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RESEARCH ARTICLE

Contributions towards the hazard evaluation of two widely used cytostatic drugs

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Abstract

Cytostatic drugs are one of the most important therapeutic options for cancer, a disease that is expected to affect 29 million individuals by 2040. After being excreted, cytostatics reach wastewater treatment plants (WWTPs), which are unable to efficiently remove them, and consequently, they will be released into the aquatic environment. Due to the highly toxic properties of cytostatics, it is particularly relevant to evaluate their potential ecological risk. Yet, cytostatics toxicity data is still not available for various species. In this work, the ecotoxicity of two widely consumed cytostatics, cyclophosphamide (CYP—as a model cytostatic) and mycophenolic acid (MPA—as a priority cytostatic), was evaluated on three freshwater species—*Raphidocelis subcapitata*, *Brachionus calyciflorus*, and *Danio rerio*, and the risk quotient (RQ) was assessed. Both drugs significantly affected the yield and growth inhibition of the microalgae, while for rotifers, the least sensitive species, only significant effects were registered for CYP. These drugs also caused significant effects on the mortality and morphological abnormalities on zebrafish. The estimation of the RQ discloses that CYP seems to pose a low risk to aquatic biota while MPA poses a very high risk. Altogether, these results emphasize the need for more complete environmental risk assessments, to properly prioritize and rank cytostatics according to their potentially toxic effects on the environment and aquatic biota.

Keywords Environmental risk assessment · Ecotoxicology · Pharmaceuticals · Aquatic contamination · Anticancer drugs · *Raphidocelis subcapitata* · *Brachionus calyciflorus* · *Danio rerio*

Introduction

According to the World Health Organization (WHO), cancer is the second leading cause of death around the world. In 2020, almost 10 million deaths were from cancer, accounting for 16.6% of all death cases (Sung et al. 2021).

Chemotherapy is one of the most used approaches for cancer treatment. Anticancer drugs (also known as antineoplastic or cytostatic drugs) are administered to oncological patients with the ultimate goal of stopping or reducing the proliferation of the fast-dividing cancer cells (National Cancer Institute 2015). However, their mechanism of action is non-selective, not distinguishing between tumour cells and healthy cells/tissues (Kummerer et al. 2016). Accordingly, many cytostatic drugs are classified as mutagenic, carcinogenic, cytotoxic, genotoxic, and teratogenic (Chu and DeVita Jr 2019, IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012) which renders them as potentially very hazardous contaminants representing a potential risk to virtually any eukaryotic living organism. This is particularly relevant for high consumption of cytostatic drugs such as cyclophosphamide (CYP) and mycophenolic acid (MPA) for which the predicted environmental concentrations are high (Booker et al. 2014; Cristóvão et al. 2020; Usawanuwat et al. 2014).

Cyclophosphamide (CYP) is one of the most widely used cytostatics worldwide, with a broad array of applications

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including the treatment of different types of cancer and its use as immunosuppressant for the treatment of autoimmune- and immune-mediated diseases (Ahlmann and Hempel 2016; Wishart et al. 2018). For this reason, in some countries, including France, Spain, or India, its consumption reaches the order of hundreds of kilogrammes per year (Besse et al. 2012; Cristóvão et al. 2020; de García et al. 2013). Cyclophosphamide mode of action is based primarily on interaction with DNA and protein synthesis, causing cross-links that inhibit DNA replication and cell division, leading to subsequent cell death (Ahlmann and Hempel 2016; Parnham 2019). On the same line of evidence, MPA and its prodrug mycophenolate mofetil (MMF) are some of the most widely and intensively administered cytostatic drugs due to their successful action in preventing organ rejection in patients receiving transplants, as well as in the treatment of various autoimmune diseases (Wishart et al. 2018). In Portugal, for example, both MPA and MMF were, respectively, the first and fourth most consumed cytostatic drugs between the period of 2007–2015, with values ranging between 1810 and 2380 kg y^{-1} for MMF and 70.4 and 289 kg y^{-1} for MPA (Santos et al. 2017). More strikingly, in Spain, the consumption of MPA alone increased from 894 to 12,296 kg y^{-1} within the period of 2010–2015 (Franquet-Griell et al. 2017b). Once administered, MMF is rapidly and completely hydrolyzed in the liver and intestine, generating its active metabolite—MPA, which acts on the activity of inosine monophosphate dehydrogenase ultimately leading to the suppression of DNA and RNA synthesis and the proliferation of T and B lymphocytes (Parnham 2019).

After administration, a portion of the drugs is excreted without being metabolized, i.e. in its original form, as CYP or MPA. This parcel is compound-dependent, being around 5–25% for CYP (Booker et al. 2014; Parnham 2019) and less than 1% in MPA (European Medicines Agency 2021).

Some of the chemotherapeutic treatments that use this type of drugs are performed in inpatients at hospitals or other specialized facilities (Cristóvão et al. 2020; Kosjek and Heath 2011), and therefore, their residues are discharged as hazardous waste. However, the majority of oncological patients are outpatients (~ 75%); as such, after urinary excretion, these drugs reach sewage systems, being posteriorly directed to wastewater treatment plants (WWTPs), and thus, effluents are currently considered the primary source of aquatic contamination by cytostatic drugs (Cristóvão et al. 2020).

Accordingly, several authors have already reported the presence of CYP and MPA in WWTPs' effluents, suggesting that these drugs are somewhat resistant to conventional treatment processes (Franquet-Griell et al. 2017c; Gouveia et al. 2020; Hartmann et al. 2020; Santana-Viera et al. 2019). Hence, these drugs have been found in treated wastewaters, surface waters, and drinking water, at concentrations ranging from nanogrammes per litre to microgrammes per litre (Azuma et al. 2016; Deere et al. 2020; Franquet-Griell et al. 2017a; Giebułtowicz and Nałęcz-Jawecki 2016; Gu et al. 2018; Hartmann et al. 2020; Moermond et al. 2018; Santos et al. 2018; Usawanuwat et al. 2014).

The fact that cytostatic drugs can reach surface waters poses an alarming threat to the environment. This is of much concern when considering the expected increase in the consumption of these drugs as a result of the predicted increase in cancer incidence (Ferlay et al. 2020). Thus, it is important to, alongside with monitoring the presence of these drugs in the environment, apply methodologies that allow to rank and prioritize anticancer drugs concerning their environment relevance and consumption trends, while generating important ecotoxicological data for the different ecological receptors. This can be achieved by the evaluation of the potentially toxic effects that these compounds might pose to the environment and specifically to aquatic organisms since they inhabit the environmental compartment that constitutes the ultimate fate of these compounds (Kummerer et al. 2016). Yet, ecotoxicity data on cytostatics is still limited, despite the increasing interest this topic has been receiving, either in freshwater or in estuarine/saltwater (Cristóvão et al. 2020; Li et al. 2021; Martins et al. 2021; Queirós et al. 2021; Ribeiro et al. 2022; Straub et al. 2019). Furthermore, most of the available data is reported as "greater than" or based on qualitative annotations. Moreover, it should be noted that in some cases for the same parameter and species, the reported effect values exhibit a high degree of variation. For example, for cyclophosphamide, Klein et al. (2021) reported an $LC_{50,48\ h}$ as > 118 mg L^{-1} for the median mortality in *Danio rerio* embryos, whereas Aderemi et al. (2020) reported an $LC_{50,96\ h}$ of 603 mg L^{-1} and Weigt et al. (2011) reported a value fourfold higher ($LC_{50,72\ h}$ of 2344 mg L^{-1}), for the same fish species. For *Daphnia magna*, Białk-Bielińska et al. (2017) indicated the $LC_{50,48\ h}$ > 100 mg L^{-1}, whereas Harris (2015) has estimated the value of 2318 mg L^{-1}. Furthermore, for MPA, the ecotoxicity data for freshwater species is still very limited (Gao et al. 2014; Jiang et al. 2016; Straub et al. 2019).

Given these important knowledge limitations, this study aims to assess the ecotoxicity of two of the most commonly used cytostatics (CYP and MPA) to freshwater organisms of different trophic levels and functional groups: the microalga *Raphidocelis subcapitata* (as a primary producer), the rotifer *Brachionus calyciflorus* (as a primary consumer), and zebrafish *Danio rerio* (as a secondary consumer). Aside of being standard species recommended by international guidelines to be used in aquatic toxicity assays (MicroBioTests Inc, OECD 2011, 2013), all species are very important biological indicators since they are quite sensitive to aquatic contamination being key-stone representatives of trophic guilds (Bellinger and Sigee 2015; Hagiwara and Yoshinaga 2017; von Hellfeld et al. 2020).

The specific objectives of this study include the following: (i) assess the lethal and sublethal ecotoxicity of the two cytostatics, namely their effects on the growth and yield inhibition rates of the *R. subcapitata*, the mortality of *B. calyciflorus*, and mortality, hatching, and morphological abnormalities of larvae of *D. rerio*; (ii) determine the risk quotient (RQ) for each studied cytostatic, to estimate their potential ecological risk. For this, their measured environmental concentrations (MECs) were retrieved from the literature and related with the ecotoxicological data obtained in the present work.

Materials and methods

Test solutions

In the present work, the ecotoxicological effects of cyclophosphamide (CYP) and mycophenolic acid (MPA) were assessed. CYP (97%) and MPA (98%) were purchased from ACROS Organics and Sigma-Aldrich (Table S1). The range of concentrations tested was based on preliminary assays that had, as a starting point, concentrations retrieved from the literature.

The cytostatics were dissolved in the different culture media of each species (MBL for microalgae, ASTM moderately hard synthetic freshwater medium for rotifers, and charcoal-activated filtered tap water for fish) to prepare the stock solutions, taking into consideration their solubility (Table S1). The stock solutions were safely stored at − 15 °C in the dark to minimize any possible degradation. Prior to each assay, they were defrosted to prepare fresh solutions, in which each concentration was achieved by dilution of the stock solution with the culture medium according to each species.

Given the toxicity of the chemicals, all laboratory procedures were carried out with as tight security measures as possible according to the current recommendations (Eitel et al. 1999; Lamerie et al. 2013, Pan American Health Organization 2013).

Organisms' maintenance

Laboratory cultures of *R. subcapitata* were maintained in the Woods Hole Marine Biological Laboratory (MBL) culture medium (Stein 1973) with aeration, under aseptic laboratory-controlled conditions of temperature (20 ± 2 °C) and light intensity (continuous cool-white, fluorescent illumination—100 µE m^2 s^{-1}). Prior to use, the medium and all the materials used to prepare the cultures were sterilized in an autoclave at 121 °C and 1 bar, for at least 20 min. Cultures were renewed once a week.

Neonates of *B. calyciflorus* were obtained from cysts available in commercial kits (MicroBioTests Inc), which were hatched at 23 °C, for 24 h, at a constant light intensity of 3000–4000 lx. Hatching was performed in ASTM moderately hard synthetic freshwater medium (American Society for Testing and Materials 2014).

Danio rerio eggs (wild-type AB) were provided by the laboratory culture kept at the Zebrafish facility at the Department of Biology from the University of Aveiro, Portugal. Adult fish, free from externally visible diseases, were maintained under controlled conditions in a ZebTEC recirculating system (Tecniplast). The culture water consisted of tap water filtered with activated charcoal and reverse osmosis, supplemented with "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA), and maintained at a temperature of 27 ± 1 °C. Conductivity was maintained at 794 ± 50 µS/cm, pH was automatically adjusted at 7.5 ± 0.5, dissolved oxygen equal or above 95% saturation, and a photoperiod cycle of 14-h:10-h light/dark. Adult fish were fed once a day with a commercially available artificial diet Gemma Micro 500 (Skretting®, Spain).

To obtain the embryos for the toxicity assay, males and females of *D. rerio* were housed in breeding aquaria, where the deposited eggs were protected from predation from adult zebrafish (Spence et al. 2008). Eggs were collected in the morning after, gently rinsed in water from the zebrafish culture system, and screened using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon) (OECD 2013). Coagulated, unfertilized, or injured eggs with obvious irregularities during cleavage were discarded.

Ecotoxicity assays

72-h growth inhibition assays with R. subcapitata

The effects of the two cytostatic compounds were assessed on the yield and population growth rate of *R. subcapitata* according to the OECD standard methodology 201 (OECD 2011), with some minor adaptations to 24-well plates (Moreira-Santos et al. 2004).

All assays were conducted at 23 ± 1 °C, under continuous white light at an intensity of 100 µE m^2 s^{-1}. Three replicates were set per concentration (cytostatics diluted in MBL medium) and six for the control group (with MBL medium only), with concentrations ranging from 500.0 to 1856 mg L^{-1} for CYP (dilution factor of 1.3×) and from 0.004 to 0.0057 mg L^{-1} for MPA (dilution factor of 1.7×) (Table S2). To all test wells, 1800 µL of the test solution and 200 µL of algal inoculum (3–4 days old, at a concentration of 10^5 cells mL^{-1} to achieve a concentration of 10^4 cells mL^{-1} at the beginning of the assay) were added. Adding to this, one replicate of the control and all cytostatics concentrations were prepared without the addition of algae to account for

possible interferences of the cytostatics in the absorbance readings.

To avoid the settling of algae and subsequent shadow effects on their growth and yield during the 72 h of the assay, all plates were daily resuspended for a few minutes on an orbital shaker (Miller and Greene 1978; OECD 2011). Absorbance (*abs*) measurements were obtained at 24, 48, and 72 h at 440 nm (Jenway, 6505 UV/VIS spectrophotometer). After subtraction of cytostatics *abs* at the same wavelength, the *abs* were then converted into cell density per volume (D, cells mL^{-1}) according to the following Eq. (1) (Venâncio et al. 2017):

$$D\left(cells\ ml^{-1}\right) = -17107.5 + (abs * 7925350) \quad (1)$$

Yield (Y, biomass produced during the test) was computed according to Eq. (2), where N_f is the biomass of the algae at the end of the assay (cell mL^{-1}) and N_i is the biomass of the algae at the beginning of the assay (cell mL^{-1}):

$$Y = N_f - N_i \quad (2)$$

The percentage of yield inhibition (I_y) was calculated according to Eq. (3):

$$I_y\ (\%) = \left(\frac{y_c - y_t}{y_c}\right) * 100 \quad (3)$$

where Y_c is the mean value for yield in the control group and Y_t is the value for yield for the treatment replicate. The population growth rate (r; day^{-1}) was also assessed according to Eq. (4):

$$r\ (day^{-1}) = \frac{ln_{Nf} - ln_{Ni}}{t} \quad (4)$$

in which *Nf* is the biomass of the algae at the end of the assay (cell mL^{-1}), *Ni* is the biomass of the algae at the beginning of the assay, and t is the time of exposure (days). The percentage of growth inhibition (*Ir*) was calculated according to the following Eq. (5), where μ_C is the mean growth rate of algae in control and μ_t is the growth rate of algae in each replicate:

$$I_r\ (\%) = \left(\frac{\mu_C - \mu_t}{\mu_C}\right) * 100 \quad (5)$$

24-h mortality assay with B. calyciflorus

The lethal effects caused by the two cytostatics on the freshwater rotifer *B. calyciflorus* were assessed by following the standard procedure for the acute Rotoxkit F® (MicroBioTests Inc). Five replicates were assigned to each treatment: control treatment (ASTM moderately hard synthetic freshwater medium) and the concentrations tested for each cytostatic (Table S2). The concentrations were selected based on preliminary experiments and ranged between 2155 and 8000 mg L^{-1} and between 10.05 and 30.00 mg L^{-1} for CYP and MPA, respectively (dilution factors of 1.3× and 1.2×, correspondingly) (Table S2). Five organisms were placed in each well of 24-well plates, which were filled with 1 mL of the test solution. The assays were conducted for 24 h at 23 °C in total darkness. After exposure, the total number of dead organisms was counted—an organism was considered dead if it did not exhibit any movement within 5 s after gentle agitation of the medium.

96-h fish embryo toxicity assay with D. rerio

Assays with embryos of *D. rerio* were performed according to the OECD guideline 236 on Fish Embryo Acute Toxicity (FET) Test (OECD 2013), with small adaptations.

Thirty eggs per treatment were transferred to 24-well plates, in which a single egg was placed in each well with 1 mL of the test solution. The ranges of concentrations tested were based on preliminary assays and were the following: 698.8 to 1716 mg L^{-1} for CYP (dilution factor of 1.2×) and 0.094 to 3.0 mg L^{-1} for MPA (dilution factor of 2.0×) (Table S2). Exposure occurred for 96 h, at 26 ± 1 °C and a 16:8-h light/dark photoperiod, and observations were made at each 24-h period with the help of a stereoscopic microscope (Zoom-SMZ 1500, Nikon Corporation). The control group consisted of tap water filtered with activated charcoal and reverse osmosis, supplemented with "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA). Several apical endpoints were evaluated, namely hatching, mortality (that included coagulated eggs, arrested development, or lack of heartbeat), and phenotypic abnormalities (such as tail and skeletal malformations, oedemas, and delayed development) (Lammer et al. 2009). Cumulative mortality, cumulative hatching, and percentage of organisms with morphological abnormalities were expressed considering the total number of embryos exposed to the different treatments.

Risk quotient (RQ)

To estimate the potential risk posed by each cytostatic to freshwater biota, the risk quotient (*RQ*) was assessed. The *RQ* was calculated according to the following equation:

$$RQ = \frac{MEC}{PNEC}$$

where *MEC* stands for measured environmental concentration and *PNEC* is the predicted no-effect concentration. As concentrations measured in superficial waters were available for both cytostatics, the highest concentration found in the literature was used as the MEC. The PNEC was computed, for each cytostatic, through a deterministic approach, i.e. by applying

an assessment factor (AF) to the lowest computed $L(E)C_{50}$ of the three tested species. Considering that short-term $L(E)C_{50}$ were here calculated for three trophic levels, an AF of 1000 was used (EC 2011). The results were interpreted according to European Commission (EC) (1996) and Sánchez-Bayo et al. (2002): negligible risk ($RQ < 0.01$), low risk ($0.01 \leq RQ < 0.1$), moderate risk ($0.1 \leq RQ < 1$), and high risk ($RQ \geq 1$).

Data analysis

The estimation of the lethal concentrations causing X% of effect (LC_x) and respective confidence limits at 95% (CL 95%) was performed by applying a regression model using the Probit software (Sakuma 1998). The estimation of sublethal concentrations causing X% of effect (EC_x) was performed by applying a non-linear model (three-parametric logistic or sigmoid curve, according to the best fit) using the Statistica for Windows 4.3 software (StatSoft, Aurora, CO, USA).

Statistical analysis was performed using SigmaPlot 14.0 (Systat Software, Inc. SigmaPlot for Windows). To mortality data sets, an Arcsin sqrt transformation was applied at first. Then, a one-way ANOVA was carried out, followed by Dunnett's test to determine potential statistical differences against control conditions. The non-lethal endpoint data sets were checked for normality and homoscedasticity, with the Shapiro–Wilk test or the Brown-Forsythe test, respectively, followed by a one-way ANOVA, and then Dunnett's test, to assess potential differences between treatments and control conditions. Whenever data sets failed one of the assumptions, a non-parametric ANOVA was carried (Kruskal–Wallis), followed by the multicomparison Dunn's test.

Results and discussion

Toxicity data

All assays fulfilled the validity criteria according to the respective guidelines (MicroBioTests Inc, OECD 2011, 2013). In the assays with *R. subcapitata*, at the end of the 72-h exposure period, the growth rate was higher than 0.92 day^{-1}, as required by the guideline (OECD 2011). All assays with rotifers fulfilled the requirement of having less than 10% of mortality on the control group (MicroBioTests Inc). Finally, concerning assays with *D. rerio* embryos, (i) the overall fertilization success of the collected eggs was higher than 70%, (ii) temperature was maintained at 26 ± 1 °C, (iii) the mortality and percentage of embryos and larvae with teratogenic effects in the control group were always below 10% during the full assay, and (iv) the hatching rate in the control group was superior to 80% at the end of the 96-h exposure period, as required for test validity (OECD 2013).

Cyclophosphamide

Cyclophosphamide significantly inhibited the yield of *R. subcapitata* at all tested concentrations (Fig. 1a; Dunnett's test: $p < 0.05$), but a significant reduction in the growth rate was only observed at concentrations equal to or higher than 845 mg L^{-1} (Fig. 1b; Dunnett's test: $p < 0.05$). The estimated $EC_{50,72\,h}$ (confidence limit (CL) 95%) for yield and growth rate were, respectively, as follows: 593.0 (510.0–676.0) mg L^{-1} and 1 108 (873.0–1 343) mg L^{-1} (Table 1). Concerning $EC_{20,72\,h}$ (confidence limit (CL) 95%), the results for yield and growth rate were, respectively, as follows: 407.0 (299.0–515.0) mg L^{-1} and 533.7 (319.0–749.4) mg L^{-1} (Table 1).

Such results are in accordance with reports from other studies, in which the majority was only able to speculate EC_{50} values to be higher than at least 100 mg L^{-1} (Białk-Bielińska et al. 2017; Česen et al. 2016; Grung et al. 2008, 2006; Russo et al. 2018) with the exception of Zounková et al. (2007) who were able to determine an $EC_{50,96\,h}$ of 930 mg L^{-1}, in a 96-h assay, and Harris (2015) who estimated an EC_{50} higher than 3000 mg L^{-1} for the effects of cyclophosphamide on the growth rate of *R. subcapitata*. In the present study, aiming at providing a dose–effect curve and effective concentration estimation, the concentrations tested were way above those normally reported for the freshwater compartment (Azuma et al. 2016; Deere et al. 2020; Franquet-Griell et al. 2017a; Gu et al. 2018; Moermond et al. 2018; Usawanuwat et al. 2014). As already hypothesized by other authors, the apparent low toxicity of CYP to these organisms may be explained by the pharmacokinetics of the compound itself, which needs to be activated by liver enzymes into active metabolites that will cause cytotoxic effects (Białk-Bielińska et al. 2017; Zounková et al. 2007). A study conducted by Mater et al. (2014) also assessed the growth inhibition of *Selenastrum capricornutum* (currently known as *R. subcapitata*) when exposed to CYP, either individually or in a combination with two other pharmaceuticals—ciprofloxacin and tamoxifen—in nominal concentrations at the microgrammes per litre level. Their results revealed that the dose–effect caused by CYP alone exhibited a U-shape form, with a slight but significant growth inhibition at a concentration of 0.01 µg L^{-1} and a significant growth promotion at the highest tested concentration of 10 µg L^{-1} (Mater et al. 2014). However, it must be emphasized that these significant changes in growth were always lower or similar to 10% when compared to the control, i.e. within the threshold of effects induced by randomness. Furthermore, it must be highlighted that the mixture of the three chemicals (thus, simulating a more realistic scenario) caused significant growth inhibition, in a dose-dependent manner, with effects at concentrations as low as 0.01 µg L^{-1} (Mater et al. 2014).

Concerning the mortality assays with *B. calyciflorus*, CYP significantly reduced the survival of rotifers at a concentration of 8000 mg L^{-1} (Fig. 2; Dunnett's test: $p < 0.05$). An $LC_{50,24\,h}$

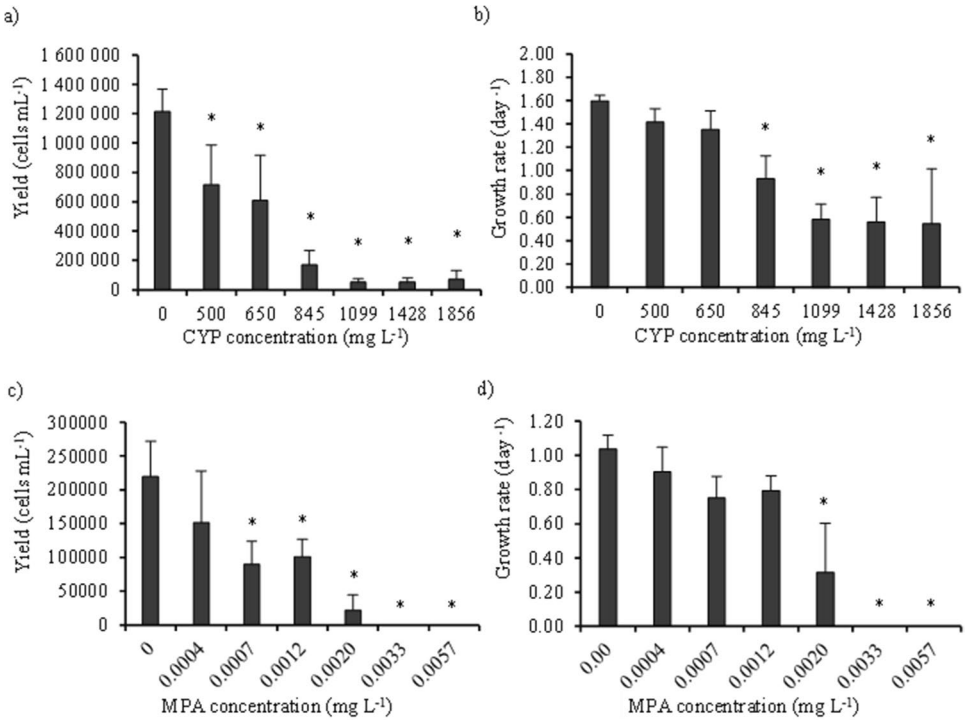

Fig. 1 Average of yield (cells mL^{-1}) (**a**) and growth rate (day^{-1}) (**b**) of *Raphidocelis subcapitata* after being exposed, for 72 h, to different concentrations of cyclophosphamide (CYP), and average of yield (cells mL^{-1}) (**c**) and growth rate ($day^{-.1}$) (**d**) of *Raphidocelis subcapitata* after being exposed, for 72 h, to different concentrations of mycophenolic acid (MPA). Vertical bars correspond to the standard deviation. * indicates a significant statistical difference in relation to control conditions (Dunnett's or Dunn's test: $p < 0.05$)

Table 1 Summary of the concentrations (mg L^{-1}) of cyclophosphamide (CYP) and mycophenolic acid (MPA), causing 20 and 50% of effect ($L(E)C_{20}$ and $L(E)C_{50}$, respectively), with the 95% confidence limits (95% CL), for the three freshwater model species, and the estimated risk quotient (RQ) of each cytostatic drug, with the indication of negligible risk (green traffic light), and high risk (red traffic sign)

Cytostatic	Species	Endpoint (hours)	Time (hours)	$L(E)C_{20}$ (mg L^{-1}) (95% CL)	$L(E)C_{50}$ (mg L^{-1}) (95% CL)	Risk quotient
CYP	*R. subcapitata*	Yield inhibition	72 h	407.0 (299.0 – 515.0)	593.0 (510.0 – 676.0)	**0.003**
		Growth inhibition	72 h	533.7 (319.0 – 749.4)	1108 (873.0 – 1343)	
	B. calyciflorus	Mortality	24 h	–	6397 (5986 – 6896)	
	D. rerio	Mortality	96 h	1219 (1157 – 1 262)	1306 (1261 – 1351)	
		Abnormalities	96 h	757.7 (614.1 – 838.1)	1030 (942.9 – 1182)	
MPA	*R. subcapitata*	Yield inhibition	72 h	0.00027 (0.00002 – 0.00053)	0.00068 (0.00035 – 0.00101)	**965**
		Growth inhibition	72 h	0.00119 (0.0008 – 0.00157)	0.00167 (0.00135 – 0.002)	
	B. calyciflorus	Mortality	24 h	>30.00	>30.00	
	D. rerio	Mortality	48 h	2.120 (1.630 – 2.440)	2.830 (2.460 – 3.440)	
			96 h	0.860 (0.660 – 1.030)	1.410 (1.180 – 1.710)	
		Abnormalities	48 h	0.170 (0.070 – 0.220)	0.220 (0.150 – 0.360)	
			96 h	0.130 (0.100 – 0.140)	0.160 (0.140 – 0.190)	
		Hatching	48 h	0.075 (0.024 – 0.126)	0.193 (0.116 – 0.269)	
			96 h	0.773 (0.663 – 0.883)	0.945 (0.760 – 1.129)	

of 6397 (5986–6896) mg L^{-1} was computed (Table 1) which, to the author's best knowledge, is the second lethality value described in the literature concerning rotifers exposed to CYP, after Russo et al. (2018) having reported an $LC_{50,24\,h}$ of 1924 mg L^{-1}. Nevertheless, it is important to highlight that these concentrations are several orders of magnitude higher than the ones usually reported in the environment, namely in surface waters which are in the nanogrammes per litre range (Azuma et al. 2016; Deere et al. 2020; Franquet-Griell et al. 2017a; Gu et al. 2018; Moermond et al. 2018) and even higher than the highest measured concentration in surface waters 1907 ng L^{-1} (Usawanuwat et al. 2014).

The results of cumulative survival and hatching in *D. rerio* embryos and larvae induced by CYP are presented in Fig. 3. Significant effects on the survival of embryos were observed at concentrations of 1430 and 1716 mg L^{-1}, and only after a period of 96 h of exposure (Fig. 3a; Dunn's: $p < 0.001$). An $LC_{50,96\,h}$ of 1306 (1261–1351) mg L^{-1} was

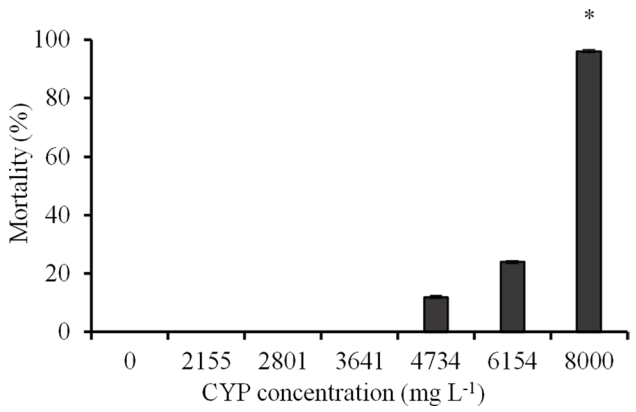

Fig. 2 Mortality percentage (%) of *Brachionus calyciflorus* after being exposed, for 24 h, to different concentrations of cyclophosphamide (CYP). Vertical bars correspond to the standard deviation. * indicates a significant statistical difference in relation to control conditions (Dunnett's test: $p < 0.05$)

computed (Table 1), which is within the range of values reported in the literature, which vary between 602.9 and 2344 mg L^{-1} (originally, 2.16 mM and 8.4 mM) (Aderemi et al. 2020; Ali et al. 2012; He et al. 2013; Klein et al. 2021; Weigt et al. 2011). Such variations may be explained due to the use of different zebrafish phenotypes, different test medium, embryos at different stages of development, and distinct times of exposure between this work and the ones cited previously. Additionally, the LC_{10} and LC_{20} were also computed ($LC_{10,96\,h}$: 1093 mg L^{-1} and $LC_{20,96\,h}$: 1219 mg L^{-1} (1157–1262)). The proximity of these values may be indicative of high mortality rates if small increments of the concentrations of these cytostatics occur in the environment. Concerning the hatching rates of *D. rerio* embryos, the exposure to CYP did not have an effect (Fig. 3b; Dunn's test: $p > 0.05$).

The sublethal endpoint concerning the presence of morphological abnormalities on the embryos and larvae was also recorded since it is an important endpoint to better demonstrate and understand the teratogenic potential of cytostatics drugs in different non-target organisms.

An $EC_{50,96\,h}$ of 1030 (942.9–1182) mg L^{-1} was computed concerning the percentage of embryos/larvae that developed some kind of morphological abnormality during the 96-h exposure to CYP (Fig. 3c, Table 1). This drug induced significant morphological abnormalities on the embryos and larvae exposed to the highest concentration from the 48 h of exposure and onwards, and on larvae exposed to lower concentrations in later stages of the assay (Fig. 3c; Dunn's: $p < 0.05$). For instance, after 48 h of exposure at concentrations of 1430 and 1716 mg L^{-1}, 33% and 21% of the live larvae exhibited severe oedemas (Fig. 4). From 72 h of exposure, at least 80% of the live larvae exhibited not only severe oedemas on the pericardial or ventral regions but also spinal cord malformations (also known as tail curvatures/bending) that remained throughout the assay (Fig. 4). These teratogenic effects were also observed in a study conducted by Weigt et al. (2011), in which almost 82% of all the zebrafish embryos

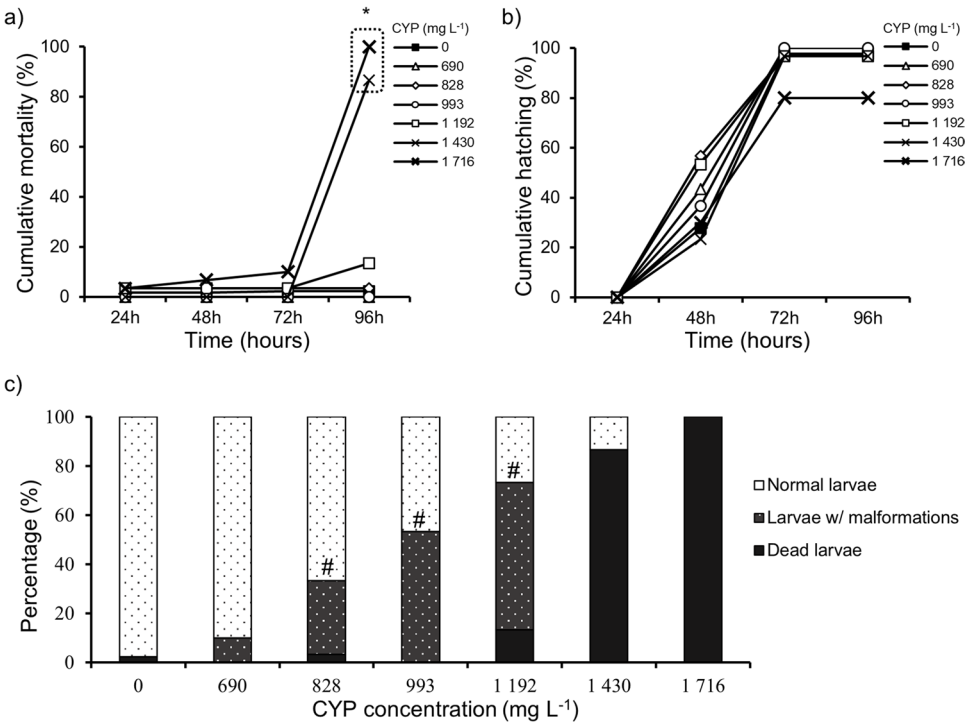

Fig. 3 Cumulative mortality (%) (**a**), cumulative hatching (%) (**b**), and general overview (%) (**c**) of the effects of different cyclophosphamide (CYP) concentrations in *Danio rerio* embryos and larvae after being exposed for 24, 48, 72, and/or 96 h. * indicates a significant statistical difference in relation to control conditions (Dunn's test: $p < 0.001$). # indicates a significant statistical difference concerning the presence of malformations in relation to control conditions (Dunn's test: $p < 0.05$). The dashed box incorporates all the statistically significant values

exposed to CYP concentrations ranging between 279.1 and 2791 mg L^{-1} (originally, 1 and 10 mM) presented chord malformations ranging from isolated lesions to complete disintegration of the chord structure. Zhu et al. (2014) also observed that CYP caused pericardial oedema and circulation defects in zebrafish larvae exposed from 48 hpf (hours post fertilization) to 72 hpf to CYP at concentrations ranging between 0.028 and 2791 mg L^{-1} (originally, 0.1 and 10,000 µM). Adding to oedemas in the pericardium and the yolk sac, spine, and tail malformations, Aderemi et al. (2020) also observed bradycardia, affected hatchability, and neurotoxic effects in zebrafish larvae exposed to CYP at concentrations varying between 212.1 and 1281 mg L^{-1} (originally, 0.76 and 4.59 mM).

One factor that could explain the increasing number of abnormalities and mortality rates in later stages of the assay (i.e. 72–96 h) is related to the hatching and organogenesis of zebrafish itself. Cyclophosphamide is a drug that needs to be activated by liver enzymes; however, it has been pointed out that zebrafish embryos do not express the necessary enzymes for the metabolism of this drug in sufficient quantities (Busquet et al. 2008). Furthermore, the vascularization of the liver and subsequent blood flow only happens around 72 hpf, being the liver fully developed only by the fifth-day post fertilization (dpf) (Chu and Sadler 2009; Ober et al. 2003). In the absence of proper metabolic activating systems, CYP is not activated, and therefore, it cannot bind and disrupt DNA synthesis, which could explain the higher rates of morphological abnormalities and mortality only in later stages of the assay (Anderson et al. 1995) (Figs. 3a, c and 4). Additionally, although the egg itself allows some exchange of molecules between the embryo and the exterior environment, it also works as a barrier, protecting the embryo from the outside conditions. Therefore, after hatching, larvae are more exposed to the outside conditions, and they will absorb small molecules diluted in the surrounding water through their gills and skin (McGrath and Li 2008). Altogether, these factors might help explain the aggravation of effects caused by CYP in hatched larvae over the course of the assay.

It was also possible to observe a significant decrease in the body length of the larvae exposed to CYP at 828.8, 993.3, and 1192 mg L^{-1} (Fig. 5; Dunn's test: $p < 0.001$). No data was available for concentrations of 1430 and 1716 mg L^{-1} since it was not possible to measure the larvae exposed at these concentrations, either because they were dead or did not hatch (Fig. 5). Some experiments with rats and mice

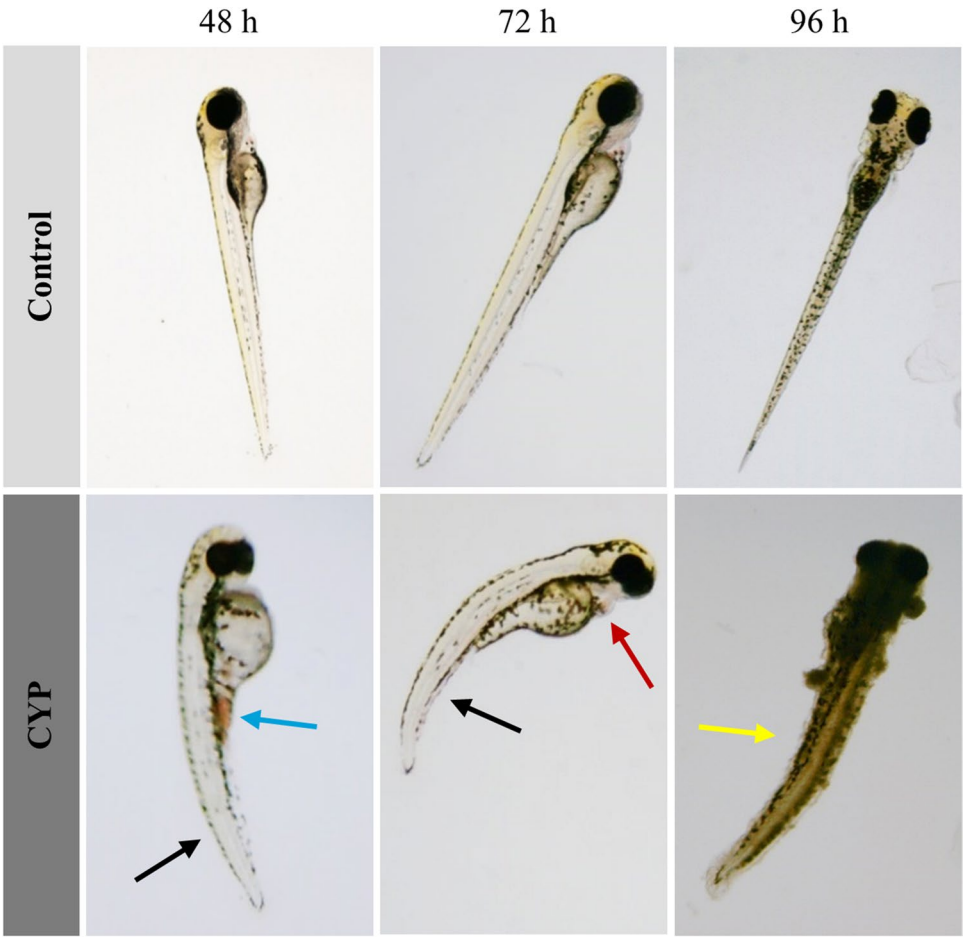

Fig. 4 Pictures illustrating the morphological abnormalities observed in embryos and larvae of *Danio rerio* after being exposed to a concentration of 1430 mg $L.^{-1}$ of cyclophosphamide (CYP), compared to larvae from the control group. Red arrows indicate oedemas in the pericardium; blue arrows indicate oedemas in the ventral region; black arrows indicate tail/spinal cord malformation; yellow arrows indicate late coagulation (magnification of 2×, pictures are not related in size)

administered with CYP also observed similar developmental anomalies, which included growth retardation events, malformed foetuses, behavioural deficits, and even structural chromosome damage (Anderson et al. 1995). Even though there are obvious morphological differences between rat foetuses and zebrafish embryos, these studies help to further demonstrate the teratogenic potential of CYP in different non-target organisms.

Mycophenolic acid

Mycophenolic acid significantly inhibited the yield of *R. subcapitata* at concentrations equal to or higher than 0.0007 mg L^{-1} (Fig. 1c; Dunnett's test: $p < 0.05$). Concerning the growth rate, this cytostatic drug only caused significant effects on *R. subcapitata* at concentrations equal to or higher than 0.0020 mg L^{-1} (Fig. 1d; Dunn's test: $p < 0.05$). The estimated $EC_{50,72\,h}$ for yield and growth rate were, respectively, 0.00068 (0.00035–0.00101) mg L^{-1} and 0.00167 (0.00135–0.002) mg L^{-1} (Table 1). To the author's best knowledge, the only data available regarding the effects of this drug on microalgae is presented in a report summary from F. Hoffmann-La Roche Ltd (2021), estimating an $EC_{50,96\,h}$ of 0.068 mg L^{-1} for growth rate inhibition and an $EC_{50,96\,h}$ of 0.017 mg L^{-1} for biomass inhibition. This lack of information emphasizes the need for more data to perform thorough environmental risk assessments. In addition to the median effective concentrations, it was also possible to estimate the $EC_{20,72\,h}$ (CL 95%), for the effects on the growth rate of *R. subcapitata*, which was 0.0018 (0.0008–0.0016) mg L^{-1}.

Concerning the results regarding the effects of MPA on the survival rate of *B. calyciflorus*, this cytostatic did not significantly impact the survival of the rotifers at any of the tested concentrations, in which the higher tested concentration was close to the limit of solubility of the compound (Table S1; Dunn's test: $p > 0.05$). To the author's best knowledge, no data is available in the literature regarding the effects of this drug in rotifers. These results suggest that concentrations expected to cause lethal effects on rotifers would be considerably higher than the ones reported in the environment; thus, at least for the mortality endpoint, this type of assay might not be the best option (Franquet-Griell et al., 2016; Franquet-Griell et al. 2017b; Giebułtowicz and Nałęcz-Jawecki 2016; Gouveia et al. 2019; Hartmann et al. 2020). Nonetheless, other endpoints such as reproduction, swimming behaviour, bioaccumulation and biomagnification potentials, biomarkers, and chronic sensitivity might be interesting to explore. For example, Martins et al. (2021) studied the ecotoxicological effects of another cytostatic drug, 5-fluorouracil, on *B. calyciflorus*, by conducting an assay to assess the lethal effects and an assay to evaluate the impacts of this drug on the inhibition of population growth. Even though it was not possible to estimate an $LC_{50,24\,h}$ for the acute assay, it was possible to estimate an $EC_{50,48\,h}$ of 10.49 µg L^{-1} concerning the effects of 5-fluorouracil on the rotifers' populational growth (Martins et al. 2021). Moreover, in terms of costs and time-effectiveness, assays with rotifers still carry some advantages and ecological importance, and thus, rotifers should not be excluded from general environmental risk assessment studies. Additionally, when studying toxic substances, such as cytostatics, these organisms provide the additional advantage of requiring lower amounts of test substances when compared with, for example, *Daphnia magna* (OECD 2012).

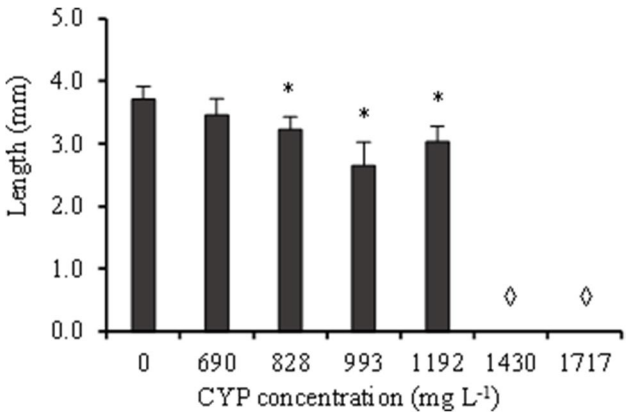

Fig. 5 Body length (mm) of *Danio rerio* larvae after being exposed for 96 h to cyclophosphamide (CYP). Vertical bars correspond to the standard deviation. * indicates a significant statistical difference in relation to control conditions (Dunn's test: $p < 0.001$). ◊ no data was available as organisms were dead or did not hatch

The results of cumulative survival and hatching in *D. rerio* larvae exposed to MPA are presented in Fig. 6. Mycophenolic acid significantly affected the survival of *D. rerio* larvae at concentrations of 1.5 mg L^{-1} after 96 h of exposure and at concentrations of 3.0 mg L^{-1} since 48 h of exposure (Fig. 6a; Dunn's test: $p < 0.001$). The estimated $LC_{50,48\,h}$ for MPA was 2.830 (2.460–3.440) mg L^{-1} and the $LC_{50,96\,h}$ was 1.410 (1.180–1.710) mg L^{-1} (Table 1). The estimated $LC_{20,96\,h}$ (threshold for effect concentration) was half of the $LC_{50,96\,h}$ (0.860 mg L^{-1}, with estimated CL 95% of 0.660–1.03 mg L^{-1}, while the estimated $LC_{10,96\,h}$ was 0.616 (0.407–0.814) mg L^{-1}. This drug affected the survival of zebrafish embryos in a dose- and time-dependent manner (Fig. 6a, c), as already observed by other authors (Gao et al. 2014; Jiang et al. 2016). However, the $LC_{50,96\,h}$ found for MPA in this work is relatively lower than other lethality values previously reported in the literature that vary from 3.9 to 17.7 mg L^{-1} (originally, 12.3 and 55.4 µmol L^{-1}, respectively) (Gao et al. 2014; Jiang et al. 2016). These differences might be due to variations in terms of the zebrafish phenotype used, techniques, methods of exposure, and duration of the assays between the different works. For example, while in this work wild-type AB zebrafish embryos were used,

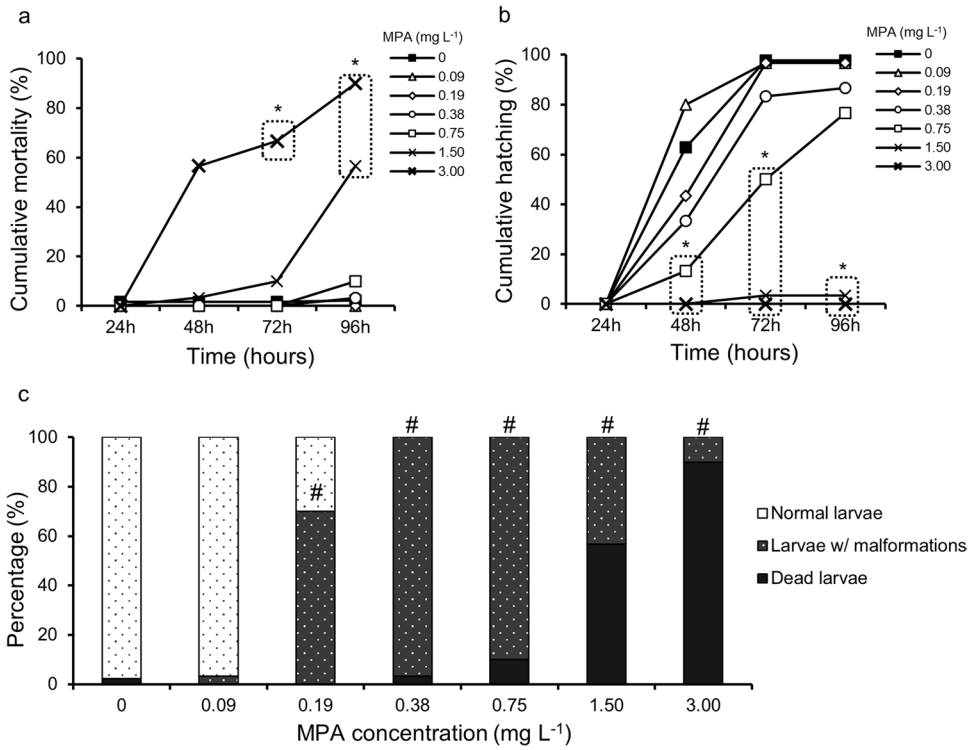

Fig. 6 Cumulative mortality (%) (**a**), cumulative hatching (%) (**b**), and general overview (%) (**c**) of the effects of different mycophenolic acid (MPA) concentrations in *Danio rerio* embryos and larvae after being exposed for 24, 48, 72, and/or 96 h. * indicates a significant statistical difference in relation to control conditions (Dunn's test: $p < 0.001$). # indicates a significant statistical difference concerning the presence of malformations in relation to control conditions (Dunn's test: $p < 0.05$). The dashed box incorporates all the statistically significant values

both Jiang et al. (2016) and Gao et al. (2014) used wild-type zebrafish embryos (Tübingen line). Moreover, Jiang et al. (2016) conducted a 70-h exposure assay with embryos at 2 hpf, while Gao et al. (2014) exposed zebrafish embryos at 72 hpf to different concentrations of MPA for 24 h.

Concerning the cytostatic effect on the hatching rate of *D. rerio*, it was observed a significant decrease in the hatching success of larvae exposed to 1.5 and 3.0 mg L^{-1} of MPA since 48-h exposure period, and at 0.75 mg L^{-1} at 48 and 72 h of exposure (Fig. 6b; Dunn's test: $p < 0.001$). An $EC_{50,48\ h}$ of 0.193 (0.116–0.269) mg L^{-1} and an $EC_{50,96\ h}$ of 0.945 (0.760–1.129) mg L^{-1} were estimated for the effects of MPA on the hatching rate of zebrafish embryos (Table 1).

Concerning the sublethal endpoint of morphological abnormalities, it was possible to observe that there were significant effects, as early as 24 h of exposure, on embryos and larvae exposed to concentrations equal and higher than 0.38 mg L^{-1} of MPA (Figs. 6c and 7; Dunn's test: $p < 0.05$). It is important to highlight that at least 97% of the live embryos exposed to 1.5 and 3.0 mg L^{-1} of MPA presented severe oedemas as early as 24 h of exposure (Fig. 7). From 48 h onwards, at concentrations equal and higher than 0.38 mg L^{-1}, 100% of the live organisms presented morphological abnormalities that mainly included severe oedemas on the yolk sac or pericardial region, tail/spine curvatures, and less often a delay in the development of the head and tail (Fig. 7). From 72 h of exposure onwards, the concentration of 0.19 mg L^{-1} of MPA also significantly induced malformations on the larvae, with 50 and 70% of the larvae affected at 72 and 96 h of exposure, respectively (Dunn's test: $p < 0.05$). Concerning this endpoint, it was possible to estimate an $EC_{50,48\ h}$ of 0.220 (0.150–0.360) mg L^{-1} and an $EC_{50,96\ h}$ of 0.160 (0.140–0.190) mg L^{-1} (Table 1).

This sublethal endpoint proved to be very important for these drugs, as they induced phenotypic changes in a dose-dependent manner (mainly oedemas in the pericardial or yolk sac regions and spinal cord malformations) (Figs. 6c and 7). Similar effects have also been reported in other studies. For example, Jiang et al. (2016) observed that zebrafish embryos exposed to concentrations of MPA ranging from 0.16 to 16.02 mg L^{-1} (originally, 0.5–50 µmol L^{-1}) for 70 h developed teratogenic defects that predominantly included tail bending and pericardial oedemas. The same authors also calculated a teratogenic index (25% lethal concentration value (LC_{25})/no observable adverse effect level ratio), which demonstrated that MPA can, in fact, be classified as a teratogen (Jiang et al. 2016). Gao et al. (2014), besides observing the same abnormalities, also noticed embryos with abnormal body shape, enlarged yolk sac, growth arrest, and motility decrease in embryos exposed to MPA at concentrations between 0.44 and 2.21 mg L^{-1} (originally, 1.38 to 6.92 µmol/L).

We hypothesize that the observed severe morphological abnormalities occurring as soon as after 24 h of exposure might have had some repercussions in the overall embryo development, since a lower number of embryos, compared to the ones from the control group, were effectively able to hatch throughout the assay (Figs. 6 and 7).

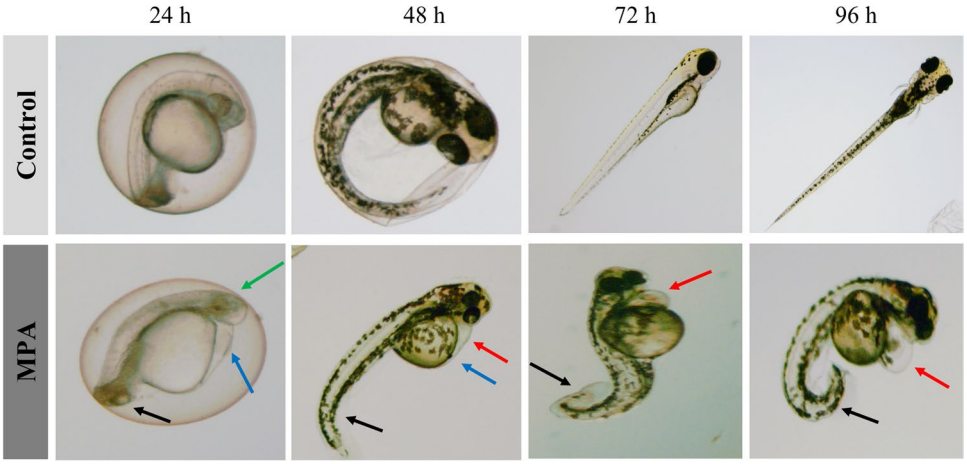

Fig. 7 Pictures illustrating the morphological abnormalities observed in embryos and larvae of *Danio rerio* after being exposed to different concentrations of mycophenolic acid (MPA). From left to right in the bottom panel: MPA = 3 mg L^{-1} (24 h); MPA = 0.38 mg L^{-1} (48 h); MPA = 0.75 mg L^{-1} (72 h); MPA = 0.38 mg $L^{-.1}$ (96 h). Red arrows indicate oedemas in the pericardium; blue arrows indicate oedemas in the yolk sac; black arrows indicate tail/spinal cord malformation; green arrows indicate delayed development (magnification of 2×, pictures are not related in size)

One possible explanation for the formation of severe oedemas is associated with the findings of Jiang et al. (2016). Considering that MPA is a strong inhibitor of IMPDH (inosine monophosphate dehydrogenase), these authors also studied *impdh* genes to decipher the mechanism of MPA toxicity. Briefly, they found that the expression of *impdh* was indeed inhibited in MPA-treated embryos, which subsequently could be causing a depletion of guanosine monophosphate (GMP) (Jiang et al. 2016). After hypothesizing that if organisms had an external guanosine supply, then the abnormal morphological traits should be prevented; they observed that some embryos treated with both mycophenolic acid and guanosine actually regained the normal phenotype (Jiang et al. 2016).

Risk quotient (RQ)

Regarding the assessment of the potential risk that these cytostatics may pose to freshwater biota, the RQ was estimated considering the highest measured environmental concentrations found in the literature and the lowest value of the toxicity data reported in this study (Table 1). The lowest L(E)C_{50} values used to calculate the RQ were both from the assays assessing the yield inhibition in *R. subcapitata*, with an $EC_{50,72\,h}$ of 593 mg L^{-1} for CYP and an $EC_{50,72\,h}$ of 0.00068 mg L^{-1} for MPA (Table 1). The highest MECs found for CYP and MPA on surface waters were, respectively, 0.0019 mg L^{-1} (in a Thai river) and 0.000656 mg L^{-1} (in a Spanish river) (Franquet-Griell et al. 2017a; Usawanuwat et al. 2014).

The RQ for each cytostatic drug was computed and is presented in Table 1. The estimated RQ for CYP was 0.003, therefore revealing that this drug poses no significant risk to these organisms (Table 1). Nevertheless, this goes according to what was expected when taking into consideration the substantial differences between the tested concentrations and the reported environmental concentrations, which have a difference of 5 orders of magnitude amongst them. Similarly, other studies have also classified CYP as posing moderate or no risk to aquatic organisms (Gouveia et al. 2019; Santos et al. 2017).

Regarding MPA, this drug seems to present a very high risk to freshwater biota, with an RQ of 965 (Table 1). However, previous studies have reported distinct classifications for MPA concerning the risk it might pose to different organisms (Franquet-Griell et al. 2017a, Franquet-Griell et al. 2017b, Giebułtowicz and Nałęcz-Jawecki 2016, Gouveia et al. 2019, Guo 2015, Saab et al. 2021, Santos et al. 2017). For example, Saab et al. (2021) compared the different risk classifications given to MPA over the years, ranging from potentially hazardous to high-risk compound. Nonetheless, while some studies also classify MPA as a high-risk compound (Giebułtowicz and Nałęcz-Jawecki 2016; Gouveia et al. 2019; Santos et al. 2017), others state that MPA poses low or no risk not only to aquatic organisms but also to organisms from higher trophic levels (Franquet-Griell et al. 2017a, 2017b, Guo 2015). These discrepancies amongst RQ might be mainly due to differences in the calculation of the PNEC values; for example, Franquet-Griell et al. (2017a) considered an EC_{50} higher than 100 mg L^{-1} for *Daphnia magna* ecotoxicity, while Giebułtowicz and Nałęcz-Jawecki (2016) calculated the PNEC value with an EC_{50} of 0.068 mg L^{-1} (originally, 68 µg L^{-1}) relative to MPA's effect on the growth inhibition of *R. subcapitata*. Moreover, while some authors consider a pharmaceutical with an RQ higher than 1 to pose a high risk, others consider an RQ between 1.0 and 10 to indicate a small potential to cause adverse effects (Franquet-Griell et al. 2017a, 2017b;

Gouveia et al. 2019). These differences in risk characterization and classification, between different works, guidelines, and countries, emphasize the need of a solid database or a widely accepted guideline with standardized methods and criteria concerning the risk assessment of different pharmaceuticals (or at least of teratogenic or carcinogenic substances) that could be used worldwide, to minimize the uncertainties associated with these classifications/prioritizations.

Conclusions

Cyclophosphamide (CYP) and mycophenolic acid (MPA) are two widely used cytostatics in cancer and autoimmune disorder treatment and/or to prevent transplanted organs rejection (Wishart et al. 2018). Based on their high consumption profiles, their measured and/or predicted environmental concentrations are relatively high, and thus, the risk they might pose towards aquatic organisms needs to be addressed. This is particularly relevant if we consider the expected rise in the use of these drugs in the future, given the increasing incidence of cancer and autoimmune diseases. Yet, the information on their ecotoxicological effects is still limited, with most of the available reports disclosing only mortality data, and generally not addressing sublethal endpoints which are highly relevant when dealing with cytostatic drugs. Furthermore, effective concentrations values are not always reported, being most of the times described as greater than, and when reported, they are highly variable between studies, with several orders of magnitude apart. These limitations pose difficulties when estimating the risk of these cytostatics. Hence, in this study, we have assessed the lethal and sublethal effects of CYP and MPA in three species representatives of different trophic and functional levels—algae, rotifers, and fish. Overall, our results disclose that MPA is more toxic to *R. subcapitata* than CYP, with MPA inhibiting both the growth and yield of *R. subcapitata* at environmental relevant concentrations. For rotifers, the concentrations for which CYP affected the mortality of *B. calyciflorus* were much higher than the usually reported environmental levels of CYP. As for MPA, it was not possible to calculate effect concentrations as this cytostatic did not result in mortality in these organisms up to the highest concentration tested (which was limited by the MPA solubility limit). To the authors' best knowledge, this is the first study to evaluate the effects of MPA on *B. calyciflorus*. Concerning the assays with zebrafish, both cytostatics resulted in lethal and sublethal effects, mainly malformations such as oedemas, which entail special importance concerning the teratogenic properties of these types of drugs. Based on the toxicity data obtained, the risk quotient was computed, and the results disclose that CYP poses low risk to aquatic biota while MPA poses a very high risk. Such results highlight the importance of developing effective removal/mitigation technologies able to prevent the introduction of these drugs into the aquatic environmental, particularly for MPA, giving the high risk it poses to biota.

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Author contribution B.M.: validation, formal analysis, investigation, writing—original draft, writing—review and editing. C.V.: validation, formal analysis, investigation, writing—review and editing. R.F.: validation, formal analysis, investigation, writing—review and editing. A.C.S.: conceptualization, methodology, writing—review and editing, visualization, supervision, funding acquisition. I.L.: conceptualization, methodology, writing—review and editing, visualization, supervision, funding acquisition.

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Data availability Data is available upon request.

Declarations

Ethics approval Not required as no human data or samples were used. All experiments with animals (non-vertebrate models and larval stages of zebra fish) were performed in compliance with the 3Rs principle.

Consent to participate Not applicable.

Consent to publication Not applicable.

Competing interests The authors declare no competing interests.

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