organisms exhibited no uptake of the coloured particles. The mean percentage inhibition of particle uptake was calculated as follows:

$$(A-B)/A\times 100 \quad (1)$$

where A is the mean percentage particle uptake in the control, and B is the mean percentage particle uptake in the sample.

2.2.2. Chronic toxicity tests

The green alga *R. subcapitata* [33,34], *B. calyciflorus* [35] and *C. dubia* were used [36].

Algal growth inhibition was evaluated by assembling 96-well plates with 100 μL of 10^3 cells/well and 200 μL of the drugs (from $1{\cdot}10^5$ μg/L to 24.41 μg/L, with a dilution factor of 2) in six replicates. NC was prepared with 200 μL/well of synthetic algal medium and 100 μL of 10^3 cells/well. The plates were incubated under shaking conditions (450 rpm) and exposed to continuous light at 6000–10 000 lux at 25 ± 1 °C. The absorbance was read at 450 nm using a microplate reader (Synergy H1, Biotek, Winooski, USA) at time zero and every 24 h for 72 h. A solvent control (0.4 % DMSO) was prepared.

The rotifer offspring reduction test was performed on 2h-old organisms hatched from cysts. One rotifer per well was transferred in 48-well plates containing 0.9 mL/well of the drugs solutions (from 30 to 0.000078 μg/L for ritonavir and nirmatrelvir, from 30 to 0.0008 μg/L for ribavirin, and from 30 to 0.0003 μg/L tenofovir, using a dilution factor of 3.2). Then, 100 μL of 10^7 cells/mL of *R. subcapitata* was used to feed the rotifers and the plates were incubated in the dark at 25 °C for 48 h. A solvent control (0.0001 % DMSO) was also prepared.

The *C. dubia* offspring reduction test was performed using organisms less than 24h-old, generated from at least the third generation, and exposed to 25 mL of different concentrations of the antivirals (ritonavir: 10 to $8.67{\cdot}10^{-6}$ μg/L, nirmatrelvir: 100 to $8.88{\cdot}10^{-4}$ μg/L, tenofovir: 3200 to $2.71{\cdot}10^{-4}$ μg/L, ribavirin: 3200 to 0.29 μg/L, with DF of 3.2) prepared in synthetic freshwater (pH 8.0 ± 0.3), over seven days at 25 ± 3 °C with a photoperiod of 16 h of light and 8 h of dark (600 lux), in ten replicates. A percentage of DMSO less than 0.01 % was used for the antivirals solubilized in this solvent. Survival was monitored and neonates born to the parent organisms were counted, recorded and then the solutions were discharged and renewed. Crustaceans were fed with the green alga *R. subcapitata*, yeast, alfalfa and food fish daily. Offspring reduction (%) was calculated relative to a negative control (synthetic freshwater only).

2.2.3. Genotoxicity

Single Cell Gel Electrophoresis, acknowledged as the Comet assay, was used to detect genotoxicity in *C. dubia* after 24 h of exposure to the four antivirals [37,38]. Comet assay was performed on single cells deriving from at least 30 whole live organisms which were exposed to 25 mL of different concentrations of the antivirals (ritonavir: 0.0001 to 10 μg/L; ribavirin and tenofovir: 0.1 to 1000 μg/L; nirmatrelvir: 0.01 to 100 μg/L, with DF of 10), in duplicates, in dark conditions at 25 °C. Specifically, the organisms were transferred post-treatment to 1 mL of phosphate-buffered saline (20 mM ethylene diamine tetra-acetic acid (EDTA); 10 % dimethyl sulfoxide) and homogenized to disintegrate the exoskeleton and to obtain single cells. Cell pellet was collected after centrifugation at 5000 rpm, resuspended in 0.7 % low melting point agarose, and spread onto microscope slides precoated with 1 % normal melting point agarose. The slides were dipped in 10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA and 1 % Triton X-100 (pH 10) for 1 h to lyse cellular and nuclear membranes. Then, the slides were dipped in alkaline solution (1 mM EDTA, 300 mM NaOH; pH ≥ 13) for 20 min at 4 °C to facilitate the unwinding of the DNA double helix. Electrophoresis was performed at 400 mA (25 V, 1 V/cm) for 20 min at 4 °C. Subsequently, the slides were dipped in 0.4 M Tris-HCl and dehydrated in 70 % ethanol. The visualization of damaged DNA was performed by fluorescence microscope (400 ×256 magnification, Eclipse 50i, Nikon, Kanagawa, Tokyo) after staining the slides with 50 μL ethidium bromide (1 mg/L). Damaged DNA appears in the shape of a comet where the head is represented by intact DNA and the tail is represented by fragmented DNA. The results were expressed as DNA in tail (%) deriving from 400 nuclei/concentration (two independent experiments).

2.2.4. ROS production

Reactive oxygen species (ROS) were measured in living *C. dubia* organisms (less than 24 h old) using the oxidative stress cell-permeant 2'7' dichlorodihydrofluorescein diacetate (H_2DCFDA) in line with Nugnes and co-workers [39] . Tests were conducted in 24-well plates, set up with 1 mL of antiviral concentrations (ritonavir: 10 to 0.0001 μg/L; ribavirin and tenofovir: 1000 to 0.1 μg/L; nirmatrelvir: 100 to 0.01 μg/L, with DF of 10) and ten organisms per well, in three replicates. After 24 h of incubation in darkness at 25 °C, the organisms were homogenized in 0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$ and 0.5 mM phenylmethylsulfonyl fluoride buffer, the solution was centrifuged at 10,000 g for 20 min at 4 °C and the ROS amount was measured in superrnatant. 96-well black plates were assembled with 50 μL PBS 1x, 50 μL H_2DCFDA 40 μM, 100 μL supernatant, (nine replicates, three replicates/well from 24-well plates) and incubated for 4 h at 20 °C in darkness. Fluorescence (λ_{ex} = 485 nm, λ_{em} = 520 nm) was measured using a fluorescence microplate reader (Synergy H1, Biotek, Winooski, VT, USA). The increase in fluorescence intensity, in reference to a negative control, yielded the ROS amount.

2.3. Data analysis

Three experiments, for acute, sub-acute and chronic assays, were performed. Statistical analysis was performed using Graphpad Prism 9 software (CA, USA) and the effective concentrations determining x% effect (L(E)Cx) were calculated by a non-linear regression (log agonist *vs.* normalized response-variable slope).

The endpoint of the acute toxicity was the median lethal concentration (LC50, concentration yielding 50 % lethality) and LC20 (concentration yielding 20 % lethality). The endpoint of the sub-acute toxicity was inhibition of red particle uptake, determined as a percentage. The endpoint of the chronic toxicity was concentration yielding 50 % (EC50), 20 % (EC20) and 10 % (EC10) inhibition of reproduction of rotifers, crustaceans and algal growth. The No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) were estimated by ANOVA and Dunnett's multiple comparison test. Comet analysis was performed by scoring the DNA in tail (%) of 400 nuclei for each concentration using Comet assay IV image analysis software (Perceptive Instrument, UK). ROS analysis was performed by studying fluorescence intensity (%). Then, for both tests, the No Observed Adverse Effect Concentration (NOAEC) and the Lowest Observed Adverse Effect Concentration (LOAEC) were estimated by ANOVA and Dunnett's multiple comparison test by significance from negative control (*$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$). Differences among test concentrations were analyzed by Tukey's HSD ($p < 0.0001$).

3. Results

3.1. Acute and sub-acute toxicity

Regarding the acute toxicity, the neonates of the rotifer *B. calyciflorus*, as well as of the crustaceans *T. platyurus* and *C. dubia* were exposed to the selected antiviral drugs for 24 h. The results, reported as LC50 with 95 % confidence intervals in brackets, were expressed in mg/L. When LC50s values were not reached, the results were reported as the percentage of lethality at the highest tested concentration (mg/L). For the sub-acute toxicity, *T. platyurus* larvae at instar II and III stages were exposed for 1 h to the antiviral drugs. At these stages the mouth and the digestive tract are fully formed, and the results are reported as the percentage inhibition of particle uptake, representing a stress condition (intoxication). These results are presented in Table 1.

Regarding *B. calyciflorus*, ribavirin and tenofovir were the most active drugs inducing mortality of 50 % of the exposed organisms at concentrations in the order of units of mg/L, diversely from ritonavir and nirmatrelvir showing LC50 in the order of tens of mg/L. Ritonavir and nirmatrelvir were tested at the highest possible concentration of 100 mg/L, taking into account the maximum percentage of DMSO equal to 0.4 % not causing lethality. In line with the EU Directive 93/67/EEC [40] (on risk assessment for new notified substances), substances are classifiable according to the L(E)C50 values. With EC50 $<$ 1 mg/L, the compound is considered very toxic to aquatic organisms; from 1 to 10 mg/L is considered toxic; and from 10 to 100 mg/L is considered harmful to aquatic organisms. Substances with an EC50 $>$ 100 mg/L result not toxic. Herein, ribavirin and tenofovir, with LC50s in the order of units of mg/L (Table 1), showed to be toxic; while ritonavir and nirmatrelvir, with LC50s at tens of mg/L, were considered harmful to *B. calyciflorus*.

Furthermore, when *C. dubia* was exposed to the selected antivirals, the median lethal concentration was obtained only for ribavirin, with a value in the order of tens of mg/L hence indicated as harmful [40] to *C. dubia* for short-term exposure. In contrast, exposure to 100 mg/L of tenofovir resulted in only 29.98 % effect, indicating not toxic effect to this freshwater organism. The highest tested concentration of ritonavir and nirmatrelvir was 2 mg/L (not exceeding the highest percentage of solvent DMSO 0.01 % for daphnids, in line with [41]) at which the percentage of effects obtained were 38.33 % and 31.67 %, respectively. Regarding *T. platyurus,* ribavirin and tenofovir showed a non-toxic effect (13.35 % and 4.98 %, respectively, at 100 mg/L). High concentration measurements for ritonavir and nirmatrelvir were not feasible due to DMSO toxicity. Specifically, the highest concentrations tested were 0.75 mg/L for ritonavir and 1.5 mg/L for nirmatrelvir with a mortality of 31.70 % and 31.65 %, respectively. Regarding sub-acute toxicity, the inhibition of particle uptake percentage in this crustacean was less than 10 % for all antiviral drugs, except for ribavirin which induced 11.30 % effect after one hour of exposure.

Table 1
Acute and sub-acute toxicity results obtained exposing rotifers and crustaceans to the antiviral drugs. The results are expressed as LC50 values (mg/L) with 95 % confidence intervals in brackets, and as effect percentage (mean ± SD, n = 3) at the highest tested concentration (mg/L).

Antiviral drugs	Acute toxicity			Sub-acute toxicity
	B. calyciflorus	*C. dubia*	*T. platyurus*	*T. platyurus*
Ritonavir	11.35 mg/L (6.92 −20.18)	38.33 ± 2.35 % (2 mg/L)	31.70 ± 7.07 % (0.75 mg/L)	7.55 ± 1.20 % (0.75 mg/L)
Ribavirin	3.23 mg/L (1.68 −6.21)	71.08 mg/L (50.46 −100.10)	13.35 ± 4.74 % (100 mg/L)	11.30 ± 0.71 % (100 mg/L)
Tenofovir	2.83 mg/L (1.91- 4.20)	29.98 ± 4.69 % (100 mg/L)	4.98 ± 2.38 % (100 mg/L)	5.60 ± 0.42 % (100 mg/L)
Nirmatrelvir	19.63 mg/L (11.38 −39.08)	31.67 ± 7.06 % (2 mg/L)	31.65 ± 2.33 % (1.5 mg/L)	6.17 ± 0.11 % (1.5 mg/L)

3.2. Chronic toxicity

To evaluate the chronic ecotoxicity of the four antiviral drugs, three freshwater organisms, *R. subcapitata*, *B. calyciflorus*, and *C. dubia*, one producer and two primary consumers, respectively, were exposed for a prolonged time and at lower concentrations than those used in acute tests. The results, represented as algal growth inhibition (72h-exposure) and as rotifer and crustacean offspring reduction (48h-exposure and 7d-exposure, respectively), are reported in Table 2 as EC50 and expressed in μg/L.

The most sensitive organisms to the four antiviral drugs during long term-exposure were the consumers showing EC50 values lower by at least three orders of magnitude compared to those obtained in the producer. In detail, regarding the alga *R. subcapitata*, ribavirin caused the highest inhibition of cell growth with EC50 at units of thousands of μg/L, revealing chronic toxic effect according to the EU Directive 93/67/EEC [40]. For ritonavir, tenofovir and nirmatrelvir EC50s were found at concentrations in the order of tens of thousands of μg/L, and resulting harmful during chronic exposure. Ritonavir caused a higher chronic effect in the rotifer, determining 50 % of inhibition of reproduction at concentrations of a few tenths of μg/L, followed by tenofovir, nirmatrelvir and ribavirin which caused the same effect at a concentration one order of magnitude higher. Similarly, exposing *C. dubia* to the antivirals, ritonavir was the most toxic ATV with EC50 values in the order of tens of ng/L followed by tenofovir and nirmatrelvir (EC50 at units of μg/L), and by ribavirin (EC50 tens of μg/L). Furthermore, considering that EC50 values found for both consumers were less than 1 mg/L, these antivirals have been classified as very toxic over extended periods of exposure.

To facilitate the understanding of the overall acute and chronic results and to identify both the most sensitive organism and most toxic antiviral, L(E)C20 values (μg/L) are presented in Fig. 1 and in Table S1.

Fig. 1 clearly shows that, for all organisms and antivirals tested, acute effects occur at concentrations much higher than those effective for chronic toxicity. The least sensitive organism was the alga *R. subcapitata*, which exhibited chronic effects at concentrations similar to those causing acute toxicity. The most sensitive organisms were the crustacean *C. dubia* and the rotifer *B. calyciflorus*. Finally, ritonavir was identified as the most toxic drug.

As the experimental long-term exposure is almost close to the environmental conditions, the concentration- effect trends may be useful to have a comprehensive idea of what happens in a real freshwater environment where organisms are extensively exposed to ATV residues. The results are illustrated in Fig. 2 where the percentages of inhibition of reproduction and cell growth are a function of the drug concentrations tested, expressed in μg/L.

For all the drugs, as the concentration tested increased, a gradual inhibition of algal cell growth and reproduction was recorded (Fig. 2). From the figure, it is evident that *B. calyciflorus* and *C. dubia* exhibited

Table 2
EC50 values (μg/L) with 95 % confidence intervals.

	Ritonavir	Ribavirin	Tenofovir	Nirmatrelvir
R. subcapitata (72 h)	$22.93{\cdot}10^{3}$ ($16.65{\cdot}10^{3}$ $-31.57{\cdot}10^{3}$)	$2.87{\cdot}10^{3}$ ($1.97{\cdot}10^{3}$ $-4.17{\cdot}10^{3}$)	$48.12{\cdot}10^{3}$ ($39.63{\cdot}10^{3}$ $-58.42{\cdot}10^{3}$)	$56.59{\cdot}10^{3}$ ($36.74{\cdot}10^{3}$ $-87.15{\cdot}10^{3}$)
B. calyciflorus (48 h)	0.20 (0.14 −0.29)	4.39 (3.24 −5.93)	1.08 (0.80 −1.45)	1.58 (0.93 −2.69)
C. dubia (7 d)	$14.32{\cdot}10^{-3}$ ($9.89{\cdot}10^{-3}$ $-20.84{\cdot}10^{-3}$)	22.78 (18.16 −28.51)	5.74 (3.72 −9.00)	3.29 (2.30 −4.82)

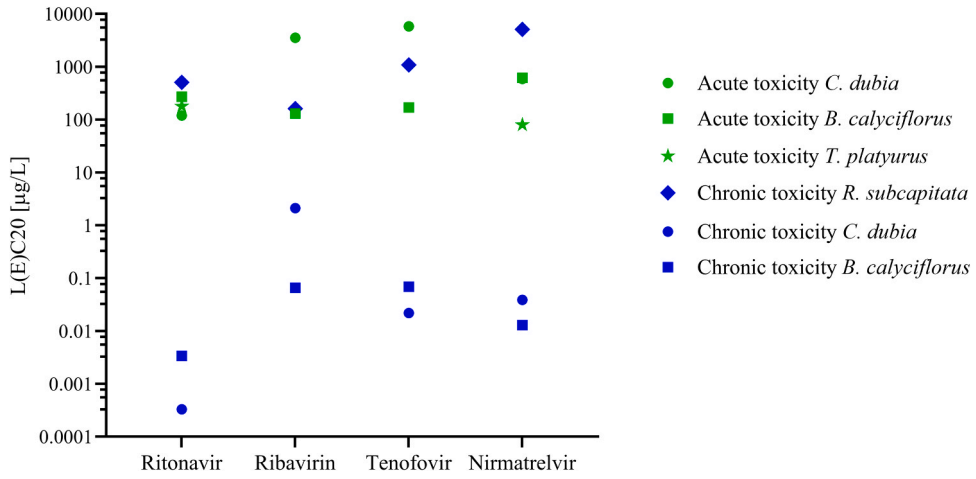

Fig. 1. L(E)C20 values expressed in µg/L coming from acute and chronic toxicity testing.

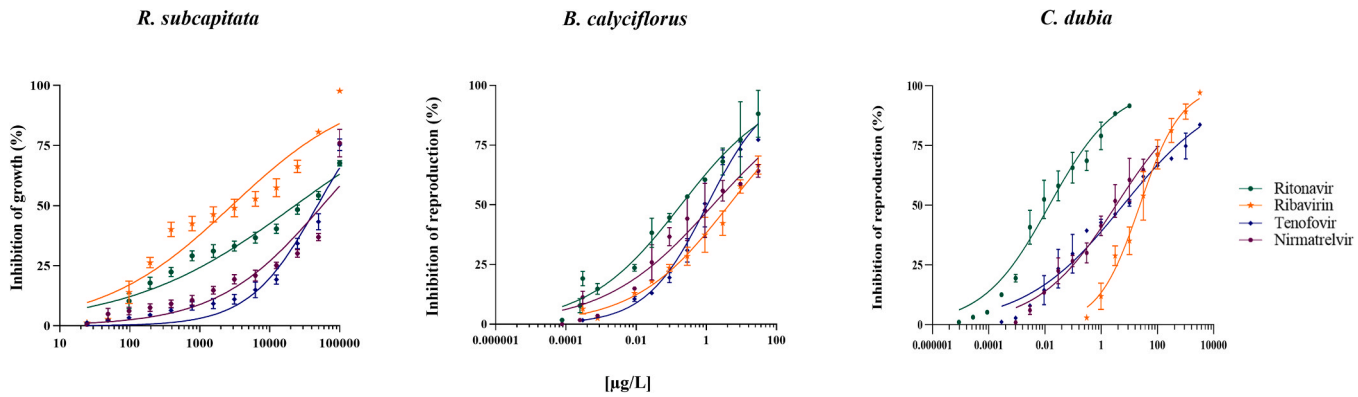

Fig. 2. Concentration/chronic effect curves (non-linear regression: log agonist *vs.* normalized response-variable slope) of the antiviral drugs in *R. subcapitata* (a), *B. calyciflorus* (b) and in *C. dubia* (c). The effect percentages of inhibition of reproduction (for *B. calyciflorus* and *C. dubia*) and algal cell growth (for *R.* subcapitata) were reported as title of y-axes, while the concentrations tested (in µg/L) were reported as title of x-axes. Bars indicate standard deviation (n = 3).

the highest sensitivity starting from very low concentrations. Specifically, the entire concentration-response curve for the effect of ritonavir in *C. dubia* shifted to the left of the graph showing a greater overall toxicity to this consumer. Differently, for the same organism, the concentration-response curve for ribavirin appears to be shifted the furthest to the right, showing less toxicity compared to the other antivirals. The same trend of both drugs just mentioned above was observed for the rotifer.

NOEC and LOEC values (Table 3), respectively the highest no-effect concentration and the lowest observed effect concentration, were obtained by One-way Anova statistical analysis, *Dunnett's multiple comparison test* ($p < 0.05$).

Although the algae are more resistant than the rotifers to antiviral drugs, for both organisms the first effective concentrations (LOECs) are of environmental concern with values at tens of µg/L for the producers and from tenths of ng/L to units of µg/L for the consumers.

Table 3
NOEC and LOEC values (µg/L) calculated using *Dunnett's test*.

		Ritonavir	Ribavirin	Tenofovir	Nirmatrelvir
R. subcapitata (72 h)	NOEC	24.41	24.41	24.41	24.41
	LOEC	48.83	48.83	48.83	48.83
B. calyciflorus (48 h)	NOEC	$0.08 \cdot 10^{-3}$	$2.73 \cdot 10^{-3}$	$2.73 \cdot 10^{-3}$	$0.85 \cdot 10^{-3}$
	LOEC	$0.27 \cdot 10^{-3}$	$8.73 \cdot 10^{-3}$	$8.73 \cdot 10^{-3}$	$2.73 \cdot 10^{-3}$
C. dubia (7 d)	NOEC	$0.28 \cdot 10^{-3}$	0.93	$8.88 \cdot 10^{-3}$	$29.10 \cdot 10^{-3}$
	LOEC	$0.91 \cdot 10^{-3}$	2.98	$28.42 \cdot 10^{-3}$	$93.13 \cdot 10^{-3}$

3.3. DNA damage

Comet assay results, obtained exposing *C. dubia* neonates *in vivo* to the antiviral drugs for 24 h, were expressed as Tail Intensity (T.I.), representing the percentage of DNA damaged present in the tail (Table 4, Fig. 3).

In Table 4, results from two independent experiments are expressed as mean values with the standard deviations. For none of the antivirals tested, the T.I values reached 20 %, indicating that these ATVs induced a moderate damage to the genetic material of the crustaceans. However, ritonavir caused 15.38 % genotoxic damage at 10 µg/L, followed by

Table 4
Effects of antiviral drugs (µg/L) on induction of DNA damage in *C. dubia* single cells. Results are expressed as Tail Intensity (T.I., % DNA in tail), mean ± SD (n = 2).

	Ritonavir	Ribavirin	Tenofovir	Nirmatrelvir
0 (NC)	1.17 ± 0.51	0.95 ± 0.46	1.23 ± 0.57	1.25 ± 0.57
0.0001	1.36 ± 0.66	-	-	-
0.001	2.02 ± 0.60	-	-	-
0.01	3.69 ± 1.27	-	-	1.50 ± 0.66
0.1	8.20 ± 1.51	1.19 ± 0.52	1.63 ± 0.59	3.48 ± 1.18
1	10.92 ± 1.46	2.17 ± 0.85	5.31 ± 0.91	7.49 ± 0.85
10	15.38 ± 1.09	4.30 ± 1.19	9.87 ± 1.18	9.11 ± 1.09
100	-	9.38 ± 0.93	11.36 ± 1.80	14.31 ± 1.36
1000	-	12.65 ± 1.34	16.63 ± 2.04	-

- no tested concentration

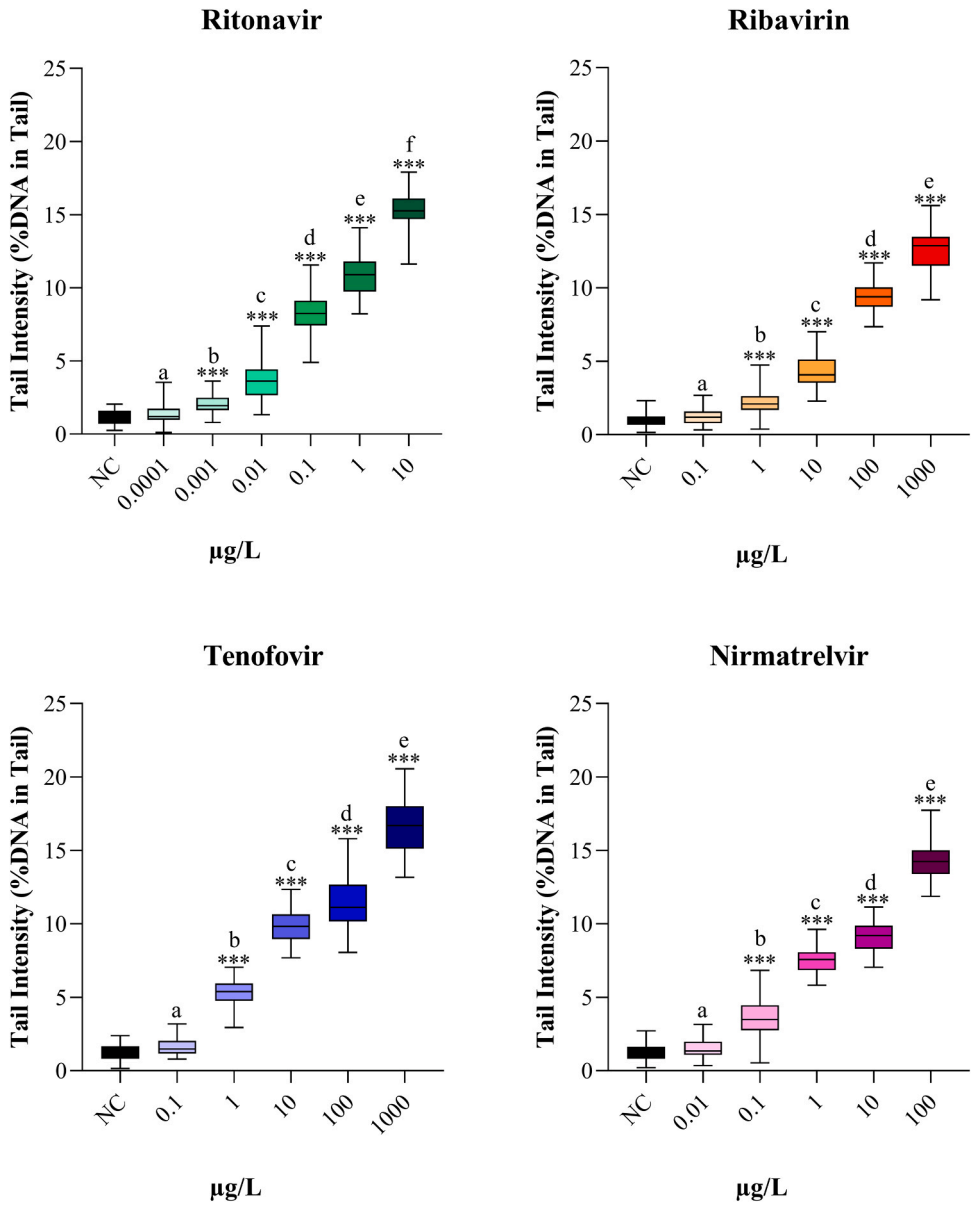

Fig. 3. DNA damage observed in *C. dubia* after *in vivo* 24h-exposure to antiviral drugs. Results, expressed as Tail intensity (% DNA in tail) of antivirals (in µg/L), are from two independent experiments (400 nuclei for each concentration). A significant difference from the control was determined by Dunnett's test (***$p < 0.0001$) and a significant difference among concentrations ($p < 0.05$; different letters) was determined by Tukey's HSD multiple comparison test.

nirmatrelvir with 14.31 % effect at 100 µg/L, and tenofovir with 16.63 % effect at 1000 µg/L. The lowest genotoxic effect was observed in *C. dubia* exposed to ribavirin (12.65 % at 1000 µg/L). Data distribution of the T.I. values, obtained from 400 data points (nuclei), is visualized by box-plots in Fig. 3.

In Fig. 3, the concentrations of the antivirals, expressed in µg/L, are on the x-axis, while the percentages of Tail Intensity, T.I (DNA in the tail) are on the y-axis. All the ATVs induced a statistically significant fragmentation of the DNA in the exposed organisms. The T.I. values increased as the concentration tested increased, showing a distinct concentration-effect trend (Tukey's test HSD multiple comparison, $p < 0.5$).

In addition, by statistically comparing T.I. values, relating to each concentration with those relating to the negative control using Dunnett's multiple comparison test, both the highest no-effect concentration (NOAEC), and the lowest effect concentration (LOAEC), were determined (Table 5).

Table 5
NOAEC and LOAEC values (µg/L) calculated using *Dunnett's test*.

	Ritonavir	Ribavirin	Tenofovir	Nirmatrelvir
NOAEC	0.0001	0.1	0.1	0.01
LOAEC	0.001	1	1	0.1

3.4. ROS production

C. dubia neonates were exposed to the antiviral drugs for 24 h to determine the production of reactive oxygen species (ROS) and the results, derived from two independent experiments, are expressed as a percentage of fluorescence (Fig. 4, Table S2).

The concentration of the antivirals, expressed in µg/L, is shown on the x-axis while the fluorescence intensity, expressed as percentage, is reported on the y-axis. None of the selected antivirals induced fluorescence that reached 20 %. Ritonavir caused 13.29 % oxidative damage at

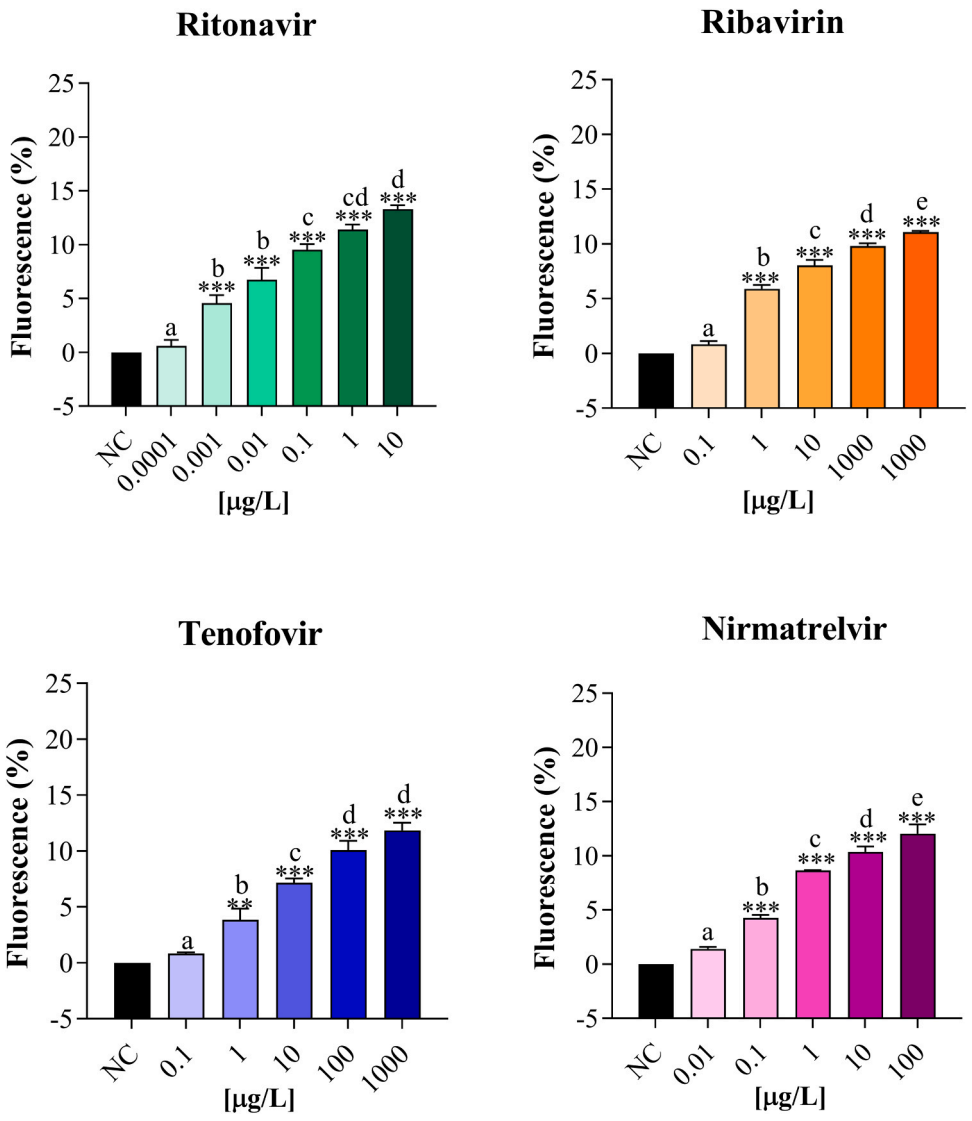

Fig. 4. ROS production in *C. dubia* after exposure to the antiviral drugs for 24 h. Data are presented as mean ± SD (n = 3). A significant difference from control was determined with Dunnett's test (** $p < 0.001$; *** $p < 0.0001$) and different letters intend significant differences for $p < 0.05$ among concentrations (Tukey's HSD multiple comparison test). A significant increase in fluorescence, proportional to the production of ROS was evident as the concentration increased. The statistical analysis (Dunnett's multiple comparison test; p ** < 0.001; *** p < 0.0001) showed that the fluorescence intensity significantly increased compared to the negative control starting from 0.001 µg/L for ritonavir, 0.1 µg/L for the nirmatrelvir, and 1 µg/L for ribavirin and tenofovir, resulting in LOAEC values that overlap with concentrations causing DNA damage (Table 5).

10 µg/L, followed by nirmatrelvir with 12.04 % effect at 100 µg/L, and tenofovir and ribavirin 11.84 and 11.06 respectively, at 1000 µg/L, inducing the lowest effect.

4. Discussion

To the best of our knowledge, data regarding the toxicity of these drugs in aquatic freshwater organisms are scarce. Nonetheless, Souza et al., [42] investigated the acute toxicity of ritonavir in three freshwater organisms, the two crustaceans *Daphnia magna* and *Hyalella azteca* (primary consumers) and the fish *Lepomis macrochirus* (secondary consumer) reporting LC50 values higher than units of mg/L, comparable to those found for the rotifer herein. The acute toxicity of ritonavir in fish, was estimated by the Ecological Structure Activity Relationships (ECOSAR) predictive model at concentrations in the order of tenths of mg/L [27], in contrast to our findings in which the LC50 of ritonavir was in the order of tens of mg/L in rotifers, and higher than tenths/units of mg/L in crustaceans.

Silva et al., [43], evaluated the acute toxicity of tenofovir in the seawater crustacean *Artemia salina*, reporting LC50 values in the order of tens of mg/L, as well as in *A. fischeri* at units of mg/L, effect concentrations that were similar to our findings for the rotifer.

Furthermore, in Mahaye and Musee [44], exposing *D. magna* to tenofovir, observed a concentration- and time-dependent immobilization at 0.25–1 mg/L after 48 h, effect concentrations lower than those found in the daphnid analyzed herein. As regards ribavirin, due to the scarcity in the literature of experimental studies on the toxicity of this drug in aquatic organisms, Kumari and Kumar [45] assessed predictive studies reporting data from the US EPA's EcoTox database 2020 (https://comptox.epa.gov/dashboard/). For this drug, toxic effect concentrations in reference organisms such as daphnia and fish were estimated in the order of thousands of mg/L, far higher than those obtained for the rotifer and cladocera crustacean in this study and from those acquired in the above-mentioned literature. Recently, Guo and collaborators [28], on studying the effect of ribavirin in the bacterium *Photobacterium phosphoreum* after 6 h of exposure, observed a significant inhibition of

bioluminescence (49 %) at tens of mg/L, in line with our results in *C. dubia*. At concentrations in the same order of magnitude, 50 % mortality was determined in *Danio rerio* embryos at the gastrula stage exposed to the drug nirmatrelvir [46]. As suggested by Mahaye and Musee [44], acute effects of antiviral drugs in aquatic organisms may be related to an increased production of ROS which induces changes in catalase and glutathione S-transferase activities, as important antioxidant response systems in non-target invertebrate species to avoid deleterious consequences at the cellular and organismal level. Regarding long term exposure, the effect concentrations were very low in consumers. Ritonavir was effective at units (for the rotifers) or tenths (for the crustaceans) of ng/L, and of all the other antiviral drugs in the order of units or tens of ng/L for both consumers, with great environmental concern considering that in surface waters the selected antiviral drugs occur from tenths to tens of ng/L [1,24,22,23]. Souza et al., [42] studied the chronic toxicity of tenofovir in the algae *R. subcapitata*, the crustacean *D. magna* and in the fish *Pimephales promelas*. In line with our results, the organism that showed less sensitivity to the antiretroviral appears to be the alga, with an EC50 value in the order of thousands of μg/L. The same authors, analyzing the toxicity of darunavir, an HIV-1 protease inhibitor with a similar mode of action to ritonavir, observed algal growth inhibition at concentrations in the same order of magnitude as those found in this experimental study. In addition, when the analysis of the chronic toxicity of some antiviral drugs was conducted considering the ecotoxicological data reported in ECOSAR in daphnia, fish and algae, ritonavir was among the most toxic antivirals [2].

Wu et al., [47] stated that after 7 days of exposure of *Danio rerio* larvae to ribavirin, significant developmental anomalies were found at concentrations that were several orders of magnitude higher (tens of mg/L) compared to those causing inhibition of reproduction of crustaceans and rotifers herein investigated, conceivably due to the increase of complexity of organisms moving up the food chain. A similar study on the toxicity of nirmatrelvir was conducted by Zizioli et al., [46] exposing *Danio rerio* larvae 120 h after fertilization and demonstrating significant sublethal effects such as reduction in length and weight, eye diameter and heartbeats at tens of mg/L. Abacavir, a drug used to treat HIV, induced the growth inhibition of the alga *R. subcapitata* with an EC50 value equal to 57 mg/L [48], in line with the chronic toxicity caused by the antiretroviral drugs ritonavir and tenofovir, used in this research. Harmful effects in *Oreochromis mossambicus* fish were demonstrated by Robson et al., [49] following exposure to the antiretroviral drug efavirenz at 10.3 ng/L, a concentration of the same order of magnitude which caused 20 % inhibition of reproduction in rotifers and crustaceans that had been exposed to the antiretroviral tenofovir.

To further contribute to scientific knowledge, we performed tests on genotoxicity and ROS production in the daphnid *C. dubia* in line with Nugnes et al. [39,50]. In detail, by exposing *C. dubia* neonates *in vivo* for 24 h to the antiviral drugs, we observed that ritonavir was the most genotoxic drug, inducing the lowest nuclear fragmentation already at concentrations (LOAEC) in the order of magnitude of units of ng/L, followed by nirmatrelvir at hundreds of ng/L and subsequently by both ribavirin and tenofovir with effects starting from units of μg/L, similar or few order of magnitude higher than those occurring in the freshwater environment [1,24,22,23]. Therefore, in the light of our outcomes, ritonavir was the antiviral that most caused genotoxicity at environmentally relevant concentrations, raising the most concern. The genotoxicity of ritonavir could be attributable to its mode of action related to the inhibition of the human CYP3A4 liver enzyme. Lee et al., [51] highlighted that even *D. magna*, and more broadly daphnids, albeit non-target organisms, possess cytochrome structures similar to those of interest which could be affected by the inhibition exerted by ritonavir and could thus be prone to alterations that similarly occur in human counterparts. As regards tenofovir, as reported by Bakare et al., [52], this drug could alter DNA by generating primary and/or secondary DNA damage, either caused by the formation of acentric chromosomal fragments or by chromosomal loss. The genotoxicity of tenofovir may be due to an imbalance of the intracellular nucleotide pool by nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), which could cause mutations, thus increasing the risk of DNA damage, reported besides by Vivanti et al., [53] and de Moraes Filho et al., [54]. To date, due to the scarcity of data, it is difficult to compare the results obtained in this research with those available in scientific literature. Nevertheless, it is possible to compare the results obtained with the data published by the same research group, Nugnes and collaborators [38], in which the genotoxic effect of the antiviral drug acyclovir, an acyclic guanosine analogue active against *Herpes simplex* virus (HSV), was evaluated by *C. dubia* for 24h-exposure. Specifically, Nugnes and collaborators [38] discovered that acyclovir induces nuclear fragmentation in *C. dubia* cells, already at concentrations in the order of hundreds of μg/L, comparable to results obtained in the present study. Considering genotoxic effects of the selected antiviral drugs in non-aquatic organisms, it is noteworthy to mention Bakare et al., [52] who investigated the genotoxic potential damage of tenofovir in the insect *Drosophila melanogaster* using the Comet assay and found the greatest percentage of Tail Intensity at 10 mg/mL, which is a concentration far higher than those of effect obtained when *C. dubia* was exposed to the same antiviral drug herein.

An increase in DNA strand breaks may be the result of ROS generation due to oxidative stress produced by xenobiotics in aquatic organisms, because such reactive oxygen species can react with DNA causing chemical changes to the nitrogenous bases or chain breaks nucleotide [55]. Interestingly, Mahaye and Musee [44] studied the effect of tenofovir on biochemical markers in *Daphnia magna* and observed an alteration in the catalase and glutathione s-transferase activity when tenofovir concentrations increased (62.5–1000 μg/L). These findings demonstrated that tenofovir determined an increase in the production of intracellular ROS resulting in oxidative stress and suppression of the antioxidant system. Further effects of tenofovir on oxidative stress were reported by Gomes et al., [24], conducted a study on the freshwater cyanobacterium *Synechococcus elongatus* and the algae *Chlorococcum infusionum*. Unlike what happens in *D. magna* and *C. dubia*, tenofovir had no significant effects on oxidative stress but showed synergy with the antiviral drug efavirenz in both species. Furthermore, Magdy et al., [56], investigated oxidative stress induced by ribavirin in Wistar rats, revealing that exposure to ribavirin, during the gestation period, induced a significant increase in oxidative stress in rats resulting in apoptosis and necrosis of placental tissues. Ribavirin was found to be teratogenic, considering that excessive production of reactive oxygen species causes damage to fetus DNA.

To understand if aquatic organisms and the entire aquatic environment could be negatively affected by the selected antiviral drugs, we assessed the environmental risk following standard guidelines [57,58] considering algae and daphnids. Therefore, the chronic risk quotient (cRQ, Table 6) was calculated as the ratio between the measured environmental concentrations (MECs) and the predicted no-effect concentrations (PNECs). The PNECs were determined by dividing the NOECs by an assessment factor (AF) which is based on the number of trophic levels and taxonomic groups. In detail, to the best of our knowledge, the most recent concentrations detected in freshwater were considered as MECs, (Table 6). Between the alga *R. subcapitata* and the daphnid *C. dubia*, the

Table 6
Chronic Risk Quotient (cRQ) calculated by measured environmental concentrations (MECs, ng/L), No Observed Effect Concentration (*C. dubia* NOECs, ng/L). and Predicted No Effective Concentration (PNEC, ng/L).

[ng/L]	Ritonavir	Ribavirin	Tenofovir	Nirmatrelvir
MEC	1 Zhao et al. [23]	52.2 Chen et al. [1]	310 Gomes et al. [24]	-
NOEC	0.28	930	8.88	29.10
PNEC	0.0056	18.60	0.1776	0.582
cRQ	178.57	2.81	1745.49	-

lowest NOECs were obtained for the daphnid *C. dubia* and were expressed as ng/L. The AF value was 50 due to two levels of the trophic chain (producers and consumers) considered in the experimental design. Only for nirmatrelvir, no RQ was determined since to the best of our knowledge, no MECs are reported in scientific literature.

We can conclude that the three antiviral drugs may represent a risk (tenofovir > ritonavir > ribavirin) for the aquatic organisms during chronic exposure, with environmental concern.

Although the standard guidelines outlined above do not take into account calculating RQ based on genotoxicity data, Mišík et al. [59], Nugnes et al. [39,50] as well as Russo et al. [34] have proposed performing it to assess the environmental genotoxic risk posed by xenobiotics to freshwater ecosystems based on the same calculation utilized for the toxic risk. Thus, the MECs are reported in Table 6; the NOAECs obtained from genotoxicity assays exposing *C. dubia* to antiviral drugs were 0.1 ng/L for ritonavir and 100 ng/L for both ribavirin and tenofovir and the AF value was equal to 100 (one trophic level only). Thus, PNECs were equal to 0.001 ng/L for ritonavir and 1 ng/L for both ribavirin and tenofovir.

The genotoxic Risk Quotient (gRQ) for the three antivirals were equal to 1000, 310, and 52.2 respectively for ritonavir, tenofovir and ribavirin indicating a potential genotoxic risk, generating environmental concern.

5. Conclusions

The outcomes of this study indicate that while antiviral concentrations causing acute and sub-acute toxicity exceed levels of environmental concern, those associated with chronic toxicity, genotoxicity as well as ROS production are of concern for *C. dubia*, with chronic and genotoxic risk quotients exceeding the threshold value of 1. However, these results should be considered preliminary and additional testing is necessary to confirm the environmental risks identified here. Although recent literature is expanding, further studies are needed to fully understand the environmental impact of antivirals and their potential unforeseen effects along the aquatic food chains.

Environmental Implications

Limited information is available regarding chronic toxicity of the most widely used antiviral drugs: ritonavir, ribavirin, tenofovir and nirmatrelvir, with no indications concerning genotoxic effects and oxidative stress in freshwater organisms (producers and primary consumers). We observed effect concentrations of chronic toxicity, genotoxicity and oxidative damage in the order of sub-ng-µg/L (concentrations occurring in surface waters). We assessed ecotoxic as well as genotoxic environmental risks, the latter being calculated for the first time for the selected molecules, and observed risk quotients exceeding the threshold of 1 with a significant environmental relevance.

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CRediT authorship contribution statement

Margherita Lavorgna: Writing – review & editing, Supervision, Conceptualization. **Chiara Russo:** Writing – review & editing, Investigation, Data curation. **Marina Isidori:** Writing – review & editing, Supervision, Conceptualization. **Elena Orlo:** Writing – original draft, Investigation, Data curation. **Roberta Nugnes:** Writing – original draft, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135947.

References

[1] Chen, X., Lei, L., Liu, S., Han, J., Li, R., Men, J., Li, L., Wei, L., Sheng, Y., Yang, L., Zhou, B., Zhu, L., 2021. Occurrence and risk assessment of pharmaceuticals and personal care products (PPCPs) against COVID-19 in lakes and WWTP-river-estuary system in Wuhan, China. Sci Total Environ 792, 148352. https://doi.org/10.1016/j.scitotenv.2021.148352.

[2] Kuroda, K., Li, C., Dhangar, K., Kumar, M., 2021. Predicted occurrence, ecotoxicological risk and environmentally acquired resistance of antiviral drugs associated with COVID-19 in environmental waters. Sci Total Environ 776, 145740.

[3] Liu, Q., Feng, X., Chen, N., Shen, F., Zhang, H., Wang, S., Sheng, Z., Li, J., 2022. Occurrence and risk assessment of typical PPCPs and biodegradation pathway of ribavirin in wastewater treatment plants. Environ Sci ecotechnology 11, 100184. https://doi.org/10.1016/j.ese.2022.100184.

[4] Netshithothole, R., Managa, M., Botha, T.L., Madikizela, L.M., 2024. Occurrence of selected pharmaceuticals in wastewater and sludge samples from wastewater treatment plants in Eastern Cape province of South Africa. S Afr J Chem 78, 7–14. https://doi.org/10.17159/0379-4350/2024/v78a02.

[5] Wang, R., Luo, J., Li, C., Chen, J., Zhu, N., 2023. Antiviral drugs in wastewater are on the rise as emerging contaminants: a comprehensive review of spatiotemporal characteristics, removal technologies and environmental risks. J Hazard Mater 457, 131694. https://doi.org/10.1016/j.jhazmat.2023.131694.

[6] Wu, H., Bin, L., Guo, P., Zhao, Y., Chen, C., Chen, Z., Tang, B., 2024. Ecological risk assessment of the typical anti-epidemic drugs in the Pearl River Delta by tracing their source and residual characteristics. J Hazard Mater 463, 132914. https://doi.org/10.1016/j.jhazmat.2023.132914.

[7] Chacca, D.E.M., Maldonado, I., Vilca, F.Z., 2022. Environmental and ecotoxicological effects of drugs used for the treatment of COVID 19. Front Environ Sci 10, 940975.

[8] Chen, M., Hong, Y., Jin, X., Guo, C., Zhao, X., Liu, N., Lu, H., Liu, Y., Xu, J., 2023. Ranking the risks of eighty pharmaceuticals in surface water of a megacity: a multilevel optimization strategy. Sci Total Environ 878, 163184. https://doi.org/10.1016/j.scitotenv.2023.163184.

[9] Diaz-Camal, N., Cardoso-Vera, J.D., Islas-Flores, H., Gómez-Oliván, L.M., Mejía-García, A., 2022. Consumption and occurrence of antidepressants (SSRIs) in pre- and post-COVID-19 pandemic, their environmental impact and innovative removal methods: a review. Sci Total Environ 829, 154656. https://doi.org/10.1016/j.scitotenv.2022.154656.

[10] Nippes, R.P., Macruz, P.D., da Silva, G.N., Neves Olsen Scaliante, M.H., 2021. A critical review on environmental presence of pharmaceutical drugs tested for the covid-19 treatment. Process Saf Environ Prot: Trans Inst Chem Eng, Part B 152, 568–582. https://doi.org/10.1016/j.psep.2021.06.040.

[11] Morales-Paredes, C.A., Rodríguez-Díaz, J.M., Boluda-Botella, N., 2022. Pharmaceutical compounds used in the COVID-19 pandemic: a review of their presence in water and treatment techniques for their elimination. Sci Total Environ 814, 152691. https://doi.org/10.1016/j.scitotenv.2021.152691.

[12] Aggarwal, N.R., Beaty, L.E., Bennett, T.D., Fish, L.E., Jacobs, J.R., Mayer, D.A., Molina, K.C., Peers, J.L., Richardson, D.B., Russell, S., Varela, A., Webb, B.J., Wynia, M.K., Xiao, M., Carlson, N.E., Ginde, A.A., 2024. Real-world use of nirmatrelvir-ritonavir in COVID-19 outpatients during BQ.1, BQ.1.1., and XBB.1.5 predominant omicron variants in three U.S. health systems: a retrospective cohort study. Lancet Reg Health Am 31, 100693. https://doi.org/10.1016/j.lana.2024.100693.

[13] Butt, A.A., Yan, P., Shaikh, O.S., Talisa, V.B., Omer, S.B., Mayr, F.B., 2024. Nirmatrelvir/ritonavir use and hospitalizations or death in a previously uninfected nonhospitalized high-risk population with COVID-19: a matched cohort study. J Infect Dis 229 (1), 147–154. https://doi.org/10.1093/infdis/jiad393.

[14] Djumaeva, N., Djumaeva, L., Akhunjanova, G., & Urunova, D. (2024). Ribavirin's Daily Dose Growth Determined as the Result of Medicament Testing May Be a Predictor of the Chronic Course of the Disease in Long COVID Syndrome Patients. https://doi.org/10.21203/rs.3.rs-4112790/v1.

[15] İşbilen, Ş., G., Onmuş, D.I.R., Çankayalı, I., Demirağ, K., Uyar, M., Çiçek, C., Yamazhan, T., Pullukçu, H., Sipahi, O.R., 2024. Tenofovir disoproxil fumarate in the treatment of COVID-19: evaluation of 78 patients. N Microbiol 47 (1), 47–51.
[16] Malden, D.E., McLaughlin, J.M., Hong, V., Lewnard, J., Ackerson, B.K., Puzniak, L., Kim, J.S., Takhar, H., Frankland, T.B., Slezak, J.M., Tartof, S.Y., 2024. Predictors of nirmatrelvir-ritonavir receipt among COVID-19 patients in a large US health system. Sci Rep 14 (1), 7485. https://doi.org/10.1038/s41598-024-57633-7.
[17] Zhao, M., Zhang, J., Li, H., Luo, Z., Ye, J., Xu, Y., Wang, Z., Ye, D., Liu, J., Li, D., Wang, M., Wan, J., 2021. Recent progress of antiviral therapy for coronavirus disease 2019. Eur J Pharmacol 890, 173646. https://doi.org/10.1016/j.ejphar.2020.173646.
[18] Biswas, P., Hasan, M.M., Dey, D., Dos Santos Costa, A.C., Polash, S.A., Bibi, S., Ferdous, N., Kaium, M.A., Rahman, M.D.H., Jeet, F.K., Papadakos, S., Islam, K., Uddin, M.S., 2021. Candidate antiviral drugs for COVID-19 and their environmental implications: a comprehensive analysis. Environ Sci Pollut Res Int 28 (42), 59570–59593. https://doi.org/10.1007/s11356-021-16096-3.
[19] Murphy, R.A., Valentovic, M.A., 2017. Factors contributing to the antiviral effectiveness of tenofovir. J Pharmacol Exp Ther 363 (2), 156–163. https://doi.org/10.1124/jpet.117.243139.
[20] Krumm, Z.A., Lloyd, G.M., Francis, C.P., Nasif, L.H., Mitchell, D.A., Golde, T.E., Giasson, B.I., Xia, Y., 2021. Precision therapeutic targets for COVID-19. Virol J 18 (1), 66. https://doi.org/10.1186/s12985-021-01526-y.
[21] Polkinghorne, A., Branley, J.M., 2022. Medications for early treatment of COVID-19 in Australia. Med J Aust 217, S7–S13. https://doi.org/10.5694/mja2.51750.
[22] Zhang, Z., Zhou, Y., Han, L., Guo, X., Wu, Z., Fang, J., Hou, B., Cai, Y., Jiang, J., Yang, Z., 2022. Impacts of COVID-19 pandemic on the aquatic environment associated with disinfection byproducts and pharmaceuticals. Sci Total Environ 811, 151409. https://doi.org/10.1016/j.scitotenv.2021.151409.
[23] Zhao, J., Kang, J., Cao, X., Bian, R., Liu, G., Hu, S., Wu, X., Li, C., Wang, D., Qi, W., Huang, C., Liu, H., Qu, J., 2024. Impacts of comorbidity and mental shock on organic micropollutants in surface water during and after the first wave of COVID-19 pandemic in Wuhan (2019–2021), China. Engineering. https://doi.org/10.1016/j.eng.2023.08.020.
[24] Gomes, M.P., Kubis, G.C., Kitamura, R.S.A., Figueredo, C.C., Nogueira, K.D.S., Vieira, F., Navarro-Silva, M.A., Juneau, P., 2022. Do anti-HIV drugs pose a threat to photosynthetic microorganisms? Chemosphere 307 (Pt 2), 135796. https://doi.org/10.1016/j.chemosphere.2022.135796.
[25] Dai, H., Wang, C., Yu, W., Han, J., 2023. Tracing COVID-19 drugs in the environment: are we focusing on the right environmental compartment? Environ Pollut (Barking, Essex: 1987) 339, 122732. https://doi.org/10.1016/j.envpol.2023.122732.
[26] Gwenzi, W., Selvasembian, R., Offiong, N.O., Mahmoud, A.E.D., Sanganyado, E., Mal, J., 2022. COVID-19 drugs in aquatic systems: a review. Environ Chem Lett 20 (2), 1275–1294. https://doi.org/10.1007/s10311-021-01356-y.
[27] Czech, B., Krzyszczak, A., Boguszewska-Czubara, A., Opielak, G., Joško, I., Hojamberdiev, M., 2022. Revealing the toxicity of lopinavir-and ritonavir-containing water and wastewater treated by photo-induced processes to Danio rerio and Allivibrio fischeri. Sci Total Environ 824, 153967. https://doi.org/10.1016/j.scitotenv.2022.153967.
[28] Guo, Z., He, H., Liu, K., Li, Z., Yang, S., Liao, Z., Lai, C., Ren, X., Huang, B., Pan, X., 2023. The photolytic behavior of COVID-19 antivirals ribavirin in natural waters and the increased environmental risk. J Hazard Mater 452, 131320. https://doi.org/10.1016/j.jhazmat.2023.131320.
[29] Khan, K., Kar, S., Roy, K., 2023. Are we ready to combat the ecotoxicity of COVID-19 pharmaceuticals? An in silico aquatic risk assessment. Aquat Toxicol (Amst, Neth) 256, 106416. https://doi.org/10.1016/j.aquatox.2023.106416.
[30] ASTM E 1440-91, 2004. Standard Guide for Acute Toxicity with the Rotifer Brachionus. American Society for Testing and Materials,, Philadelphia PA, USA, reapproved.
[31] ISO 14380 (2011). Water quality — Determination of the acute toxicity to Thamnocephalus platyurus (Crustacea, Anostraca).
[32] US EPA, 1993. Waters to Freshwater and Marine Organisms. Methods for Measuring the Acute Toxicity of Effluents and Receiving, fourth ed. US Environmental.Protection Agency,, Washington DC. EPA-600-4-90.
[33] OECD 201 (2011). Test No. 201: Freshwater alga and cyanobacteria, growth inhibition test. OECD Guidelines for the Testing of Chemicals, Sections 2, 2, 25.
[34] Russo, C., Nugnes, R., Orlo, E., di Matteo, A., De Felice, B., Montanino, C., Lavorgna, M., Isidori, M., 2023. Diclofenac eco-geno-toxicity in freshwater algae, rotifers and crustaceans. Environ Pollut 335, 122251. https://doi.org/10.1016/j.envpol.2023.122251.
[35] ISO 20666 (2008). Water quality — Determination of the chronic toxicity to Brachionus calyciflorus in 48h.
[36] ISO 20665, 2008. Water quality-determination of chronic toxicity to *Ceriodaphnia dubia*. 7 Days-population Growth Inhibition Test. International Organization for. Standardization,, Geneva, Switzerland.
[37] Collins, A., Møller, P., Gajski, G., Vodenková, S., Abdulwahed, A., Anderson, D., Bankoglu, E.E., Bonassi, S., Boutet-Robinet, E., Brunborg, G., Chao, C., Cooke, M.S., Costa, C., Costa, S., Dhawan, A., de Lapuente, J., Bo', C.D., Dubus, J., Dusinska, M., Duthie, S.J., Azqueta, A., 2023. Measuring DNA modifications with the comet assay: a compendium of protocols. Nat Protoc 18 (3), 929–989. https://doi.org/10.1038/s41596-022-00754-y.
[38] Nugnes, R., Russo, C., Di Matteo, A., Orlo, E., De Rosa, E., Lavorgna, M., Isidori, M., 2024. Acyclovir eco-geno-toxicity in freshwater organisms. Ecotoxicol Environ Saf 278, 116437. https://doi.org/10.1016/j.ecoenv.2024.116437.
[39] Nugnes, R., Russo, C., Orlo, E., Lavorgna, M., Isidori, M., 2023. Imidacloprid: Comparative toxicity, DNA damage, ROS production and risk assessment for aquatic non-target organisms. Environ Pollut (Barking, Essex: 1987) 316 (Pt 2)), 120682. https://doi.org/10.1016/j.envpol.2022.120682.
[40] EU Directive, 1996. Commition of the European Communities: technical guidance document in support of commition directive 93/67/EEC on risk assessment for existing substances, Part II- Environmental rsk assessment (Brussels, Belgium).
[41] ISO 10706, 2000. Water quality - Determination of long-term toxicity of substances to *Daphnia magna* Straus (Cladocera, Crustacea). Published (Edition 1, 2000).
[42] Souza, C.R., Ribeiro, M.S., Soares, M.D., Santos, S.F., Pereira, C.A., Mol, M.P., Silveira, M.R., 2022. Environmental elimination estimates and literature review of ecotoxicological aspects of the main widely used antiretrovirals in Brazil. Res, Soc Dev. https://doi.org/10.33448/rsd-v11i10.32975.
[43] Silva, S., Barbosa, F., Mol, M., Magalhães, S., 2019. Toxicity for aquatic organisms of antiretroviral tenofovir disoproxil. J Environ Prot 10, 1565–1577. https://doi.org/10.4236/jep.2019.1012093.
[44] Mahaye, N., Musee, N., 2022. Effects of two antiretroviral drugs on the crustacean daphnia magna in river water. Toxics 10 (8), 423. https://doi.org/10.3390/toxics10080423.
[45] Kumari, M., Kumar, A., 2022. Environmental and human health risk assessment of mixture of Covid-19 treating pharmaceutical drugs in environmental waters. Sci Total Environ 812, 152485. https://doi.org/10.1016/j.scitotenv.2021.152485.
[46] Zizioli, D., Ferretti, S., Mignani, L., Castelli, F., Tiecco, G., Zanella, I., Quiros-Roldan, E., 2023. Developmental safety of nirmatrelvir in zebrafish (Danio rerio) embryos. Birth Defects Res 115 (4), 430–440. https://doi.org/10.1002/bdr2.2128.
[47] Wu, X., Zhang, J., Hu, S., Zhang, G., Lan, H., Peng, J., Liu, H., 2022. Evaluation of degradation performance toward antiviral drug ribavirin using advanced oxidation process and its relations to ecotoxicity evolution. Sci Total Environ 850, 157851. https://doi.org/10.1016/j.scitotenv.2022.157851.
[48] Minguez, L., Pedelucq, J., Farcy, E., Ballandonne, C., Budzinski, H., Halm-Lemeille, M.P., 2016. Toxicities of 48 pharmaceuticals and their freshwater and marine environmental assessment in northwestern France. Environ Sci Pollut Res 23, 4992–5001.
[49] Robson, L., Barnhoorn, I.E.J., Wagenaar, G.M., 2017. The potential effects of efavirenz on Oreochromis mossambicus after acute exposure. Environ Toxicol Pharmacol 56, 225–232. https://doi.org/10.1016/j.etap.2017.09.017.
[50] Nugnes, R., Russo, C., Lavorgna, M., Orlo, E., Kundi, M., Isidori, M., 2022. Polystyrene microplastic particles in combination with pesticides and antiviral drugs: toxicity and genotoxicity in *Ceriodaphnia dubia*. Environ Pollut (Barking, Essex: 1987) 313, 120088. https://doi.org/10.1016/j.envpol.2022.120088.
[51] Lee, B.Y., Choi, B.S., Kim, M.S., Park, J.C., Jeong, C.B., Han, J., Lee, J.S., 2019. The genome of the freshwater water flea Daphnia magna: a potential use for freshwater molecular ecotoxicology. Aquat Toxicol (Amst, Nether Lands) 210, 69–84 https://doi. org/10.1016/j.aquatox.2019.02.009.
[52] Bakare, Adekunle, Akinseye, Kehinde, Aminu, Bayonle, Ofoegbu, Francis, Saheed Olaide, Anifowose, Abruda, Stork, Ogunsuyi, Opeoluwa, Ogunsuyi, Olusegun, Oyeyemi, Ifeoluwa, Alabi, O., Adetona, Olusola, Alimba, Chibuisi, 2020. Genetic and reproductive toxicity of lamivudine, tenofovir disoproxil fumarate, efavirenz and their combination in the bone marrow and testicular cells of male mice. Ann Sci Technol 5. https://doi.org/10.2478/ast-2020-0001.
[53] Vivanti, A., Soheili, T.S., Cuccuini, W., Luce, S., Mandelbrot, L., Lechendec, J., Cordier, A.G., Azria, E., Soulier, J., Cavazzana, M., Blanche, S., André- Schmutz, I., 2015. Comparing genotoxic signatures in cord blood cells from neonates exposed in utero to zidovudine or tenofovir. AIDS 29, 1319–1324. https://doi.org/10.1097/QAD.0000000000000564.
[54] de Moraes Filho, A.V., de Jesus Silva Carvalho, C., Verçosa, C.J., Gonçalves,vi, M. W., Rohde, C., de Melo E Silva, D., Cunha, K.S., Chen-Chen, L., 2017. In vivo genotoxicity evaluation of efavirenz (EFV) and tenofovir disoproxil fumarate (TDF) alone and in their clinical combinations in *Drosophila melanogaster*. Mutat Res Genet Toxicol Environ Mutagen 820, 31–38. https://doi.org/10.1016/j.mrgentox.2017.05.012.
[55] Sahlmann, A., Wolf, R., Holth, T.F., Titelman, J., Hylland, K., 2017. Baseline and. oxidative DNA damage in marine invertebrates. J Toxicol Environ Health A 80 (16-18), 807–819. https://doi.org/10.1080/15287394.2017.1352179.
[56] Magdy, M., El Ghareeb, A.E.W., Eldebss, T.M.A., Abd El Rahman, H.A., 2023. Investigation of the embryo-toxicity of the antiviral drug "Ribavirin" in Wistar rats during different gestation periods. Egypt J Basic Appl Sci 10 (1), 396–409. https://doi.org/10.1080/2314808X.2023.2217650.
[57] EMA, 2024. Guideline on the Environmental Risk Assesment of the Medicinal Products for Human Use. European Medical Agency. EMEA/CHMP/SWP/4447/00.
[58] EU TGD, 2003. Technical Guidance Document, De Bruijn, J., Hansen, B., Johansson, S., Luotamo, M., Munn, S., Musset, C., Olsen, S., Olsson, H., Paya-Perez, A., Pedersen, F., Rasmussen,K., Sokull-Kluttgen, B., 2003. Technical Guidance Document on Risk Assessment. Part 2. EUR 20418 EN 2, 3–328, 2002. JRC23785.
[59] Mišík, M., Filipic, M., Nersesyan, A., Kundi, M., Isidori, M., Knasmueller, S., 2019. Environmental risk assessment of widely used anticancer drugs (5-fluorouracil, cisplatin, etoposide, imatinib mesylate). Water Res 164, 114953. https://doi.org/10.1016/j.watres.2019.114953.