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Individual and mixed effects of anticancer drugs on freshwater rotifers: A multigenerational approach

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ABSTRACT

Human population growth has led to an increased release of chemical contaminants into aquatic environments. Emerging chemical contaminants (ECCs) are of increasing concern because they can affect non-target organisms in aquatic ecosystems. The application of anticancer drugs is increasing because of enhanced cancer rates and use of chemotherapy. We assessed the impacts of two widely used anticancer drugs known for their distinct modes of action, namely 5-fluorouracil (5-FU) and doxorubicin (DOX), on the freshwater rotifer *Brachionus calyciflorus* across generations. Rotifer mortality (24 h) and population growth (48 h) were assessed to determine initial lethal and sub-lethal effects. Exposure of rotifers to 5-FU (up to 200 mg L^{-1}) did not cause mortality, while DOX caused mortality at high concentrations (EC_{50} = 15.6 mg L^{-1}). Effects of 5-FU on population growth rate was higher than DOX (5-FU EC_{50} = 10.49 μg L^{-1}, DOX EC_{50} = 8.78 mg L^{-1}). The effects of the drugs in binary mixture on population growth rates were dose dependent; significant antagonistic effects were found when 5-FU was present in the mixture at high concentrations. Finally, a transgenerational assay for five generations revealed that rotifers were able to recover their population growth rate after fourth generation when exposed to 5-FU; however, population became non-viable after the second generation of exposure to DOX. At the cellular level, accumulation of reactive oxygen species and plasma membrane damage were observed at EC_{10} and increased at EC_{50} for both drugs. After exposure of rotifers to 5-FU across generations, there were signs of oxidative stress recovery, as shown by a decrease in ROS accumulation and plasma membrane damage. Our results showed for the first time that the adverse effects of anticancer drugs on freshwater rotifer populations are drug and dose dependent and can persist or be attenuated along generations.

1. Introduction

Emerging chemical contaminants (ECCs) have raised a huge concern about their potential impacts on environmental and human health (Ahmed et al., 2017). ECCs are divided into several groups, including pharmaceuticals, personal care products, endocrine disruptors, surfactants, pesticides and engineered nanoparticles (Ahmed et al., 2017; Luo et al., 2014). The potential impacts of some ECCs in aquatic systems are still uncertain, and only few data is available on their long-term effects at environmentally relevant concentrations. Various governmental and non-governmental organizations are establishing directives and legal frameworks to protect the quality of freshwater resources from ECCs (Esplugas et al., 2007; Valbonesi et al., 2021).

Increasing consumption of pharmaceuticals by increasing world population leads to an inevitable release of pharmaceuticals into aquatic environments, raising concern on their possible threats to non-target organisms because of their high biological activities (Ferrari et al., 2004; Fent et al., 2006; Santos et al., 2010; Martins et al., 2012;). The elevated cancer rates and consequent increase of chemotherapy (Johnson et al., 2008) contribute to an increased concern of pharmaceutical contamination. In recent years, cocktail administration of drugs revealed to be more effective, and their application is mainly in outpatient settings (at home) (Lenz et al., 2007; Shi et al., 2012), increasing the possible routes of drug entry into the aquatic environments.

Cytostatic anticancer drugs interfere with the structure of cell DNA with lack of specificity to target tumour cells; therefore, it is expected that in the contact with non-target organisms, these drugs would exhibit a similar effect posing risks to aquatic organisms (Parrella et al., 2014).

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5-fluoruoracil (5-FU) represents one of the most consumed anticancer drugs (Besse et al., 2012); it is a uracil analogue that misincorporates fluoronucleotides into RNA and DNA, disrupting their synthesis and repair, and inhibiting the activity of thymidylate synthase (Longley et al., 2003). On the other hand, doxorubicin (DOX) is a cytotoxic antibiotic of the anthracycline class, commonly used in chemotherapy, with the ability to cause DNA strand breakage, inhibiting DNA and RNA synthesis and activity of topoisomerase II, leading to DNA damage and induction of apoptosis (Johnson-Arbor and Dubey, 2020). Traces in aquatic environments of 5-FU (hospital effluent: $<$ 8.6–124 μg L^{-1}, Mahnik et al., 2007; municipal wastewater 4.7–14 ng L^{-1}, Kosjek et al., 2013) and DOX (hospital effluent: $<$ 0.26–1.35 μg L^{-1}, Mahnik et al., 2007; wastewater effluent: 3.4 ng L^{-1}, Martin et al., 2011) are reported; but only few information is available on their ecotoxicity individually (Parrella et al., 2014), or in presence of other ECCs (Brezovšek et al., 2014), and the mechanisms of toxicity. Some studies have reported about their availability, stability and degradation. Degradation of DOX is common and mainly dependent on pH and availability of light (Janssen et al., 1985; Beijnen et al., 1986; Wu and Ofner, 2013). However, DOX adsorption to various surfaces including glass, polyethylene, polytetrafluoroethylene, and other substances is also well known (Wood et al., 1990; Bapat, 1991; Wu and Ofner, 2013). On the other hand, 5-FU is highly resistant to biodegradation and it has low adsorption to suspended solids in water matrices (Zhang et al., 2013; Governo et al., 2017); therefore, it is expected to persist in aquatic environments.

Rotifers are relatively small ($<$ 200 μm), non-arthropodan, metazoan, and ecologically important freshwater invertebrates that are harmless to humans (Dahms et al., 2011). They represent a substantial part of the secondary production in aquatic systems thanks to their large population size (Wallace, 2002), high turnover rate and optional reproduction by parthenogenesis (Snell and Janssen, 1995; Hagiwara et al., 1997, 2007). These characteristics, together with ease of cultivation (Gómez et al., 2002), have made rotifers a widespread model for ecotoxicological studies.

Most of the laboratory-based ecotoxicity studies focus on mortality and reproduction (population growth rate); but other endpoints, such as behaviour, physiology, bioaccumulation and biochemistry (Dahms and Hellio, 2009) are also relevant and easy to assess in the rotifer model. Furthermore, the short generation time of rotifers allows for testing the impacts of new compounds at short times (24–48 h), and across generations, providing quick and reliable data to assess their ecotoxicity. The monogonont rotifer *Brachionus calyciflorus* is a well-established freshwater bioassay model species (ASTM, 2004), and it has been used for assessing toxicity of a large variety of xenobiotics, including metals, nanoparticles and pesticides (Marcial et al., 2005; Snell and Hicks, 2011; Martins et al., 2020).

Standard testing for lethal and sub-lethal effects of ECCs and their transgenerational impacts on terrestrial and freshwater invertebrates are important to better know their environmental effects (Yu et al., 2013; Castro et al., 2018). In a recent study, *B. calyciflorus* recovered its population growth rate after the second generation of exposure to CuO-NPs and the third generation of exposure to Ag-NPs, despite the persistence of oxidative stress due to accumulation of reactive oxygen species (ROS) up to the fourth generation (Martins et al., 2020). However, the knowledge on transgenerational effects of ECCs is limited (Yu et al., 2013; Castro el, 2018; Martins and Guilhermino, 2018; Martins et al., 2020), particularly for pharmaceuticals (Minguez et al., 2015; Kim et al., 2017). Although the persistence of ECCs in the environment and mutagenic properties of some anticancer drugs have been shown, trophic transfer (via food supply) and transgenerational effects have not yet been properly studied; but these aspects are crucial for understanding their impacts on aquatic ecosystems.

This study aims to evaluate the lethal and sub-lethal effects of two anticancer drugs (5-FU and DOX) on rotifer population by assessing if and how i) concentrations of the drugs detected in effluents can affect the fitness and population size alone or in binary mixtures, ii) future generations can be affected by parental exposure, and iii) population can adapt to a continuous exposure scenario. We hypothesised that i) 5-FU would promote higher toxicity than DOX due to its lower adsorption and/or degradation ability, ii) both drugs would show an additive effect in binary mixtures due to their distinct mechanisms of action, and iii) effects would persist along generations after releasing rotifers from the anticancer drugs.

2. Materials and methods

2.1. Selection and maintenance of rotifers

B. calyciflorus dormant cysts were obtained from MicroBioTests Inc. and stored at 4 ºC in dark to be hatched before experiments. A standard freshwater medium (Table S1) was prepared with moderately hard water (U.S. Environmental Protection Agency, 1985) using deionized water for rotifer hatching and to prepare test dilutions. Hatching was performed in Petri dishes with 10 mL of standard freshwater in a climate chamber for 16–18 h at 25 ºC with continuous lighting.

In order to provide food for rotifers, *Raphidocelis subcapitata* cultures were maintained weekly in 2-L flasks in COMBO algae modified medium (Table S2) at 20 ºC, with constant aeration (filtered through a 0.2 μm syringe filter) and continuous light. COMBO medium and flasks were sterilised (30 min, 120 ºC, 5 bar). At the exponential growth, algae were centrifuged in cycles of 7500 rpm, at 4 ºC for 5 min to remove the COMBO medium and re-suspend the algae pellet in a small volume of freshwater medium to concentrate the food stock.

2.2. Stock solutions

The anticancer drugs, 5-Fluorouracil (5-FU; $\geq$ 99% HPLC grade) [2,4-Dihydroxy-5-fluoropyrimidine, CAS Number: 51–21–8] and Doxorubicin hydrochloride (DOX; 98.0–102.0% HPLC grade) [Hydroxydaunorubicin hydrochloride, CAS: 25316–40–9] were obtained from Sigma-Aldrich. Stock solutions were prepared in the test medium with vigorous mixing and 30 min sonication in dark, until total solubility of the compounds was attained.

2.3. Quantification of 5-fluorouracil and doxorubicin

Samples were collected for quantification of anticancer drugs in the test medium at different conditions: T0 (at the beginning of the experiment), T24 (after 24 h), and T48 (after 48 h), i) in the absence of rotifers and algae; ii) in the presence of rotifers but absence of algae; iii) in the presence of rotifer and algae. For each condition, 15 mL of medium (in triplicates) was filtered (through 0.22 μm syringe filter) to remove the organisms. Each sample was quantified for drugs through High Performance Liquid Chromatography coupled with Mass Spectrometry (HPLC-MS/MS) at Scientific and Technological Research Assistance Centre, University of Vigo, Spain.

2.4. Ecotoxicological assays for individual drugs

Mortality of *Brachionus calyciflorus* (ISO, 2001) caused by 5-FU or DOX was assessed by 24 h rotifer mortality tests. Rotifers were hatched for 18 h and no feeding was provided prior to or during the tests. After hatching, 5 juvenile rotifers were placed in each well of the 24 multiwell test plates (1 mL total volume per well). For each drug, 8 concentrations (5-FU: 11.71–200 mg L^{-1}; DOX: 5.93–30 mg L^{-1}) and a negative control were used (4 replicates). Plates were incubated at 25 ºC in the absence of light. After 24 h, the number of living and dead rotifers per well was counted under a dissection microscope.

Inhibition of population growth rate of *B. calyciflorus* (ISO, 2001) caused by 5-FU or DOX was assessed through 48 h population growth test. After 18 h hatching period, rotifers were pre-fed for 2 h with Roti-Rich pre-feeding mixture and then fed with a fresh suspension of

R. subcapitata (2×10^6 cells per mL) during the 48 h of experimental period. Then, one juvenile of *B. calyciflorus* was placed in each well of 24-multi-well test plates (1 mL total volume per well), with 8 concentrations of the drugs (5-FU: 3.91–500 μg L^{-1}; DOX: 0.12–15 mg L^{-1}) and a negative control as mentioned above (8 replicates). Plates were incubated and the number of living and dead rotifers per well was counted. Rotifer population growth rate (r) was calculated as follows: $r = (\ln N_{final} - \ln N_{start} / T)$, where N_{final} represents mean (average) number of rotifers after 48 h incubation, N_{start} represents mean number of rotifers at the beginning of the experiment (T = 1); T represents time of exposure in days (T = 2) (Martins et al., 2020).

To achieve the test validation, mean rotifer population growth rate in control has to be equal or superior to 0.55 d^{-1}, reproduction must occur in at least 7 out of the 8 control replicates and the percentage of effect in the lowest concentration should be inferior to 50% (Martins et al., 2020).

2.5. Effects of anticancer drugs in mixtures on population growth

Following the 48 h population growth test, an exposure experiment with binary mixtures of both anticancer drugs was conducted. To that end, two effect concentrations were selected (EC_{10} and EC_{50}) for each drug with a total of four mixed exposure treatments as follows: i) 5-FU EC_{10} + DOX EC_{10}, ii) 5-FU EC_{10} + DOX EC_{50}, iii) 5-FU EC_{50} + DOX EC_{10}, and iv) 5-FU EC_{50} + DOX EC_{50}. A negative control with clean medium and four positive controls with individual drugs (5-FU EC_{10}, DOX EC_{10}, 5-FU EC_{50} and DOX EC_{50}) were performed and used in binary mixture predictions. Each treatment was prepared prior to the start of the experiment and then properly mixed before pouring into the 24 multi-well test plates (1 mL total volume per well). Rotifers were then added to each plate to start the experiment following the same experimental conditions described in the Section 2.4 for the 48 h population growth test.

2.6. Reactive oxygen species assessment by epifluorescence microscopy

Following the method used by Martins et al. (2020), epifluorescence microscopy was used to monitor ROS accumulation and plasma membrane damage in rotifers. Rotifer population, exposed to EC_{10} or EC_{50} of each anticancer drug or to binary mixture of EC_{10} of both drugs, were collected after 48 h of exposure and frozen in liquid nitrogen. After defrosting, rotifers were treated with a mixture of fluorescence markers composed of 5 μM CM-H_2DCFDA, 15 μM of propidium iodide and 100 μL of anti-fading reagent containing 4′,6-diamidino-2-phenylindole (DAPI) as described in Martins et al. (2020). After loading the samples onto microscope slides, each slide was monitored under epifluorescence microscope (200 ×, Leica DM5000B for single generation and Olympus BX63F2 for multigerations), and images were acquired in bright field and different fluorescence modes with a digital camera (Leica DFC 350 FX R2 and Olympus DP74) using the software LAS AF (Ver 1.4.1) and the software cellSens (Ver 1.18) for each microscope and camera respectively. Fluorescence data for drug mixtures were obtained only at EC_{10} concentrations, due to the loss of rotifer integrity in other treatments.

2.7. Transgenerational assay with individual and mixed drug exposures

Following the experimental design in Martins et al. (2020), we assessed generational impacts of anticancer drugs in *B. calyciflorus* over a period of 10 days. Modelled EC_{50} of each drug alone or in mixture were used. At the start of the experiment, F0 generation (8 replicates per treatment, 1 randomly selected rotifer per replicate) was exposed to EC_{50} concentration of each drug (5-FU and DOX) for 48 h. After 48 h, freshly born rotifers (< 24 h old) were transferred into new wells to start the exposure of the F1 generation. At the F1 generation, 8 replicates were placed in a exposure treatment and another 8 were placed in fresh (unexposed) medium. At the end of the exposure period, exposed F1 offspring with < 24 h was transferred to contaminated medium for 48 h exposure, while fresh F1 offspring with < 24 h was transferred to a non-contaminated fresh medium and incubated for 48 h (Fig. S1). This procedure was repeated up to the F4 generation. In cases where there were not enough rotifers to start all 8 replicates, only the available replicates were performed. Live and dead rotifers were recorded under a dissection microscope to estimate population growth rate, and rotifers older than 24 h were sampled and frozen in liquid nitrogen for assessing the intracellular ROS accumulation and plasma membrane damage as described in Section 2.6.

2.8. Data analysis

To test if the concentration of each anticancer drug in the medium varied with time and in the presence of living organisms, we used a two-away ANOVA (Table S3) followed by Tukey's multiple comparison post-hoc tests. Effect concentrations (EC_{10} and EC_{50}; with 95% confidence limits) of the drugs for rotifer mortality were determined by Probit regression (Finney 1971; using SPSS statistics 17.0) and for population growth rate were calculated by logistic regression (using STATISTICA 8.0). The LOEC (lowest-observed-effect-concentration) and NOEC (no-observed-effect-concentration) of each contaminant for mortality and population growth rate were determined by one-way ANOVA (Table S4), followed by the Dunnett's test to assess significant differences between the treatments and the control. The effects of each drug and their binary mixture on exposed or pre-exposed rotifers for each generation in the transgenerational test were determined by one-way ANOVAs (Table S5), followed by Dunnett's tests.

To estimate mixture toxicity population growth rate was calculated and converted into inhibition of population growth rate. Using data from the effect concentrations of each individual drug, we estimated the toxicity of the binary mixtures using a mathematical model based on the theory of probabilities (Kungolos et al., 1997; Tsiridis et al., 2006) as follows: $P(E) = P1 + P2 - (P1 \times P2) / 100$, where P1 is the inhibition of population growth rate caused by contaminant A P2 is the inhibition caused by contaminant B, and P(E) is the theoretical expected additive inhibition of population growth rate caused by drug mixture. Multiple *t*-tests were used to test whether the predicted and observed effects differed significantly.

ANOVAs and *t*-tests were performed in GraphPad Prism 7.

3. Results

3.1. Characterization of 5-fluorouracil and doxorubicin

In the absence of rotifers and algae, the concentration of 5-FU in the medium did not vary with time (T0 = 431 ± 8 μg L^{-1}, T24 = 378 ± 24 μg L^{-1}, T48 = 421 ± 21 μg L^{-1}; $P > 0.05$; Fig. 1A). At T0, 5-FU concentration in the medium did not change in the presence of rotifers ($P > 0.05$) or by the further addition of algae ($P > 0.05$; Fig. 1A). The addition of rotifers and algae resulted in a decrease in 5-FU concentration at T24, (284 ± 21 μg L^{-1}; $P < 0.05$; Fig. 1A), and a further decrease at T48 (207 ± 42 μg L^{-1}; $P < 0.05$). A significant decrease in 5-FU concentration at T48 was observed in the medium when the rotifers were alone (334 ± 16 μg L^{-1}; $P < 0.05$; Fig. 1A).

In the absence of rotifers and algae, DOX concentration in the medium decreased significantly with time (T0 = 455 ± 23 μg L^{-1}, T24 = 247 ± 22 μg L^{-1}, T48 = 146 ± 13 μg L^{-1}; $P < 0.05$; Fig. 1B). At T0, the addition of rotifers reduced the DOX concentration in the medium (247 ± 16 μg L^{-1}; $P < 0.05$; Fig. 1B); and the concentration was reduced further with the addition of algae (146 ± 13 μg L^{-1}; $P < 0.05$; Fig. 1B). At T24, the presence of rotifers resulted in the reduction of DOX concentration in the medium (120 ± 20 μg L^{-1}; $P < 0.05$), but the addition of algae did not result in a further decrease in concentration ($P > 0.05$; Fig. 1B). At T48, there was no significant differences in DOX concentration caused by the presence of rotifers and algae (T48 with

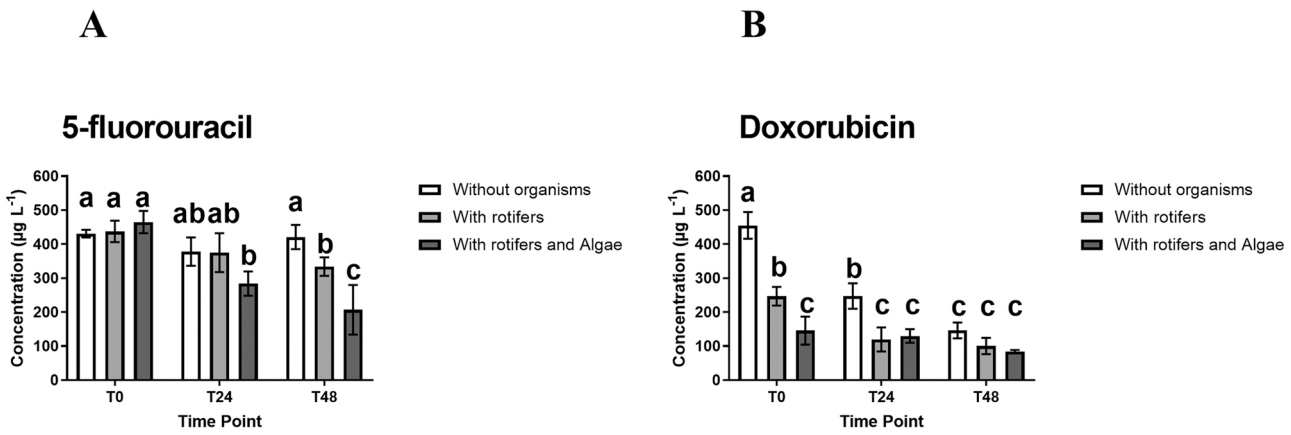

Fig. 1. Concentrations of 5-fluororuacil (A) and doxorubicin (B) measured in the medium along time in the absence or presence of rotifers and/or algae. Initial concentration of contaminants was 0.5 mg L^{-1}. Mean ± SE; n = 3; significant differences ($P < 0.05$) are represented by different letters. T0 = 0 h, T24 = 24 h, T48 = 48 h.

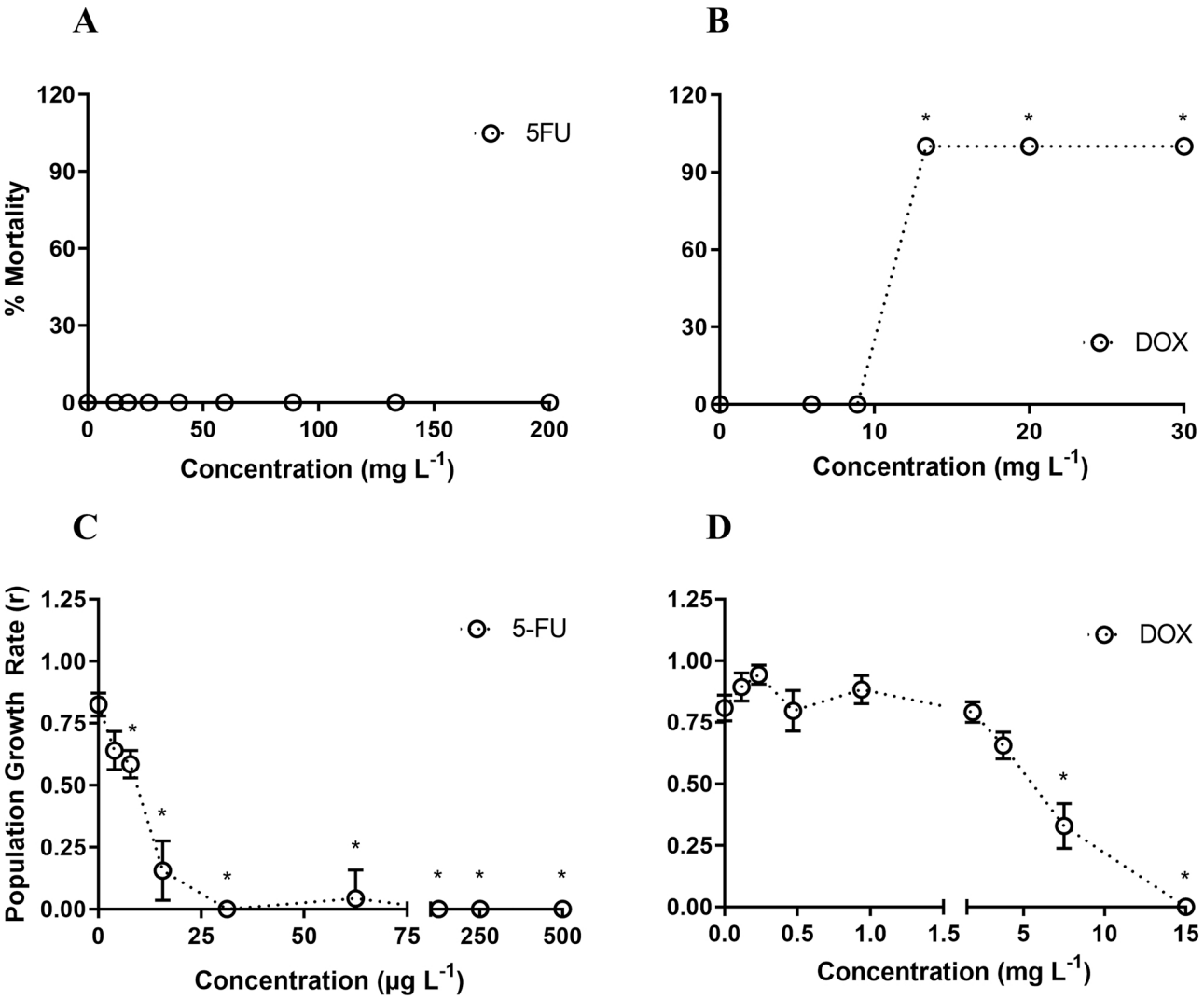

Fig. 2. Mortality (%) of *B. calyciflorus* upon exposure to 5-fluorouracil (A) and doxorubicin (B), and effects on population growth rate after exposure to increasing concentrations of 5-fluorouracil (C) and doxorubicin (D). Mean ± SE; n = 3; significant differences from control ($P < 0.05$) are represented by asterisks.

rotifer = 100 ± 14 μg L^{-1} vs T48 with rotifer and algae = 84 ± 3 μg L^{-1}; $P > 0.05$; Fig. 1B).

3.2. Effects of anticancer drugs on rotifer mortality and population growth rate

The anticancer drug 5-FU had no lethal effects on rotifers, even at the highest tested concentration (200 mg L^{-1}; Fig. 2A, Table 1). On the other hand, DOX led to 100% mortality at the lowest observed effect concentration (LOEC = 13.3 mg L^{-1}) (Fig. 2B, Table 1).

The exposure of rotifers to 5-FU significantly affected the population growth rate at low concentration (LOEC = 7.8 μg L^{-1} and EC_{50} = 10.5 μg L^{-1}; Fig. 2C; Table 1). DOX was also capable of inhibiting the rotifer population growth rate, but toxicity was > 800 × lower (LOEC = 7.5 mg L^{-1}; EC_{50} = 8.8 mg L^{-1}; Fig. 2D; Table 1).

In binary mixtures, the addition of DOX at concentrations of EC_{10} and EC_{50} to 5-FU at EC_{10} resulted in slightly higher inhibition of the population growth rate (59.6% and 82.3%, respectively) than that predicted (42.4% and 64%, respectively; Fig. 3), although the differences were not significant (*t*-tests, $P > 0.05$). Conversely, the addition of EC_{10} and EC_{50} of DOX to EC_{50} of 5-FU resulted in significantly lower inhibition of population growth rate (37% and 65.4%, respectively) than that predicted (83.7% and 96.5%, respectively) (*t*-tests, $P < 0.05$).

3.3. ROS accumulation and plasma membrane damage

The 48 h exposure of rotifers to both anticancer drugs at EC_{10} and EC_{50} concentrations led to accumulation of ROS and damage on plasma membranes (Fig. 4). ROS accumulation and plasma membrane damage appeared to increase in a dose-dependent manner, but not at the same extent for each drug. The effects were visually stronger after exposure to 5-FU at EC_{50} compared to DOX at the same effect concentration. In drug mixtures at EC_{10}, the levels of ROS and plasma membrane seemed slightly higher than that observed for the individual drugs.

3.4. Transgenerational effects of anticancer drugs

At all generations, the population growth rate of rotifers was higher than the minimum required 0.55 d^{-1} for validation (F0 = 0.74 d^{-1}, F1 = 0.84 d^{-1}, F2 = 0.76 d^{-1}, F3 = 0.68 d^{-1}, F4 = 0.71 d^{-1}; Fig. 5), and no evidence of parental mortality occurred in controls. At F0, the inhibition of population growth rate by modelled EC_{50} concentrations of each drug alone was significant ($P < 0.05$; Fig. 5) and close to the expected 50% inhibition, with some parental mortality occurring in all treatments. At F0, the effects in mixtures did not differ significantly from those observed for each drug alone ($P > 0.05$; Fig. 5). At F1, the population growth rate of rotifers continually exposed to each drug was inhibited ($P < 0.05$; Fig. 5). Rotifers released from contaminants showed signs of recovery from exposure to 5-FU ($P > 0.05$ compared to unexposed controls; Fig. 5) but not from exposure to DOX ($P < 0.05$; Fig. 5). At F1, rotifer population, released from the contaminants in mixtures, failed to grow ($P < 0.05$; Fig. 5). At F2-F4, rotifers continuously exposed to or released from 5-FU recovered their population growth rate to control levels ($P > 0.05$; Fig. 5). At F2-F4, rotifers exposed to DOX did not recover their population growth rate and had high levels of parental mortality even after their release from the drug ($P < 0.05$; Fig. 5).

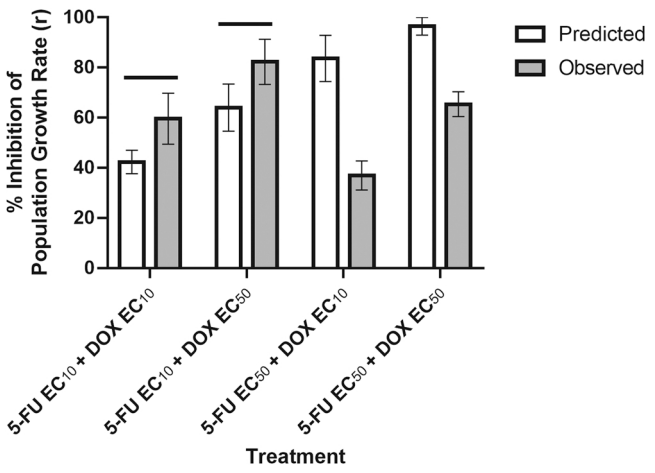

Fig. 3. Comparisons between the predicted and observed inhibition (%) of population growth rate of *B. calyciflorus* caused by the exposure to binary mixtures of 5-fluorouracil (5-FU) and doxorubicin (DOX). Mean ± SE; n = 3. Horizontal lines indicated no significant differences between the predicted and observed effects ($P > 0.05$).

Due to the low availability of rotifers, ROS accumulation and plasma membrane damage could not be assessed in all treatments from all generations. Fluorescence markers showed ROS accumulation and plasma membrane damage in all treatments at F0 (Fig. S2). At F1, rotifers continuously exposed to 5-FU showed signs of recovery in terms of ROS accumulation and plasma membrane damage. At F1, rotifers released from 5-FU showed lower levels of ROS accumulation and plasma membrane damage when compared to the exposed ones (Fig. S2).

4. Discussion

In our study, the two tested anticancer drugs, 5-fluorouracil and doxorubicin, were expected to exhibit different levels of toxicity, and, indeed, the magnitude of differences in their toxicity was considerably high. DOX showed ability to kill half of the rotifer population and inhibit 50% the population growth rate at 15.6 mg L^{-1} and 8.8 mg L^{-1}, respectively. These values are higher than those detected in the environment so far (0.3–1.4 μg L^{-1} at hospital effluents; Mahnik et al., 2007; and 4.5 ng L^{-1} in wastewater influent; Martín et al., 2011), but are very close to the EC_{50} values determined by Parrella 12.7 and 7.7 mg L-1, respectively) et al. (2014) for crustaceans, including rotifers. In our study, 5-FU did not cause mortality to rotifers at the maximum tested concentration (500 μg L^{-1}), but inhibited the population growth rate > 800 × in comparison to DOX (5-FU EC_{50} = 10.5 μg L^{-1} vs DOX EC_{50} = 8.8 mg L^{-1}; Table 1). EC50 values of 5-FU for population growth rate were very close to the concentrations detected in aquatic systems (11.5–122 μg L^{-1} in hospital sewage, Fürhacker et al., 2006; 8.6–124 μg L^{-1} in hospital effluents, Mahnik et al., 2007). We found that 5-FU showed much higher toxicity for rotifers than that found in other studies (EC_{50} = 100 μg L^{-1}, Zounkova et al., 2010; 322 μg L^{-1}, Parrella et al., 2014), however, the LOEC of 5-FU for population growth rate of rotifers (7.8 μg L^{-1}) was closer to that of *Daphnia magna* (16 μg L^{-1}) as

Table 1
Effect concentrations for ECCs used in this study. In brackets are indicated the 95% confidence limits of EC_{10} and EC_{50} values, whenever available. n/e stands for not estimated.

Contaminant	Endpoint	Unit	EC_{10}	EC_{50}
5-Fluorouracil	Mortality	μg L^{-1}	n/e	n/e
	Population Growth Rate	μg L^{-1}	5.37 (3.60–7.14)	10.49 (8.83–12.16)
Doxorubicin	Mortality	mg L^{-1}	13.06 (11.88–13.91)	15.56 (14.71–16.75)
	Population Growth Rate	mg L^{-1}	6.48 (5.24–7.72)	8.78 (7.15–10.40)

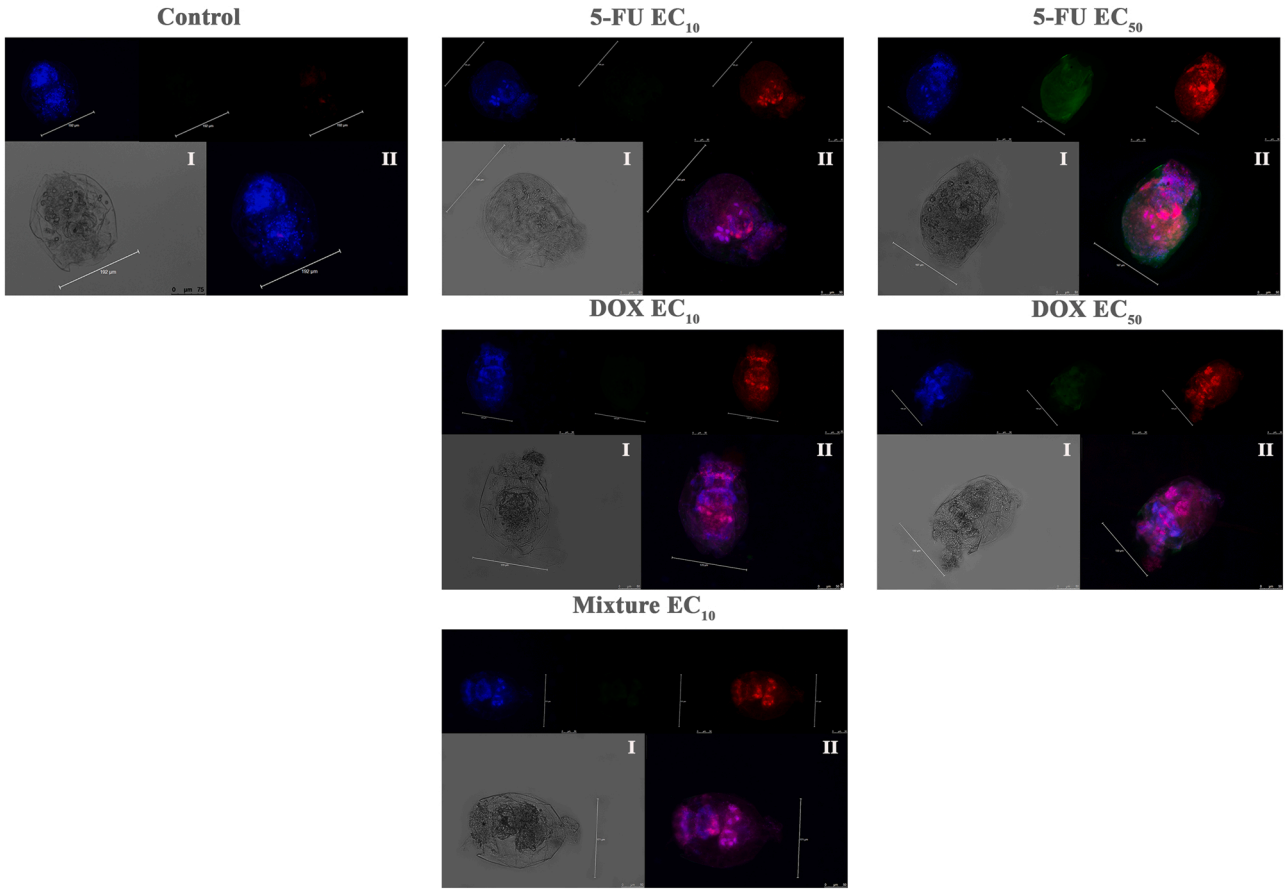

Fig. 4. Epifluorescence microscopic images of rotifers after 48 h exposure to EC_{10} and EC_{50} concentrations of 5-fluorouracil (5-FU), doxorubicin (DOX) and mixtures of both; bright field images (panel I); composite images with the 3 different markers (panel II). Blue fluorescence shows co-localized nuclei after staining with anti-fading reagent containing DAPI; green fluorescence shows cellular accumulation of reactive oxygen species (ROS) after staining with the indicator CM-H_2DCFDA; red fluorescence shows plasma membrane damage after propidium iodide staining.

reported by Straub (2010). Moreover, the EC_{50} for rotifers was lower than that found for *Daphnia magna* ($EC_{50} = 26.4\ \mu g\ L^{-1}$) and lower than the toxicity found for *Ceriodaphnia dubia* ($EC_{50} = 3.4\ \mu g\ L^{-1}$; Parrella et al., 2014).

In our study, both anticancer drugs showed adverse effects on *B. calyciflorus* at the cellular level by triggering oxidative stress, which might have contributed to the inhibition of rotifer reproduction. Moreover, the accumulation of reactive oxygen species and plasma membrane damage occurred even at concentrations of the drugs where no significant effects on reproduction of rotifers were visible. These results are consistent with those for cisplatin, which induced oxidative stress in digestive glands and gills of *Mytilus galloprovincialis*, DNA damage in hemocytes and changes in antioxidant enzyme activities (Trombini et al., 2016).

An apparent synergistic effect, although not significant, was observed in our study for binary mixtures of DOX and 5-FU at the lower concentration of 5-FU; but the effect turned to significantly antagonistic when the concentration of 5-FU increased. To the best of our knowledge, no information about the effects of DOX in mixtures is available for aquatic organisms; however, very few data were found for 5-FU. Brezovšek et al. (2014) reported synergistic effects for the mixture of 5-FU and imatinib mesylate (IM) on the freshwater algae *R. subcapitata* and antagonistic effects on the cyanobacteria *Synechococcus leopoliensis*. Differences in pharmacological action mechanisms and pharmacokinetics (i.e., absorption, accumulation, elimination) of 5-FU and IM in binary mixtures may account for the compound-specific and species-specific synergistic and antagonistic effects (Brezovšek et al., 2014). Similarly, effects of three anticancer drugs in mixtures (5-FU, IM and etoposide (ET)) depend on the drug, the tested species, and the dose (Jia et al., 2009; Elersek et al., 2016). Effects were synergistic for mixtures of the drugs at $\leq EC_{50}$ and antagonistic at higher concentrations (EC_{90}) (Elersek et al., 2016). The antagonism was explained by the suppressive effect of ET on the toxicity of 5-FU (Jia et al., 2009; Xie, 2012; Elersek et al., 2016). 5-FU is a pyrimidine analogue that interferes with DNA synthesis, and therefore ET might block the incorporation of 5-FU metabolites into DNA (Jia et al., 2009). Similar to ET, DOX is a topoisomerase II inhibitor and interferes with DNA replication; hence, in our study, DOX might have induced a similar suppression to 5-FU, explaining the observed antagonistic effects at higher 5-FU concentrations in the binary mixtures with DOX.

In a recent transgenerational study, rotifers showed high levels of oxidative stress under exposure to metal nanoparticles and metal ions that persisted along multiple generations under continuous exposure, but rotifers were able to recover their population growth rate (Martins et al., 2020). The population growth rate of rotifers showed a similar pattern under continuous exposure along multiple generations and when released from 5-FU exposure. However, unlike metal nanoparticles and metal ions (Martins et al., 2020), the continuous exposure to 5-FU showed a decreased level of oxidative stress. On the other hand, rotifers showed no signs of recovery when continuously exposed or released from DOX exposure, with a high parental mortality that led to a non-viable population.

Pharmacokinetics and drug availability to organisms might have played a key role in generational patterns but this was not investigated in our study. However, accumulation and/or adsorption by rotifers was higher for DOX than for 5-FU, especially at T0; whereas, the

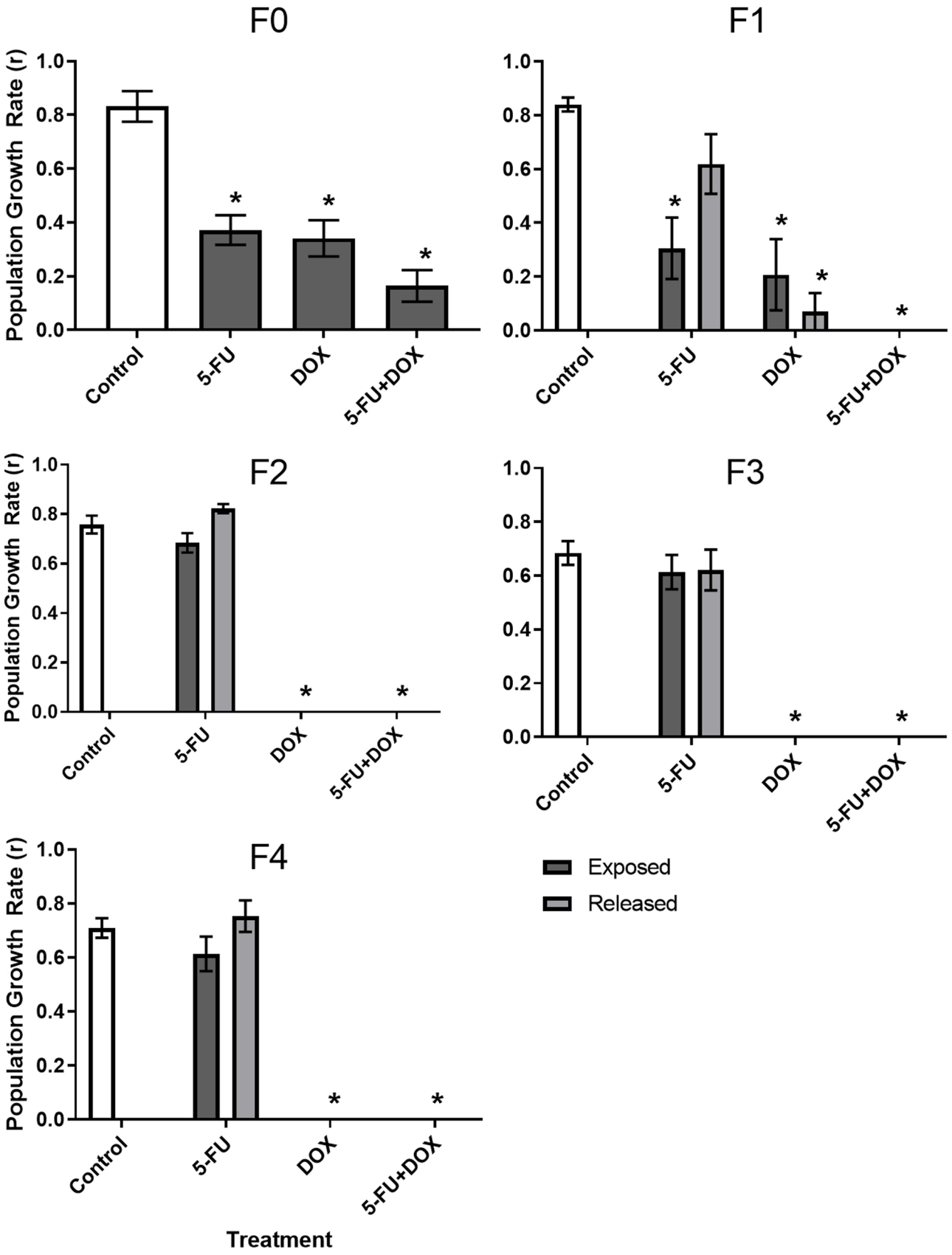

Fig. 5. Effects of 5-fluorouracil (5-FU) and doxorubicin (DOX), alone or in binary mixtures, on population growth rate of rotifers, exposed and pre-exposed to the drugs, along generations (F0-F4). Mean ± SE; n = 8; significant differences from control ($P < 0.05$) are represented by asterisks.

accumulation/and or adsorption of 5-FU by rotifers and algae increased over time. Unlike 5-FU that shows high stability (Zhang et al., 2013; Governo et al., 2017), DOX concentration in the medium decreased with time in the absence of organisms. This suggests a degradation of DOX and/or adsorption to the surface of the plastic wells probably due to its instability in the medium (Wood et al., 1990; Wu and Ofner, 2013). Bioaccumulation of 5-FU (as an analogue of uracil) might have contributed to the reduction of 5-FU concentration in the medium with time, probably due to the rapid and facilitated transport mechanism of uracil (Longley et al., 2003), while DOX might be more easily adsorbed to rotifers due to its tendency to adsorb to multiple surfaces (Wood et al., 1990; Wu and Ofner, 2013). The higher DOX removal by rotifers from the medium at T0 together with the possible parental exposure might have resulted in offsprings with high levels of accumulated and/or adsorbed DOX with its metabolites contributing to the low population growth rate of rotifers even after the release from DOX exposure. On the other hand, when released from 5-FU exposure, full recovery of population growth rate was achieved at F1, suggesting very low to no transmission from parental exposure. At the generational level, these dynamics changed as DOX toxicity increased while 5-FU toxicity decreased at the EC_{50} modeled concentration from F0 to F1 generation, leading to 100% inhibition in mixtures at F1.

Different mechanisms of action might have accounted for the distinct toxicity of the tested anticancer drugs across generations (Minguez et al., 2015). 5-FU is involved in the DNA synthesis S-phase, by preventing DNA replication (Longley et al., 2003). In our study, 5-FU led to a drop in the population growth rate and the visible presence of non-hatched eggs in the affected generations probably because the S-phase was tightly regulated and conserved with checkpoints to ensure that cell cycle events could occur correctly in order to prevent replication of damaged DNA (Takeda and Dutta, 2005). As rotifer offsprings are the result of rotifers that managed to repair DNA from 5-FU exposure, this effectiveness in the repair process might be inherited in the newer generations, probably explaining why rotifers became less sensitive to 5-FU after F2 generation. On other hand, DOX can intercalates within DNA base pairs, causing DNA strand breaks and inhibition of both DNA and RNA syntheses (Johnson-Arbor and Dubey, 2020), which may lead to cell death. This mode of action together with the discussed expected bioaccumulation of DOX across generations might have accounted for the increased effects of DOX in F1 and F2 generations that led to the loss of population viability in F2.

To the best of our knowledge, no data is available on the effects of anticancer drugs at the generational level on aquatic organisms, except for transcriptional responses of *Daphnia magna* at multiple generational exposures to the antibiotic tetracycline (Kim et al., 2007). In our study, we showed that transgenerational effects of anticancer drugs were able to compromise population viability. This will compromise functions ensured by living organisms in ecosystems, therefore transgenerational studies are important when assessing the toxicity of pharmaceuticals to avoid underestimating their impacts. As such, the inclusion of transgenerational data might become a crucial step for proper environmental risk assessment of anticancer drugs.

5. Conclusions

We found differences in the modes of action of 5-FU and DOX and on their lethal and sub-lethal effects to rotifer populations: i) 5-FU showed higher stability in the medium compared to DOX and promoted higher toxicity as shown by a reduction in the population growth rate, ii) effects in binary mixtures varied with the drug concentration, and antagonistic effects were found at higher concentrations of 5-FU, iii) the individual effects of 5-FU did not persist across generations, as predicted; instead, a full recovery in population growth rate was reached at F2 under continuous exposure, and at F1 when the rotifers were released from the drug. However, the individual effects of DOX persisted along generations and led to population extinction at F2 under continuous exposure even when the rotifers were released from DOX. Mixture toxicity assessment showed to be crucial to understand the mechanisms of action of anticancer drugs and their possible impacts under more realistic exposure scenarios, while transgenerational data clearly pointed to an alteration in interpreting the impacts of anticancer drugs when compared to standard acute and chronic tests. Overall, our study represents a step forward that helps to fill the gap of information on anticancer drug effects beyond individual and F0 generation to better assess anticancer drug impacts on freshwaters.

CRediT authorship contribution statement

Nuno Martins: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Writing - original draft. **Arunava Pradhan**: Conceptualization, Methodology, Formal analysis, Supervision, Writing - review & editing. **Cláudia Pascoal**: Conceptualization, Supervision, Writing - review & editing. **Fernanda Cássio:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

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.

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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.2021.112893.

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