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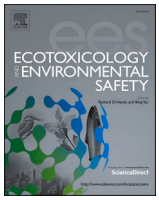

Acyclovir eco-geno-toxicity in freshwater organisms

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ABSTRACT

This study explores the eco-geno-toxic impact of Acyclovir (ACV), a widely used antiviral drug, on various freshwater organisms, given its increasing detection in surface waters. The research focused on non-target organisms, including the green alga *Raphidocelis subcapitata*, the rotifer *Brachionus calyciflorus*, the cladoceran crustacean *Ceriodaphnia dubia*, and the benthic ostracod *Heterocypris incongruens*, exposed to ACV to assess both acute and chronic toxicity. The results indicate that while acute toxicity occurs at environmentally not-relevant concentrations, a significant chronic toxicity for *C. dubia* (EC_{50} = 0.03 μg/L, NOEC = $0.02{\cdot}10^{-2}$ μg/L), highlighted substantial environmental concern. Furthermore, DNA strand breaks and reactive oxygen species detected in *C. dubia* indicate significant increase at concentrations exceeding 200 μg/L. Regarding environmental risk, the authors identified chronic exposures to acyclovir causing inhibitory effects on reproduction in *B. calyciflorus* at hundreds of μg/L and hundredths of μg/L for *C. dubia* as environmentally relevant environmental concentrations. The study concludes by quantifying the toxic and genotoxic risks of ACV showing a chronic risk quotient higher than the critical value of 1and a genotoxic risk quotient reaching this threshold, highlighting the urgent need for a broader risk assessment of ACV for its significant implications for aquatic ecosystems.

1. Introduction

Acyclovir (ACV), a synthetic analogue of purine nucleoside, is an antiviral drug, widely used over time to treat infections caused by the herpes virus family (including *herpes simplex*, *Epstein Barr*, *Varicella zoster*, and cytomegalovirus) (Almeida et al., 2021; Gupta et al., 2021). ACV, after its conversion into acyclovir triphosphate by viral and cellular enzymes, inhibits virus replication by competitively preventing the formation of viral DNA through inhibition of the viral DNA polymerase (Hassan et al., 2016; Fernandez et al., 2021). Administered in numerous forms (tablet, suspension, intravenous injection, ophthalmic ointment), ACV is excreted by patients as an unchanged compound from 45 % to 75 % up to 92 %, while only a negligible portion of the remaining administered dose is metabolised or bioaccumulated in the human body; ACV residues are mainly found in the urine given that the kidneys serve as primary organs to eliminate the drug (Schlüter-Vorberg et al., 2015; Hassan et al., 2016; Almeida et al., 2021; Gupta et al., 2021; Almeida et al., 2023). ACV, similarly to other antivirals, passes through the urban drainage network following therapeutic use and reaches waste water treatment plants (WWTPs) which often exhibit limited effectiveness in completely removing the drug, allowing access to surface water bodies (Schlüter-Vorberg et al., 2015; Zhou et al., 2015; Seitz and Winzenbacher, 2017; Jia et al., 2019; Almeida e al., 2021). The studies on this subject are limited and somewhat contradicting, in fact Prasse and co-authors (2010) reported high removal (> 95 %) of acyclovir in German conventional WWTPs while Peng and coauthors (2014) in traditional Chinese WWTPs, found that the concentration of ACV in influents was not dissimilar from its concentration in effluents (up to hundreds of ng/L). As reported in Table 1, ACV has been detected at concentrations in the order of units of ng/L to units of μg/L.

To the best of our knowledge, there are no recent measured concentrations (MECs) of ACV in surface waters more recent than those shown in the table above mentioned, so it would be better to refer to the predicted environmental concentrations (PECs) given the high increase in the use of antivirals following the COVID-19 pandemic. Almeida and collaborators (2023) used the data obtained from consumption patterns,

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Table 1
Occurrence of acyclovir in different aquatic matrices.

Matrix	Concentration [μg/L]	Reference
Yodo River - Kansai region – Japan	0.010–0.023	Azuma et al. (2016)
Hospital WWTP effluent – Japan	0.020–0.600	
Fourmile Creek (tributary stream) - USA	0.738–1.590	Bradley et al. (2014)
WWTP effluent - USA	1.360	
Groundwater - USA	0.013–0.059	
German WWTP effluents	0.044–0.650	Funke et al. (2016)
German Neckar, Weschnitz, Modau, Schwarzbach, Rodau, Main, Nahe, Lahn, Rhein surface waters	< 0.010	
USA WWTP effluents	0.154	McCurry et al. (2014)
Pearl River Delta - China	0.009–0.113	Peng et al. (2014)
Chinese WWTP effluents	0.114–0.205	
Lenne river - Germany	0.020	Prasse et al. (2010)
Wenne river -Germany	0.003	
Volme river - Germany	0.006	
Rivers and streams from the Hessian Ried region - Germany	0.002–0.190	
German WWTPs effluent	0.027–0.053	
German surface waters	0.005–0.025	Prasse et al. (2011)
German WWTPs effluent	0.121–0.148	

Table 2
Ecotoxic effect of ACV in freshwater organisms.

Non-target organism	Ecotoxicological effects	Reference
Raphidocelis subcapitata	Growth inhibition IC_{50} = 3.612 mg L^{-1} (96 h)	Almeida et al. (2021)
Ceriodaphnia dubia	Inhibition of reproduction EC_{50} = 3.062 mg L^{-1} (8 d)	
Photobacterium phosphoreum	12.3 % Luminescence inhibition at 225 mg L^{-1} (15 min)	An et al. (2015)
Raphidocelis subcapitata	4.8 % Growth inhibition at 225 mg L^{-1} (96 h)	
Daphnia magna	35.1 % Acute immobilization at 225 mg L^{-1} (48 h)	
Daphnia magna	Acute toxicity > 92.1 mg L L^{-1}	Gupta et al. (2021)
Raphidocelis subcapitata	Growth inhibition EC_{50} >100 mg L^{-1} (72 h)	Minguez et al. (2016)
Daphnia magna	Immobility EC_{50} = 64.12 mg L^{-1} (48 h)	
Raphidocelis subcapitata	Growth inhibition < 10 % at mg L^{-1} (72 h)	Russo et al. (2017)
Daphnia magna	% Immobility <10 % at 1.2 mg L^{-1} (24 h and 48 h)	
Daphnia magna	% Survival < 60 % at 1.2 mg L^{-1} (21 d)	
Raphidocelis subcapitata	No growth inhibition up to 100 mg L^{-1} (72 h)	Schlüter-Vorberg et al. (2015)
Daphnia magna	No inhibition of reproduction up to 92.1 mg L^{-1} (21 days)	
Danio rerio	No embryo toxicity up to ~100 mg L^{-1} (96 h)	

inhabitants number, human metabolism and rates of excretion to evaluate the PECs of ACV for Portuguese surface waters in 2021, obtaining estimated concentrations of hundreds of ng/L (from 200 to 740 ng/L). Considering the expected increase in ACV occurrence within surface waters (Almeida et al., 2021; Gupta et al., 2021) and, that few ecotoxicological data exist regarding the effects of ACV in aquatic organisms belonging to different levels of the freshwater trophic chain it would be necessary to amplify the knowledge of ACV effects in aquatic producers and consumers. Relevant, albeit scarce data refer to acute and chronic toxicity tests performed on exposure of *in vivo* organisms to the drug (Table 2), as well as data indicating predictions by means of the Ecological Structure Activity Relationships (ECOSAR), yielding in both cases such effects at concentrations from tens to thousands of mg/L for acute toxicity and, at concentrations from units to tens of mg/L for chronic toxicity (Almeida et al., 2021; Li et al., 2016; Ding et al., 2023; Minguez et al., 2016; Schlüter-Vorberg et al., 2015).

No genotoxicity testing with ACV was performed on aquatic organisms until 2022, when Nugnes and collaborators conducted the comet assay on freshwater consumers observing effects at hundreds of μg/L and finding chronic effects in *Ceriodaphnia dubia* at concentrations of environmental relevance (tens of ng/L). Furthermore, no data are available regarding the oxygen radical species produced in aquatic organisms exposed to ACV. In the light of these findings, the primary aim of this work was to evaluate the acute and chronic toxicity in sentinel species of the freshwater ecosystem *in vivo* exposed to ACV. The organisms, selected for their sensitivity, abundance in fresh water, simplicity of cultivation, genetic uniformity, were the green alga *Raphidocelis subcapitata*, the rotifer *Brachionus calyciflorus*, the cladoceran crustacean *Ceriodaphnia dubia,* and the benthic ostracod *Heterocypris incongruens* that was exposed for the first time to this drug. Furthermore, to explore the genotoxic effects of ACV, the secondary aim of this research was to expose the crustacean *C.dubia* to ACV to study the potential DNA damage as well as the reactive oxygen species (ROS) production. The third aim of this study was to assess the ACV environmental risk according to standard guidelines (EU Technical Guidance Document, 2003; EMA guidelines, 2006) and to evaluate the ACV potential genotoxic environmental risk for the first time, using procedures previously adopted by Mišík and collaborators (2019), Nugnes and collaborators (2023) and Russo and collaborators (2023).

2. Materials and methods

2.1. Test compound

Acyclovir (ACV, CAS: 59 277–89–3, purity ≥ 99 %, solubility limit in water of 1600 mg/L at 22–25°C) was purchased from Sigma Aldrich (Milano, Italy). Stock solution (500 mg/L) was prepared by dissolving the powder in Milli-Q water. Test solutions were freshly prepared by mixing the appropriate volumes of the aqueous stock solution and standard synthetic media. Differences between nominal and actual concentrations of ACV in test solutions were analyzed by Total Organic Carbon Analyzer TOC-L 153 CSN (Shimadzu, Kyoto, Japan) and by LC-MS/MS. Analyses were performed using Thermo Scientific™ UltiMate™ 3000UHPLC coupled to TSQ Fortis Triple Quadrupole MS. Details are reported in Supplementary Materials.

2.2. Acute toxicity tests

The acute assays were performed in the rotifer *B. calyciflorus*, the crustacean *C. dubia* and the benthic ostracod *H. incongruens* in line with standard guidelines (ASTM E, 1440–91, 2004; US EPA-600–4–90, 1993; ISO 14371, 2012). The acute toxicity test endpoint was mortality. The concentrations for *B. calyciflorus* and *C. dubia* exposure were between 12.5 and 200 mg/L, with a geometric progression (GP) of 2, while for *H. incongruens* exposure concentrations were from 1.56 to 400 mg/L, with a GP of 2. The effective percentages were calculated by the ratio between the number of organisms obtained in the test solutions with the number of organisms in the negative controls (synthetic fresh water only); percentages for the *C. dubia* acute assay were calculated by using ToxRat Professional software, Version 2.10.05 (Alsdorf, Germany). The

obtained effective percentages were used to estimate the median lethal concentration producing 50 % effect (LC_{50}). The details of the tests are reported in Supplementary Material.

2.3. Chronic and sub-chronic toxicity tests

The green alga *R. subcapitata*, the rotifer *B. calyciflorus* and the cladoceran crustacean *C. dubia* were used for chronic toxicity testing (ISO 20666, 2008; ISO 20665, 2008; OECD 201, 2011), while *H. incongruens* was used for sub-chronic toxicity testing (ISO 14371, 2012).

R. subcapitata growth inhibition test was performed testing ACV from $12.4{\cdot}10^3$ to $36.9 \cdot 10^3$ μg/L, GP of 1.2, for 72 h-exposure. *B. calyciflorus* offspring reduction test was performed exposing neonates for 48 h to ACV from 0.08 to $1{\cdot}10^4$ μg/L, GP of 3.2. *C. dubia* offspring reduction test was performed over 7 days and neonates were exposed to ACV from 0.00026 to 10 μg/L, GP of 3.2. *H. incongruens* growth inhibition test was performed as described for the acute assay, exposing organisms for 6 d to the selected drug.

Effective Concentrations (ECx) were calculated comparing effect rates obtained in test solutions with negative controls. The details of the tests are reported in Supplementary Material.

2.4. Genotoxicity testing and ROS production

The crustacean *C. dubia,* was the organism selected for genotoxicity testing (Comet assay, Collins et al., 2023) and for ROS production (Nugnes et al., 2023) as this organism had proven to be the most sensitive to ACV. The Comet assay, commonly known as Single Cell Gel Electrophoresis was performed in alkaline conditions using electrophoresis at 4°C, 400 mA (25 V, 1 V/cm). The ROS production assay was performed using the general oxidative stress cell-permeant fluorescein diacetate.

Tests were performed on cells deriving from at least 20 neonates placed in glass beakers and exposed to polystyrene for 24 h, in darkness at 25 °C exposed to ACV at concentrations starting from 200 000–20 μg/L and arranged in a geometric series with a factor 10. Cell viability was checked by trypan blue staining and always higher than 80 %.

The details of the tests are reported in Supplementary Material.

2.5. Data analysis

Ecotoxicity results were derived from three independent tests. The effect percentages were calculated and then interpolated using a non-linear regression (log agonist *vs.* normalized response-variable slope) by the software Graphpad prism 5 Analysis (Graphpad Inc, CA, USA). For acute toxicity, the LC_{50} value was calculated. For chronic toxicity, EC_{50} values were calculated with 95 % confidence limits. Lowest Observed Effect Concentration (LOEC), indicating the minimum concentration at which a statistically significant effect is observed, and the No Observable Effect Level (NOEC), indicating the maximum concentration of no effect, were obtained by ANOVA statistical analysis and Dunnett's multiple comparison.

Genotoxicity and ROS production results were from two independent tests. Tail intensity (T.I.) value (the percentage of DNA in tail) scoring 200 cells, 50 cells per slide for each assay (2 slides per each concentration prepared in duplicate, 4 slides/concentration) using Comet assay IV image analysis software (Perceptive Instrument, UK). Tukey's Test HSD and Dunnett's multiple comparison test by ANOVA were used for statistical analysis. Details of data analysis are described in Supplementary Material.

3. Results and discussion

The nominal total organic carbon of ACV, detected by TOC analyzer, differed from the measured by less than 5 % at T_0 and after 24 h. The results of HPLC analysis showed that the nominal concentrations in distilled water and in test solutions differed from the actual ones of an uncertainty factor from 0.14 to 0.15. In the guideline OECD 202 (OECD 202, 2004) it is reported that if the concentration of the test substance has been satisfactorily maintained within 20 per cent of the nominal or measured initial concentration throughout the test, then the results can also be based on nominal initial values. Furthermore, ACV has a good thermal stability and a low photolytic degradation in water for some days (Sinha et al., 2007; Hasanovic et al., 2009); such chemical physical properties are required by EU TGD, 2003 for the acceptance of the nominal concentrations as actual ones.

Table 3
Acute toxicity data reported as LC_{50} (median lethal concentration) values expressed in mg/L, with 95 % confidence range.

	B. calyciflorus (24 h)	*C. dubia (24 h)*	*H. incongruens (6 d)*
LC_{50}	> 200	> 200	149.10 (129.10–172.20)

3.1. Acute toxicity testing

The acute toxicity data, described as LC_{50} values and expressed in mg/L, are reported in Table 3.

Acyclovir caused the highest acute effect in the crustacean *H. incongruens*, with median lethal effect at concentrations in the order of hundreds of mg/L (149.10 mg/L). Regarding *C. dubia* and *B. calyciflorus*, the statistical determination of the LC_{50} values could not be achieved since the highest concentration levels (200 mg/L) did not reach 50 % mortality. Precisely, on exposure of *C. dubia* to 200 mg/L, the obtained percentage of mortality was equal to 10 %, while on exposure of *B. calyciflorus* at the same concentration, 43.3 % of lethal effect was reached. Following a scale of increasing sensitivity to ACV, the organisms may be arranged in the following order: *C. dubia* < *B. calyciflorus* < *H. incongruens.* The benthic organism was found to be more resistant to the drug than pelagic organisms during short-term exposure, although respective guidelines specify that the detection of acute effects in *C. dubia* and in *B. calyciflorus* will last for 24 h and in *H. incongruens* for 6 d. Limited evidence is available in literature, and to the best of our knowledge, no existing data are forthcoming regarding ostracods and rotifers. Our research contributes, therefore, to expand on existing data with reference to the acute effects of ACV in freshwater primary consumers. Current data on ACV acute toxicity are referred to bacteria, daphnids and fish. In 2015, An and collaborators exposing the gram-negative bacterium *Photobacterium phosphoreum* (15 min-exposure) and the cladoceran crustacean *Daphnia magna* (48 h-exposure) to ACV at 225 mg/L, found 12.3 % and 35.1 % of toxicity, respectively. Considering that *D. magna*, similarly to *C.dubia,* analyzed herein, is a cladoceran crustacean, we suggest that our outcomes (10 % mortality at 200 mg/L) are in line with the findings of previous analysis (An et al., 2015). Moreover, in 2015, Schlüter-Vorberg and collaborators did not observe acute toxic effects in fish *Danio rerio* and in the daphnid *D. magna* up to 100 mg/L. Alternatively, in 2016, Minguez et al. found an EC_{50} of 64.12 mg/L exposing *D. magna* for 48 h to ACV. In the same year, Li and collaborators (2016), using the ECOSAR program, predicted acute toxicity in fish (96 h-exposure) and daphnids (48 h-exposure) calculating the median toxic effects at 1760 and at 69.2 mg/L respectively. Ding et al. (2023) also used the ECOSAR software and obtained EC_{50} values higher than 100 mg/L for both fish and daphnids. Our analysis clearly conforms with existing literature reporting evidence of the impact on freshwater organisms solely at high concentrations (from tens to thousands mg/L), far from levels of environmental concern. Results obtained regarding short-term exposure can be collected and utilised to improve databases for further estimations, including Quantitative structure-activity relationship (QSAR) predictive modelling.

Table 4
Chronic toxicity data reported as EC_{50}, EC_{20}, EC_{10} (growth inhibition for *R. subcapitata*, offspring reduction for *B. calyciflorus* and *C. dubia*), and expressed in μg/L with 95 % confidence intervals.

	R. subcapitata (72 h)	*B. calyciflorus (48 h)*	*C. dubia (7d)*
EC_{50}	$2.65{\cdot}10^5$ ($2.52{\cdot}10^5$-$2.77{\cdot}10^5$)	230.70 (149.90–354.80)	0.03 (0.02–0.05)
EC_{20}	$1.86{\cdot}10^5$ ($1.73{\cdot}10^5$-$1.99{\cdot}10^5$)	4.07 (1.72–8.81)	$0.02{\cdot}10^{-1}$ ($0.08{\cdot}10^{-2}$-$0.03{\cdot}10^{-1}$)
EC_{10}	$1.51{\cdot}10^5$ ($1.36{\cdot}10^5$-$1.68{\cdot}10^5$)	0.38 (0.12–1.23)	$0.05{\cdot}10^{-2}$ ($0.03{\cdot}10^{-2}$-$0.01{\cdot}10^{-1}$)

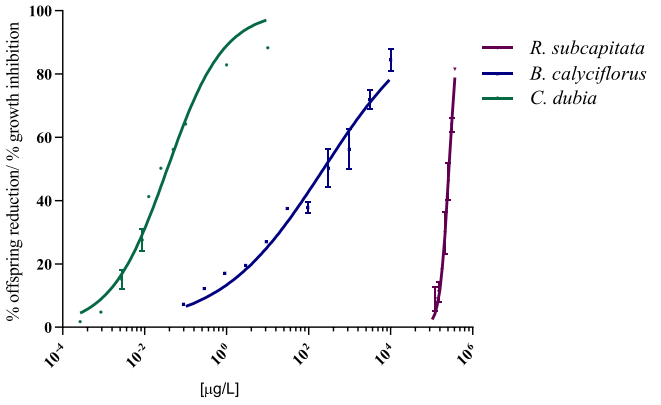

Fig. 1. Concentration effect curves (non-linear regression: log agonist vs. normalized response-variable slope) of acyclovir in *R. subcapitata* (% growth inhibition), *B. calyciflorus* ((% offspring reduction) and *C. dubia* (% offspring reduction). Bars indicate standard deviation (n=3).

According to the EU Directive 93/67/EEC, chemical substances may be classified according to the EC_{50} values. With $EC_{50} < 1$ mg/L, the compound is very toxic to aquatic organisms; 1–10 mg/L is considered toxic; and 10–100 mg/L is considered harmful to aquatic organisms. Substances with an $EC_{50} > 100$ mg/L may be considered non-toxic. As in the present study LC_{50}s are > 100 mg/L, ACV is non-toxic for all freshwater organisms tested for short-term exposure.

Considering that aquatic organisms may be exposed to ACV for a prolonged period in relation to its extensive release in the aquatic environment, incomplete removal from conventional WWTPs and pseudo persistence (An et al., 2015, Jia et al., 2019), we evaluated long-term toxicity of ACV to enhance knowledge regarding its environmental fate.

3.2. Chronic and sub-chronic toxicity testing

The chronic toxicity data of ACV, reported as EC_{50} and expressed in μg/L, are illustrated in Table 4.

From data collected, *C. dubia* was the most sensitive organism after 7 d-exposure, with EC_{50} and EC_{20} in the order of tens and units of ng/L respectively, showing 50 % and 20 % inhibition of reproduction at concentrations of environmental concern. Less sensitive than daphnids, rotifers showed 50 % and 20 % of inhibition of reproduction at hundreds and units of μg/L after 48 h-exposure. Among all exposed organisms, green algae were the most resistant to ACV, with effect concentrations in the order of tens-hundreds of mg/L. According to the EU Directive 93/67/EEC, ACV can be classified as very toxic for daphnids and rotifers ($EC_{50} < 1$ mg/L), and non-toxic for algae ($EC > 100$ mg/L) following chronic exposure. For a better understanding of the overall response trend, concentration-chronic effect curves with the respective standard deviation were reported for each organism (Fig. 1).

The figure shows that *C. dubia* reproductive inhibition started at tenths of ng/L (EC_{10}) with an evident concentration effect trend while *B. calyciflorus* inhibition started at hundreds of ng/L with a slow increase in effect as the concentrations increased. Despite a rapid increase in toxicity as concentrations increased, *R. subcapitata* was definitely less sensitive to the drug.

Table 5
H. incongruens growth inhibition test. Survival and inhibition of growth are expressed in percentage, length is expressed in μm. Results are presented as mean ± SD (n=3). Significance from negative control was determined by Dunnett's multiple comparison tests (***p<0.0001).

μg/L	Survival (%)	Length (μm)	Inhibition of growth (%)
0	100 ± 0	376.67 ± 28.97	-
$1.56{\cdot}10^4$	90 ± 0	374.65 ± 31.32	8 ± 0
$3.12{\cdot}10^4$	85 ± 5	363.27 ± 26.02	9 ± 1
$6.25{\cdot}10^4$	72 ± 7	262.50 ± 17.68***	80 ± 1

Table 6
NOEC and LOEC values expressed in μg/L obtained in chronic/sub-chronic assays.

	R. subcapitata (72 h)	*B. calyciflorus* (48 h)	*C. dubia* (7 d)	*H. incongruens* (6 d)
LOEC	$2.14{\cdot}10^5$	29	$0.08{\cdot}10^{-2}$	$6.25{\cdot}10^4$
NOEC	$1.78{\cdot}10^5$	9	$0.02{\cdot}10^{-2}$	$3.12{\cdot}10^4$

Existing literature, albeit contradictory, is available on chronic toxicity of ACV in relation to daphnids and algae. Almeida and collaborators in 2014 (Almeida et al., 2014) exposed *C. dubia* to ACV for 8 days observing 50 % toxic effect at units of mg/L while, more recently, Nugnes et al. (2022) revealed a median toxic effect in the same organism at tens of ng/L. In 2021, Schlüter-Vorberg did not observe chronic toxicity in *D. magna* up to the concentration of 92 mg/L while, Li et al. (2016) and Ding et al. (2023) found median effects at units and tens of mg/L respectively regarding the same consumer, using ECOSAR software. As far as *R. subcapitata* is concerned, Schlüter-Vorberg et al. (2015) as well as Minguez et al. (2015) did not observe median toxic effect up to 100 mg/L, while, Almeida et al. (2021) found median toxicity at units of mg/L, finally Li et al. (2016) and Ding et al. (2023) reported median effects at units and tens of mg/L respectively, with ECOSAR modelling. To the best of our knowledge, no information of ACV chronic/sub-chronic effect is available neither in rotifers nor in ostracods. In this context, we investigated the sub-chronic toxicity in the benthic ostracod *H. incongruens* after 6 days of exposure to ACV, and data are reported in Table 5.

According to standard guidelines, the body length of the ostracods was recorded when mortality was < 30 %. Statistically significant morphological modifications, in terms of length reduction, were observed in ostracods only following exposure to high concentrations of ACV (thousands of μg/L), leading to an inhibition of growth equal to 80.3 %. Fifty percent of growth inhibition was found at $4.78{\cdot}10^4$ μg/L and according to the EU Directive 93/67/EEC, ACV may be considered harmful to ostracods after sub-chronic exposure (10 mg/L < EC_{50} < 100 mg/L). To compare all toxicity results obtained in chronic/sub-chronic assays, the values of the No Observed Effect Concentration (NOEC) and of the Lowest Observed Effect Concentration (LOEC), obtained by ANOVA statistical analysis and Dunnett's multiple comparison, are reported in Table 6.

Noteworthy is the scale of increasing sensitivity of the organisms to ACV, that may be arranged as follows: *C. dubia* < *B. calyciflorus* < *H. incongruens* < *R. subcapitata,* with the algae found to be the most resistant organism after long-term exposure to the selected drug. The higher resistance of *R. subcapitata* (producer) to xenobiotics compared to consumer organisms is underlined by the physiological and transcriptional mechanisms of Selenastraceae associated with a development of tolerance towards xenobiotics which leads to adaptive mechanisms that are not yet fully understood (Gómez-Martínez et al., 2023).

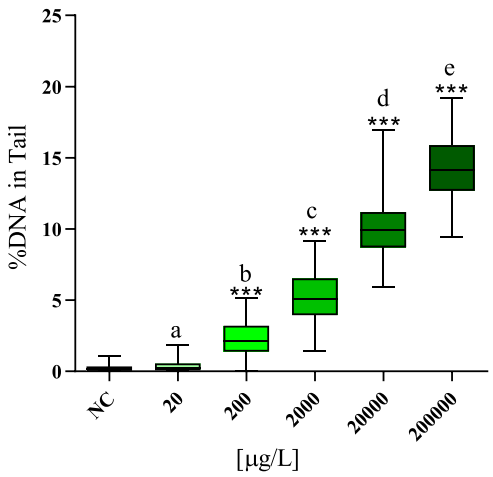

Fig. 2. Dataset of the effects of acyclovir (µg/L) on induction of DNA strand breaks in *C. dubia* cells is shown into equal fourths (box plots), the center line is the median. Results are expressed as % DNA in tail and are from two independent experiments (400 nuclei for each concentration). A significant difference from control was determined with Dunnett's test (***$p < 0.0001$) and a significant difference among concentrations ($p < 0.05$; different letters) was determined with Tukey's HSD multiple comparison test.

Contrastingly, to date, the molecular mechanisms involved in the chronic toxicity effects observed in non-target consumers exposed to ACV are a challenging issue with a complete lack of information present in literature. Hence, to better understand the mode of action concerning ACV, we conducted numerous bioassays on non-target organisms, performing assays on genotoxicity and ROS production.

3.3. Genotoxicity testing and ROS production

C. dubia, the most sensitive organism to ACV in acute and chronic assays, was exposed to the drug for 24 hours to evaluate DNA damage (Fig. 2, Fig. 3 and Table 7) and ROS production (Fig. 4). For both assays, statistically significant (***$p<0.0001$) DNA damage and ROS production were observed starting from the concentration of 200 µg/L (LOEC). The NOEC was 20 µg/L. In addition, for both assays, significant differences among concentrations were found (different letters, *Tukey's HSD multiple comparison test*), indicating a clear concentration-response relationship.

Nugnes and collaborators (2022) found DNA damage in *C. dubia* neonates starting from hundreds of µg/L, but to the best of our knowledge, no information on ROS production in aquatic invertebrates exposed to ACV is present in literature. Our results may be may be explained an increase in the DNA strand breaks as a result of reactive oxygen species (ROS) generation due to the oxidative stress produced by xenobiotics in aquatic organisms as stated by Sahlmann and collaborators (2017).

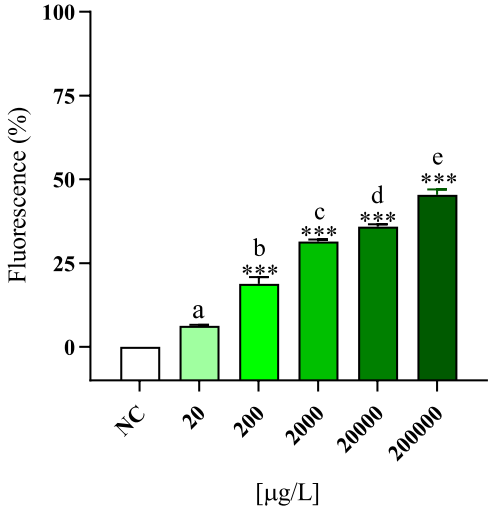

Fig. 4. ROS production in *C. dubia* after exposure to acyclovir for 24 h. Data are presented as mean ± SD (n = 3). A significant difference from control was determined with Dunnett's test (***$p < 0.0001$) and different letters intend significant differences for $p < 0.05$ among concentrations (Tukey's HSD multiple comparison test).

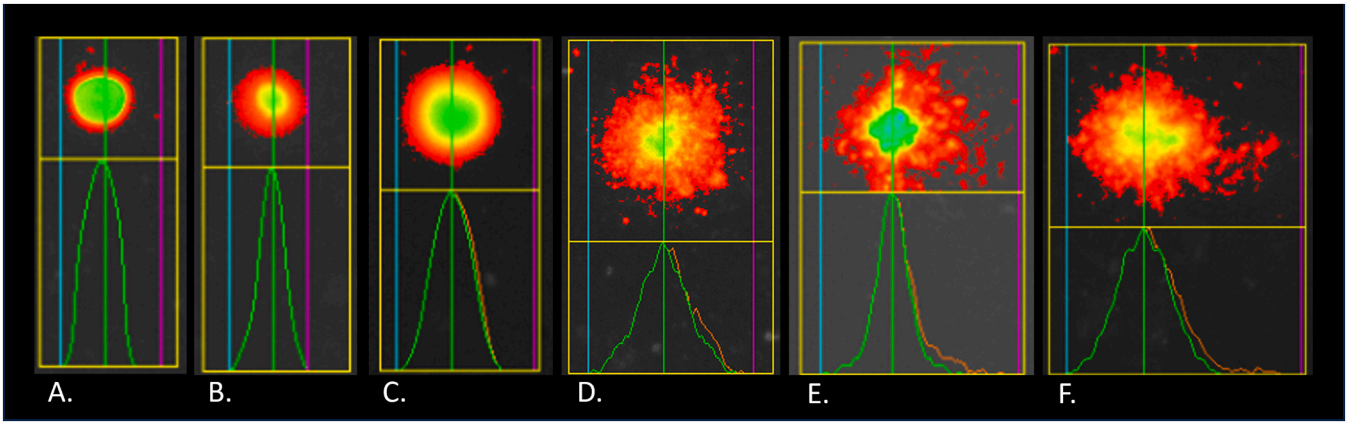

Fig. 3. Examples of the most representative nuclei observed exposing *C. dubia* to acyclovir for 24 h (A. NC; B. 20 µg/L; C. 200 µg/L; D. 2000 µg/L; E. 20000 µg/L; F. 200000 µg/L). Comets (frammented DNA in tail) were observed using Comet assay IV image analysis software (Perceptive Instrument, UK) using a fluorescence microscope (400× 256 magnification, Eclipse 50i, Nikon, Kanagawa, Tokyo) after staining with ethidium bromide (10 mg/L).

Table 7
Effects of acyclovir (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).

	NC	20	200	2000	20000	200000
T.I.	0.23 ± 0.02	0.38 ± 0.03	2.26 ± 1.46	5.26 ± 2.47	10.08 ± 2.81	14.22 ± 0.22

3.4. Environmental risk assessment

In light of our findings, we also assessed the environmental risk posed by ACV, following standard guidelines (EU TGD, 2003; EMA, 2006). The Risk Quotient (RQ) was calculated by the ratio between MEC or PEC and the Predicted No-Effect Concentration (PNEC). The latter value denotes the concentration of a substance below which the likelihood of adverse effects occurring during the exposure is negligible, calculated by the ratio between NOEC and an Assessment Factor (AF). The size of AF depends on the toxicity data available, the number of trophic levels and taxonomic groups (Table S1). Considering the lack of updated MEC data of surface waters, for the RQ calculation, we chose the most recent and the lowest PEC value regarding European surface waters sampled in 2021 in Portugal, equal to 200 ng/L (Almeida et al., 2023). Among all chronic assays performed, the lowest NOEC value was obtained exposing *C. dubia* to ACV for 7 days and was equal to 0.2 ng/L (Table 6). The AF value was 50 (Table S1) because of two levels of the trophic chain (producers and consumers) considered in experimental design. Thus, $_{chronic}$RQ was substantially higher than the threshold value of 1, deemed to be of remarkable environmental concern.

Standard guidelines do not consider the calculation of RQ based on genotoxicity data. Although standard guidelines do not consider the calculation of RQ based on genotoxicity data, Mišík et al. (2019), Nugnes et al.(2023) and Russo and collaborators (2023) have suggested performing calculations to understand the environmental genotoxic risk that xenobiotics can pose to freshwater environment. The NOEC value, obtained in genotoxicity testing, was 20 μg/L and, since in this study genotoxicity was evaluated considering only one trophic level, the AF value was, therefore, equal to 100 (Table S1). The $_{genotoxic}$RQ was equal to 1, corresponding to the threshold value, implying thereby an issue of potential environmental concern.

4. Conclusions

ACV, excreted by patients mainly as unchanged compound, gets into the environment as the WWTPs often are not able to remove drugs. The findings of the present study underscore the necessity to proceed with research in assessing the risk of ACV residues in water bodies. The chronic toxicity in non-target organisms exposed for their entire lifespan to drugs is to be accounted for as well as its genotoxicity investigated herein. Such toxic effects occur at low concentrations and the employed approach has allowed a first estimation of both chronic and genotoxic risks that would benefit from the study of diverse aquatic and benthic species along with additional occurrence data that is crucial for conducting risk assessment.

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CRediT authorship contribution statement

Chiara Russo: Writing – original draft, Investigation, Formal analysis, Data curation. **Roberta Nugnes:** Writing – original draft, Investigation, Formal analysis, Data curation. **Elena Orlo:** Writing – original draft, Investigation, Formal analysis, Data curation. **Angela Di Matteo:** Investigation. **Marina Isidori:** Writing – review & editing, Supervision, Conceptualization. **Margherita Lavorgna:** Writing – review & editing, Supervision, Conceptualization. **Elvira De Rosa:** Investigation.

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.ecoenv.2024.116437.

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