Aquatic ecotoxicology of anticancer drugs: a systematic review

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Abstract:

Anticancer drugs in the aquatic environment have drawn a lot of attention in the last decade. Since wastewater treatment plants proved to be inefficient to fully eliminate trace concentrations of anticancer drugs, these compounds are continuously discharged into the aquatic environment. Subsequently, non-target organisms such as the aquatic biota are directly exposed to a variety of anticancer drugs. To understand the potential impact on the aquatic organisms, a systematic review was conducted in compliance with the PRISMA guidelines. The results acquired from the 152 included studies were analysed and sorted into four categories: the impact of each included anticancer drug, the effect of metabolites, the effect of a mixture of drugs, and risk assessment. Findings showed that risk on the aquatic biota was unlikely to occur as the concentrations needed to induce effects were much higher than those detected in the environment. However, these data were based on acute toxicity and included only basic toxicity endpoints. The concentrations that produced significant effects were much lower when tested in the long-term or in multi-generational studies. Variabilities in results were also observed, these depended on the organism tested, the assessment adopted, and the endpoints selected. In this systematic review, an overall view

of the research studies was generated by which all the variability factors to be considered were reported and recommendations to guide future studies were proposed.

Keywords: Anticancer drugs; Aquatic ecotoxicology; Systematic review; Water pollution

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1. Introduction

Pharmaceuticals, including anticancer drugs, are considered an environmental threat due to their constant release into the aquatic environment (Jureczko and Kalka, 2020). With anticancer drugs being administered mainly in outpatient departments, households are currently regarded as one of the critical discharge sources along with hospitals, pharmaceutical industries and solid waste disposal (Johnson et al., 2008; Jureczko and Kalka, 2020; Toolaram et al., 2014). Also, since parent compounds and metabolites of anticancer drugs are discharged in domestic sewage, it would be useful to pre-treat the water before it enters the wastewater treatment plants (Balcerzak and Rezka, 2014). The removal rate of anticancer drugs in wastewater treatment plants (WWTPs) could range from less than 20% to approximately 90% depending on the compound and the type of treatment applied. Hence, the detection of these compounds and their metabolites in water resources is demonstrated in numerous studies (Zhang et al., 2013).

A recent systematic review demonstrates that the most detected anticancer drugs in the aquatic environment are cyclophosphamide (0.05-22100 ng/L), tamoxifen (0.01-740 ng/L), ifosfamide (0.14-86200 ng/L), and methotrexate (1.6-4756 ng/L) (Nassour et al., 2019). The concentrations observed could be explained by the high stability of some anticancer drugs, as reported in Negreira et al. (2014) study, which could be problematic and detrimental to the aquatic biota (Negreira et al., 2014).

Anticancer drugs are different classes of chemotherapy agents with the primary aim to disrupt, at various points, the cancer cells life cycle. Therefore, they can be classified according to their cell cycle effects or their biochemical properties (Dickens and Ahmed, 2018). Besides treating tumour sites, anticancer drugs can cause considerable toxicity as side

effect since their mode of action is not specific to cancer cells, and they can alter normal cells' function (Gajski et al., 2018). Hence, environmental and occupational exposure to anticancer drugs could potentially harm humans and non-target organisms, including the aquatic biota (Fabbri, 2015; Ladeira et al., 2014).

In this systematic review, the impact of anticancer drugs and their metabolites are assessed exclusively on the aquatic biota. The results are divided into four categories: exposure to one anticancer drug, exposure to metabolites, exposure to a mixture of drugs and risk assessment. Apart from reporting the included studies' outcomes, this review discusses several points such as the reasons for research findings' heterogeneity, the different issues to be considered when evaluating the risk of anticancer drugs in the aquatic environment and proposes several recommendations for future research. Finally, this report is conducted in compliance with the PRISMA (Preferred Reporting Items for Systematic and Meta-Analysis) checklist, and to the best of the authors' knowledge, such study has not been previously reported in the literature.

2. Search strategy and inclusion criteria

A search strategy (PROSPERO registration: CRD42020191754) was formulated to collect as many significant publications as possible. The key search terms were defined and combined using Boolean operators and wildcards: (Anticancer$ OR Cytotoxic$ OR Cytostatic$ OR Chemotherapeutic$) AND (Ecological Risk Assessment OR Ecotox* OR Acute OR Chronic OR Toxic) AND (*Water* OR Environment* OR Aquatic*). A search was then conducted on two initial databases: PubMed and OpenGrey, on April 3, 2020. The search was refined by

Languages = English (on PubMed and OpenGrey) and Sort by = Best Match (on PubMed). In parallel, the same research was conducted on ScienceDirect to confirm that no additional studies were available.

All original studies written in English, peer-reviewed and published or not (Grey literature) that assess the effect of anticancer drugs on any aquatic biota *in vivo* were included in this review with no restriction on the study year. The final outcome of the screening and selection process was the inclusion of 108 studies. In addition to that, 44 studies were added to the included studies from bibliography searches. More details about the selection process are presented in **Supplementary Information**.

All types of endpoints and findings were reported, such as survival and growth rates, behavioural and physiological changes, physical malformations, etc. The data extracted from the included studies were arranged in four different tables (**Supplementary Information**). **Table S2** shows the effect of a single exposure of aquatic organisms to anticancer drugs; **Table S3** shows the impact of a combination of drugs; **Table S4** shows the effect of anticancer drug metabolites on non-target organisms. Finally, **Table S5** presents the calculated/predicted risk of anticancer drugs on various aquatic organisms. Outcomes of the different tests conducted were presented as reported in the studies, including the concentrations which induced the stated effects.

The risk to the aquatic biota resulting from exposure to anticancer drugs in the aquatic environment will be discussed in the below sections, considering the different species tested and the endpoints evaluated.

3. Exposure to one anticancer drug (**Table S1**)

3.1 Antineoplastic agents:

3.1.1 Alkylating agents:

Alkylating agents target the cancer cells at any point of the cycle by binding to the DNA and preventing the cell replication (Yan and Gulbis, 2019). In the included studies, cyclophosphamide and cisplatin were the most studied alkylating agents compared to ifosfamide. In Zounková *et al.* (2007) study, no effect was observed by cyclophosphamide on the growth and bioluminescence of all the organisms tested for up to 1000 and 100 mg/L, respectively, except *Pseudokirchneriella sucapitata,* where EC_{50} was found to be 930 mg/L (Zounková et al., 2007). However, in another study by Russo et al (2018), cyclophosphamide proved to inhibit crustacea and rotifera's reproduction in chronic tests with EC_{50} ranging between 58.03 and 89.84 mg/L (Russo et al., 2018c). In addition to that, perturbation of the burrowing behaviour and the activity of antioxidant and biotransformation enzymes was observed in *Nereis diversicolor* at concentrations ranging between 10 and 1000 ng/L (Fonseca et al., 2018). And as expected, cyclophosphamide-induced significant micronucleus formation in mollusca (18-180 mg/L), echinodermata (32-56 mg/L) and fish (20 mg/kg) (Canty et al., 2009; Grisolia and Cordeiro, 2000) in addition to other nuclear abnormalities and mutagenic damages. Ifosfamide, which was less studied, has only been shown to induce mortality in crustacea and rotifera with LC_{50} values ranging between 986.6 and 1924 mg/L, and reproduction inhibition with EC_{50} ranging between 15.84 and 76.05 mg/L (Russo et al., 2018c).

Cisplatin inhibited the growth of bacteria, cyanobacteria, algae, rotifera and aquatic plant with EC_{50} varying between 440 µg/L and 1.52 mg/L (Brezovšek et al., 2014; Parrella et al.,

2014b; Supalkova et al., 2008; Zounková et al., 2007). It has also affected the reproduction of crustacea by reducing the offspring percentage, the number of eggs and the population growth rate at low concentrations (Grzesiuk et al., 2019; Parrella et al., 2014c, 2014a; Russo et al., 2018b). As demonstrated by Parrella et al. (2015) study, significant DNA damage was produced at concentrations starting from 30 ng/L (Parrella et al., 2015). Moreover, cisplatin perturbed the activity of enzymes such as AChE, SOD, CAT and GST at 100 ng/L and increased the oxidative damage demonstrated by high levels of lipid peroxidation (Fonseca et al., 2017; Trombini et al., 2016).

3.1.2 Antimetabolites:

Antimetabolites are specific to the cell's cycle phase S. They act by inhibiting the DNA synthesis's key enzymes or by causing strand breaks in DNA and RNA or premature chain termination (Lind, 2011). Most of the studies were conducted for the antimetabolites 5-fluorouracil, followed by methotrexate and capecitabine.

Methotrexate which is the most administered antifolate has shown to inhibit bioluminescence, growth and reproduction of aquatic organisms with EC_{50} between 0.08 mg/L for *Lemna minor* (Białk-Bielińska et al., 2017) and 1220 mg/L in *Vibrio fischeri* (Henschel et al., 1997). It has also significantly increased the activities of detoxification enzymes such as EROD (phase I) at concentrations starting from 100 ng/g and 0.2 mM, and GST (phase II) at 1000 ng/g and 0.08-10 mM in *Ampelisca brevicornis* and *Elliptio complanata*, respectively (Martín-Díaz et al., 2009; Moreira et al., 2016).

5-Fluorouracil has been shown to suppress the bioluminescence and growth of different species at lowest concentrations compared to methotrexate with EC_{50} between 0.016 and 48 mg/L (Załeska-Radziwiłł et al., 2014; Zounkova et al., 2010). It also inhibited the reproduction

of the algae *Pseudokirchneriella subcapitata* and the crustaceans *Daphnia magna* and *Ceriodaphnia dubia* with a significant reduction in the percentages of offspring. In fish and amphibia, malformations of larvae and embryos were observed, such as a significant dose-dependent increase of body length starting from 5 ng/L (Ng et al., 2020), incomplete closure of the choroid fissure at 1000 mg/L (Kovács et al., 2016), abdominal oedema, axial flexure and head, eyes and gut malformations at 50 mg/L (Isidori et al., 2016). Furthermore, 5-fluorouracil caused significant DNA damage in *Ceriodaphnia dubia* at 0.06 µg/L (Parrella et al., 2015), *Unio pictorum* and *Unio tumidus* at 0.4 µM (Gačić et al., 2014) and *Danio rerio* at 1 µg/L (Kovács et al., 2015); in addition to micronuclei induction and nuclear abnormalities in fish and amphibia (Araújo et al., 2019; Kovács et al., 2015).

Capecitabine, a pro-drug which could be activated to 5-Fluorouracil (Lind, 2011), has shown the same results in crustacea, rotifera and amphibia. However, it somehow produced effects at higher concentrations compared to 5-FU. For instance, growth inhibition was caused with EC_{50} equal to 15.4 mg/L (Parrella et al., 2014b) and reproduction was inhibited with an EC_{50} ranging between 2.4 and 20.5 mg/L (Parrella et al., 2014b). Also, DNA damage occurred in *Daphnia magna* and *Ceriodaphnia dubia* at 22.5 and 120 µg/L, respectively. In *Xenopus laevis* embryos, the same malformations were reported at 20 mg/L (Isidori et al., 2016).

Only a few studies were conducted for the three antimetabolites, azaserine, cytarabine and gemcitabine. For azaserine, the EC_{50} obtained for the bioluminescence inhibition assay in *Vibrio fischeri* were 0.151 mg/L and 0.83 µmol/L (Backhaus et al., 2000; Backhaus and Grimme, 1999). Cytarabine inhibited bacteria and algae's growth with EC_{50} ranging between 17 and 53 mg/L, respectively, and the reproduction of crustacea with EC_{50} equal to 10 mg/L. Finally, gemcitabine caused growth inhibition of bacteria and algae with EC_{50} ranging between

45 and 100 mg/L; and the immobilisation of *Daphnia magna* with EC_{50} equal to 110 mg/L (Zounkova et al., 2010).

3.1.3 Plant alkaloids and other natural products:

Vinca alkaloids such as vincristine act by disturbing the dynamics of microtubules (Chu and Rubin, 2018). In crustacea organisms, vincristine caused immobilisation of *Daphnia magna* with EC_{50} equivalent to 7.74 mg/L (Jureczko and Przystaś, 2019).

On the other hand, etoposide, an inhibitor of topoisomerase II and which acts by inducing DNA strand breaks (Makin, 2018), caused immobilisation of *Daphnia magna* with EC_{50} = 30 mg/L (Zounková et al., 2007). It inhibited the growth of bacteria, algae and rotifera with EC_{50} ranging between 3.7 and 351.1 mg/L (Parrella et al., 2014b; Zounková et al., 2007) and the reproduction of crustacea with EC_{50} between 204 and 239 µg/L (Parrella et al., 2014b), in addition to a reduction in the number of offspring for up to 90.4% at 473.7 µg/L (Parrella et al., 2014a). Furthermore, as reported in Isodiri et al. (2016) study, etoposide caused DNA damage in crustacea at lower concentrations and embryonic malformations in fish and amphibia, starting from 30 mg/L (Isidori et al., 2016).

3.1.4 Cytotoxic antibiotics:

Anti-tumour antibiotics intercalate sequences of DNA and cause strand breakage (Fernando and Jones, 2015). Cytotoxic antibiotics were not extensively examined: bleomycin, doxorubicin and mitomycin C were investigated in two, three and five studies, respectively. Bleomycin has shown to inhibit bacteria and aquatic plant growth with EC_{50} equivalent to 0.2 and 7.27 mg/L, respectively and has caused immobilisation of *Daphnia magna* with EC_{50} equal to 0.77 mg/L (Jureczko and Przystaś, 2019). The anthracycline doxorubicin prevented rotifera

and algae's growth with EC_{50} ranging between 7.7 and 10 mg/L, respectively. In crustacea, the EC_{50} obtained was between 2 and 2.14 mg/L for the immobilisation test, and DNA damage has occurred starting from 0.02 µg/L. Doxorubicin has also caused mortality in crustacea and rotifera with LC_{50} ranging between 0.31 and 12.69 mg/L (Parrella et al., 2015, 2014b; Zounková et al., 2007). Finally, in Nakano et al. (2003) study, mitomycin C elicited offspring malformations and germ cells mutations at concentrations ranging between 1 and 100 µM in mollusca (Nakano et al., 2003). It has also induced dose and time-dependent micronuclei formation in fish with concentrations ranging between 0.25 and 10 mg/kg (Bahari et al., 1994; Das and Nanda, 1986; Winter et al., 2007).

3.1.5 Tyrosine kinase inhibitor:

Tyrosine kinase inhibitors block the receptor tyrosine kinases that promote cell division and survival (Gustafson and Bailey, 2019). Imatinib, the only anticancer drug tested in this class, inhibited the bioluminescence and bacteria growth with EC_{50} ranging between 5.36 and 23.06 mg/L (Białk-Bielińska et al., 2017; Brezovšek et al., 2014). It has also inhibited the growth of algae, rotifera, crustacea and aquatic plant with EC_{50} ranging between 0.74 and 72.43 mg/L (Białk-Bielińska et al., 2017; Parrella et al., 2014b). Mortality was recorded in crustacea, rotifera and fish with LC_{50} ranging between 3.82 and 65.9 mg/L (Kovács et al., 2016; Parrella et al., 2014b). Reproduction inhibition was observed in algae and crustacea with EC_{50} between 0.115 and 5.08 mg/L and a reduction in the % of offspring for up to 56.5 % at 0.514 mg/L in crustacea (Białk-Bielińska et al., 2017; Parrella et al., 2014b, 2014a). Additionally, DNA damage significantly increased in crustacea, starting from 0.3 µg/L (Parrella et al., 2015), and feeding behaviour significantly decreased in rotifera at 1.2 mg/L (Yan et al., 2017). In fish and amphibia, embryonic deformities were perceived starting at 20 mg/L with the most reported

deformations being: tail thinning, deformed yolk sacs, change in pigmentation, head, eyes gut and heart malformations, etc. (Isidori et al., 2016; Kovács et al., 2016).

3.2 Endocrine therapy

Endocrine therapy is typically adapted for cancers that depend on hormones for their growth, such as breast, prostate and endometrial cancers. The treatment consists of administering agents that bind to the hormone receptors and alter their expression in the cell (Archampong and Sweetland, 2015; Hanratty and Sweetland, 2012). Endocrine-disrupting chemicals (EDCs) attracted greater attention in the last couple of decades in terms of investigating their occurrence in the aquatic environment and their adverse effects on the aquatic biota (Rodenas et al., 2015). In the included studies, the most studied EDCs for the two classes were diethylstilbestrol and tamoxifen, respectively.

3.2.1 Hormones and related agents:

In crustacea, diethylstilbestrol has affected the offspring rate and the reproduction of the second generation of *Daphnia magna,* starting from 0.2 mg/L, as demonstrated in Brennan et al. (2006) study (Brennan et al., 2006). It was also lethal at 1.5 mg/L and caused immobilisation with EC_{50} equal to 1.55 mg/L (Baldwin et al., 1995; Brennan et al., 2006). The moulting frequency, which is linked to crustaceans' growth, has been significantly reduced, starting from 0.5 mg/L (Baldwin et al., 1995). Similarly, body length was decreased at 0.54 mg/L and, more specifically, in female daphnids at 3 µM (Baldwin et al., 1995; Olmstead and LeBlanc, 2000). Additionally, diethylstilbestrol has significantly inactivated testosterone and increased glucosyltransferase activity at 0.5 mg/L (Baldwin et al., 1995). In amphibia,

232 diethylstilbestrol has significantly increased the mortality rate and caused the total death of
233 *Xenopus laevis* embryos after stage 32 of the tadpole's life cycle at 10^{-5} M. It has also induced
234 malformations in embryos and retarded their development after stage 38 at the same
235 concentration (Nishimura et al., 1997). Finally, it has increased the production of vitellogenin
236 in adult males of amphibia and reptilia at 1 µg/g (Palmer and Palmer, 1995).

237 In Roepke *et al.* (2005) study, progesterone has inhibited echinodermata embryos'
238 development with EC_{50} equivalent to 546.6 ng/ml and increased the number of embryos with
239 delayed development (Roepke et al., 2005). Testosterone has caused mortality and a
240 decrease in moulting frequency of crustacea with LC_{50} = 5.6 mg/L and EC_{50} = 1.5 mg/L
241 (Andersen et al., 2001). Moreover, a significant reduction in the number of fertilised sexual
242 females was observed, in Preston *et al.* (2000) study, in rotifera at 10 µg/L with 96-h
243 fertilisation NOEC equal to 1 µg/L (Preston et al., 2000). In mollusca, testosterone has
244 triggered the development of male sex organs in females with a significant increase of
245 endogenous testosterone levels at 500 ng/L (Bettin et al., 1996).

246 3.2.2 Hormone antagonists and related agents:

247 Tamoxifen, which has been extensively studied in different aquatic species, has caused
248 bioluminescence inhibition of bacteria with EC_{50} = 330 mg/L (Aguirre-Martínez et al., 2016).
249 In algae, tamoxifen has affected the growth with IC_{50} ranging between 470 and 980 µg/L
250 (Orias et al., 2015a) and has accumulated in *Pseudokirchneriella subcapitata* at
251 concentrations for up to 100 µg/L (Orias et al., 2015b). Besides that, tamoxifen has influenced
252 the swimming behaviour of crustacea with EC_{50} between 0.21 and 1.53 mg/L (Dellagreca et
253 al., 2007; Orias et al., 2015a) and the reproduction, significantly, starting from 5.26 µg/L
254 (Borgatta et al., 2016). It has also modified the expression of genes related to oogenesis,

moulting, early development and metamorphic transitions such as vmo1, ecrb, usp and cyp314, respectively, at concentrations ranging between 50 and 100 µg/L (Jo et al., 2018). In rotifera, tamoxifen caused mortality and growth inhibition with LC_{50} = 0.97 mg/L and EC_{50} = 0.25 mg/L, respectively (Dellagreca et al., 2007). As reported in Fonseca *et al.* (2019) study, tamoxifen has affected the burrowing behaviour of polychaeta, at low concentrations starting from 25 ng/L. It has also induced neurotoxicity and altered the activities of antioxidant and biotransformation enzymes accompanied by a significant increase in lipid peroxidation levels and % of DNA in tail at concentrations ranging between 0.5 and 100 ng/L (Fonseca et al., 2019). The same effects were observed in mollusca at concentrations ranging between 1 and 50 µg/L; in addition to a significant decrease of vitellogenin levels and endocrine disruption in females and males (Aguirre-Martínez et al., 2018, 2016, 2015; Fonseca et al., 2019). At higher trophic levels, tamoxifen has caused malformations, a reduction in body length and a decrease in the heart rate of fishes. In addition to that, it has altered the expression of several genes related to the endocrine system, metabolism and morphology at concentrations ranging between 0.5 and 500 µg/L (Xia et al., 2016). Tamoxifen has shown to significantly alter the structure and function of gonads, especially in females where a decrease of vitellogenin levels in plasma and reduction of egg production were observed (Chikae et al., 2004; Sun et al., 2007a; Van Der Ven et al., 2007; Williams et al., 2007). However, in males, an increase of vitellogenin levels in plasma was observed in Sun *et al.* (2007) study, which caused a decrease of fecundity and fertility at 625 µg/L and a modification in sex ratio starting from 25 µg/L (Sun et al., 2007a). In amphibia, tamoxifen has altered the expression of hormones mRNA in females, such as the luteinising hormone and the follicle-stimulating hormone (Urbatzka et al., 2006). Also, it has altered the expression of biomarker and aromatase mRNA in females, increased the level of estradiol-17β in plasma of both sexes, and modified the anatomy of the

gonads at 0.01 µM (Cevasco et al., 2008; Massari et al., 2010; Urbatzka et al., 2007). Finally, it has inhibited the growth of the aquatic plant, *Lemna minor*, with EC_{50} ranging between 0.18 and 0.23 mg/L (Białk-Bielińska et al., 2017).

Bicalutamide has caused a reduction of nuptial tubercle prominence in adult male fishes at 100 µg/L. However, embryos from first-generation were more affected at the same concentration; for instance, survival significantly decreased, and a gonadal lesion with the inability to spawn was detected in females. Also, increased body weight and length and a perturbation of the reproduction were observed in females (Panter et al., 2012).

Cyproterone acetate has delayed the maturation of crustacea, decreased the moulting and body length and reduced the number of offspring starting from 1.2 µM (Leblanc and Mclachlan, 1999). In mollusca, the size of the penis sheath and the spermatogenesis of adult males significantly diminished, and the level of free estradiol increased at 1.25 mg/L (Santos et al., 2005; Tillmann et al., 2001). In echinodermata, cyproterone acetate has decreased the testosterone levels significantly starting from 300 ng/L and hindered the regenerative cell proliferation (Lavado et al., 2006; Sugni et al., 2008). Lastly, in fish, body weight and length decreased at 1 µg/L and alteration of the gonadal anatomy was observed in females and males starting from 1 µg/L (Kiparissis et al., 2003). Furthermore, Sharpe *et al.* (2004) demonstrated that the steroid plasma levels decreased significantly, such as testosterone and 11-ketotestosterone in males and testosterone and estradiol in females at concentrations ranging between 250 and 1000 ng/L (Sharpe et al., 2004).

The anticancer drug fadrozole, tested only on fish, has decreased the vitellogenin levels starting from 2 µg/L and the reproduction rate (Ankley et al., 2002; Zerulla et al., 2002). It has also reduced the steroid levels in females' plasma and increased them in males at

302 concentrations ranging from 2 to 50 µg/L. Additionally, it has altered the gonadal histology
303 and the brain aromatase activity in both sexes and modified the gene expression in females'
304 ovarian and brain tissue (Ankley et al., 2002; Fenske and Segner, 2004; Kuhl and Brouwer,
305 2006; Villeneuve et al., 2009).

306 Flutamide has caused mortality, immobilisation, and decreased moulting of crustacea with
307 LC_{50} = 5.4 mg/L, EC_{50} = 2.7 mg/L and EC_{50} = 0.48 mg/L, respectively (Andersen et al., 2001;
308 Haeba et al., 2008). As well as affecting the reproduction in females by decreasing the
309 offspring counts, suppressing the maternal organisms and delaying their maturation at 1 mg/L
310 (Haeba et al., 2008). Similarly, in rotifera, it has altered females' reproductive functions with
311 NOEC = 0.1 µg/L (Preston et al., 2000). In fish, flutamide has diminished the fecundity and
312 decreased embryos hatch at 500 µg/L (Jensen et al., 2004). Vitellogenin levels in plasma
313 seemed to increase, and variation in the sex steroid levels in plasma was observed in both
314 sexes. In addition to that, changes in the sexual behaviour of females and males were noticed
315 at concentrations ranging between 100 and 1000 µg/L (Sebire et al., 2008). Body length and
316 weight reduction in males and gonadal histology alterations in both sexes were also
317 highlighted, among other effects. In amphibia, alterations in gonadal structures were
318 observed in males and females with an increase of spermatogenic nests number in males and
319 absence of such in 100% of females at 0.01 µM (Cevasco et al., 2008). Also, the aromatase
320 mRNA expression was modified in both sexes at the same concentration (Massari et al., 2010).

321 Letrozole increased the hepatosomatic index in males and decreased it in females starting
322 from 25 µg/L. The liver has a role in the ovarian development of fish, which is why the
323 hepatosomatic index is correlated with the gonadosomatic index and has increased in both
324 sexes after exposure to letrozole starting from 125 µg/L. Gonadal histology was also altered

at higher concentration (625 µg/L) in both sexes, and vitellogenin levels were significantly reduced in females starting from 25 µg/L. Consequently, fecundity and fertility significantly decreased in females, and the males' proportion significantly increased (Sun et al., 2007b). Moreover, expression of steroid hormones receptor and synthesis-related genes were altered in the brain, liver and gonads of males and female fishes (Sun et al., 2011a).

3.3 Immunosuppressants

Prednisone is a corticosteroid that suppresses the immune system by interacting with the glucocorticoid receptor of the hematopoietic cancer cells and induce apoptosis (Gustafson and Bailey, 2019). In algae, it has inhibited the growth with IC_{50} = 85.5 µM and induced mortality of rotifera with LC_{50} = 152.2 µM. Finally, in crustacea, prednisone caused immobilisation at 279 µM and mortality at 447 µM (DellaGreca et al., 2003).

4. Sensitivities of different organisms

From the results obtained, it was apparent that the range of concentrations causing potential effects is broad. This could be explained by the different sensitivities of the aquatic organisms to anticancer drugs. In fact, these sensitivities could be influenced by several factors such as the species tested, strain, exposure history, age, size, health and handling procedures (DeYoung et al., 1996).

Studies have shown that the toxic effects of anticancer drugs were enhanced when the trophic level increased. For instance, in a study by Russo *et al.* (2018), ifosfamide achieved higher toxicity in crustaceans compared to rotifers and algae (Russo et al., 2018c). Also, the

toxicity assessed in Sanderson *et al.* (2003) study increased, moving from algae to daphnids to fish (Sanderson et al., 2003). However, this hypothesis was not applicable in all cases as some species appeared to be more or less sensitive depending on the anticancer drug tested. In the fish *Pimephales promelas*, cyclophosphamide failed to induce the formation of micronuclei in erythrocytes at 400 mg/kg while it has significantly increased the micronucleus frequencies at 20 mg/kg in other species of fish such as *Tilipia redalli*, *Oreochromis niloticus* and *Cyprinus carpio* (Grisolia and Cordeiro, 2000; Winter et al., 2007). Furthermore, the daphnids crustaceans showed higher sensitivity to anticancer drugs such as imatinib, cisplatin, and etoposide than other organisms, including fish (Parrella et al., 2014b; Russo et al., 2018a).

Sensitivity could also be affected by the gender of the same species, especially when exposed to endocrine-disrupting compounds. For example, the growth of male fish treated with flutamide was hindered, and a significant demasculinisation was demonstrated, with no effect observed in females at the same concentrations (Bayley et al., 2002; León et al., 2007). Other anticancer drugs like tamoxifen, methyldihydrotestosterone and letrozole also have a gender-specific effect on the aromatase mRNA expression, which is related to the reproduction and brain development of vertebrate, among other transcriptional responses (Massari et al., 2010; Sun et al., 2011a; Urbatzka et al., 2006).

Other studies demonstrated that results might be affected by the level of maturity of the organism tested. In a study by Sun *et al.* (2007), letrozole had no effect on larvae and embryos development for up to 3125 µg/L. However, when exposed to adults, letrozole caused significant reproductive effects with a lower concentration range (25 – 625 µg/L), which could presume the importance of adult exposure (Sun et al., 2007b). Contrary to what was obtained, in another study conducted in 1995, juvenile daphnids appeared to be more sensitive to

diethylstilbestrol effects than adults (Baldwin et al., 1995). Hence, multi-generational exposure could possibly eliminate age-related ambiguities. Multi-generational studies is when exposure to a selected anticancer drug is extended to several broods from the same parent generation (F0) (Borgatta et al., 2016). From the included studies that performed this procedure, it was apparent that sensitivity increased over the generations, especially in terms of development and reproductive endpoints such as body length and weight, and the number of offspring per female (fecundity) (Borgatta et al., 2016, 2015; Brennan et al., 2006; Kovács et al., 2015; Van Der Ven et al., 2007).

5. Choice of a suitable test: Acute vs Chronic tests

Ecotoxicology of anticancer drugs has recently attracted a lot of attention; however, studies conducted revealed a lack of sufficient information compared to other pharmaceuticals. Several guidelines and assays were established to test the effects of the chemicals on aquatic organisms, such as the OECD guidelines and the FETAX assay. Nevertheless, limitations were reported as protocols were more available for acute assays and also, *in vivo* tests mainly were carried out with lower-level organisms (Fent et al., 2006; Kovács et al., 2016).

Acute assays can generate relevant preliminary data of anticancer drugs' toxicity and form a background for chronic tests. In general, studies performing acute toxicity tests have obtained significant effects at concentrations in the range of µg/L and mg/L, which is relatively higher than the concentrations detected in the aquatic environment (Kovács et al., 2016; Martín-Díaz et al., 2009). Hence, these results could only represent occasional events such as spillages (Fent et al., 2006).

Anticancer drugs are released continuously into the environment; in consequence, acute assays would underestimate the potential toxicity of these compounds in the aquatic environment. Accordingly, chronic tests would be able to reproduce the reality of aquatic exposure to anticancer drugs while investigating more sensitive and specific endpoints (Henschel et al., 1997; Martín-Díaz et al., 2009; Załeska-Radziwiłl et al., 2011; Zounkova et al., 2010). Numerous researchers compared the two types of tests and showed that chronic toxicity occurred at lower concentrations (Andersen et al., 2001; Henschel et al., 1997; Russo et al., 2018a; Załeska-Radziwiłl et al., 2011; Zounkova et al., 2010). For instance, in Parrella *et al.* (2014) study, acute toxicity of 5-fluorouracil recorded an EC_{50} between 20.84 and 501 mg/L for the organisms tested (Crustaceans and Rotifera); however, following chronic exposure, the range of EC_{50} significantly decreased to 3.35 – 322 µg/L. The same trend was also observed for cisplatin, imatinib, etoposide, doxorubicin and capecitabine (Parrella et al., 2014b).

Furthermore, it is worth mentioning that long-term toxicity tests could reveal various phenomena such as bioaccumulation, hormesis and adaptation:

a) Bioaccumulation: Bioaccumulation is the intake of an emerging contaminant and its concentration in the exposed organism. High bioaccumulation caused by the chemical's persistence or constant exposure could lead to the endangerment of the exposed organisms (Orias et al., 2015c). Tamoxifen proved to accumulate in gonads, liver and muscles of *Danio rerio* in a concentration-dependent manner (Orias et al., 2015b). In the algae, *Pseudokirchneriella subcapitata*, high bioconcentration of tamoxifen was also recorded but not in a concentration-dependent manner (Orias et al., 2015c). The same effect was observed for cisplatin in *Danio rerio* and the macroalgae *Ulva lactuca* (Easton et al., 2011; Hung et al., 2019). Finally, 5-fluorouracil accumulated instantly in the green

415 microalgae cells and integrated into the DNA and the RNA of the specie. This could lead
416 to the transfer of the genome from one organism to another, and the DNA might
417 reintegrate into the genome of predators moving to the top of the food chain (Asad et al.,
418 2012).
419
420 b) Hormesis: Hormesis is when an organism reacts to low concentrations of a compound,
421 and higher concentrations inhibit this reaction. This is due to the organism's adaptive
422 response to moderate environmental stresses (Mater et al., 2014). For example,
423 cyclophosphamide increased the growth/reproduction of *C. dubia* and *P. subcapitata* only
424 at the lowest concentration tested (10 mg/L) (Russo et al., 2018c). It has also inhibited the
425 growth of the algae *S. capricornutum* at the lowest concentration (10 µg/L) and induced
426 the proliferation at the highest concentration tested (100 µg/L) (Mater et al., 2014). For
427 tamoxifen, AChE activity was not induced at 10 ng/L in the polychaete *N. diversicolor,* and
428 antioxidant enzymes activity was increased at 0.5 ng/L; however, the opposite activities
429 were observed at higher concentrations (Fonseca et al., 2019).
430
431 c) Adaptation: Adaptation was observed when an organism was re-exposed to the same
432 chemical. Here, the sensitivity of the organism might decrease due to an adaptation to
433 the toxicant. For instance, when the rotifer *B. calyciflorus* was re-exposed to the same
434 concentration of imatinib, the inhibition rate of the feeding behaviour decreased
435 compared to the first exposure (Yan et al., 2017).
436
437 ## 6. Choice of suitable endpoints

To evaluate the adverse effects of a drug, multiple endpoints could be selected. The included studies covered endpoints from conventional tests: mortality, inhibition of mobility/growth and reproduction, to more advanced endpoints such as genotoxicity (DNA damage), neurotoxicity (AChE activity), oxidative stress (by testing biomarkers such as SOD, CAT, T- and SE-GPx, GST and lipid peroxidation), physical abnormalities (body weight and length, embryonic deformities), physiological effects (heart rate, fecundity and fertility), behaviour (locomotion, swimming performance, feeding, burrowing activity), in addition to the expression of several genes related to gonads and brain development, and histological studies; among others (Bhatia et al., 2014; Trombini et al., 2016; Yan et al., 2017).

Nevertheless, the right endpoint selection depends on the sensitivity of the organism tested (discussed in the above section) and on the compound to be tested, considering their different modes of action. For example, in Urbatzka *et al.* (2007) study, the selected genes allowed only the detection of the adverse effects of anti-estrogenic exposure. However, no response was recorded for anti-androgenic compounds as androgen and anti-androgen are regulated by different genes (Urbatzka et al., 2007). It is also essential to link any detectable effects (e.g. physiological or behavioural) with genotoxic effects as they might be connected by a cascade of events (Canty et al., 2009). The feeding behaviour, which could cause a reduced energy intake if depressed, appeared to be an important indication of survival, growth and fecundity irregularities at individual and population levels (Yan et al., 2017). Another example is Ankley *et al.* (2002) study, where fadrozole inhibited the brain aromatase activity, which caused a reduction in E2 and vitellogenin production, affected the maturity of the ovaries (target tissues) and finally, caused a response in the whole organism that was demonstrated by a reduced fecundity in female fish (Ankley et al., 2002).

7. Exposure to anticancer drugs' metabolites/transformation products

Human metabolites of anticancer drugs are excreted through faeces and urine alongside the parent compounds as they are not completely metabolised in the human body. Additionally, throughout the water treatment, parent compounds undergo numerous degradation processes, which might produce transformation products (Borgatta et al., 2015; Česen et al., 2016; Zounkova et al., 2010). Consequently, metabolites and transformation products are also released into the aquatic environment and might cause a potential risk to the aquatic biota. Only a few studies analysed the effects of anticancer drugs' metabolites, and most of the tested compounds caused toxic effects on different organisms (**Table S3**).

Carboxy-cyclophosphamide, the only metabolite of cyclophosphamide detected in surface water, induced chronic toxicity by inhibiting the rotifera *B. calyciflorus*' reproduction EC_{50} = 23.66 mg/L, and the growth of the cyanobacteria *S. leopoliensis* with EC_{50} = 17.1 mg/L. It also appeared that carboxy-cyclophosphamide was more toxic than its parent compound as cyclophosphamide inhibited the reproduction of *B. calyciflorus* with EC_{50} = 89.84 mg/L and did not affect the growth of *S. leopoliensis* (Česen et al., 2016; Russo et al., 2018c). On the contrary, the human metabolite of 5-fluorouracil showed lower toxicity than the parent compound in all the organisms tested (*P. putida*, *D. subspicatus* and *D. magna*). For instance, in the growth inhibition test with *P. putida*, 5-fluorouracil caused effect with EC_{50} = 48 mg/L, and FBAL inhibited the growth with EC_{50} = 80 mg/L (Zounkova et al., 2010). The active metabolites of tamoxifen (4-hydroxy-tamoxifen and endoxifen), known to have higher potency and affinity for estrogen receptors and estrogen-related receptors, affected the

survival and the reproduction of *D. pulex*. However, compared to the parent compound's toxicity, tamoxifen proved to be more potent (Borgatta et al., 2016, 2015).

Looking at the results, it was evident that evaluating the ecotoxicity of the metabolites and the transformation products was necessary as some compounds are more toxic than their corresponding parent compounds. In addition to that, anticancer drugs are activated by liver enzymes to produce pharmacologically active metabolites and cause adverse effects. In case the parent compound is not activated in the organism tested, no effects will be observed. Therefore, it is essential to assess the metabolites' impact together with their parent compounds to avoid any inaccurate low activities (Białk-Bielińska et al., 2017).

8. Mixture effects

As a matter of fact, aquatic organisms are exposed to a complex combination of different pharmaceuticals, including anticancer drugs, their metabolites, and other chemical pollutants, and rarely to one individual drug. Consequently, to avoid underestimating the real impact, it is essential to assess the combinatorial effects as adverse responses might be more intense than the responses of a single compound exposure (Česen et al., 2016; Chakrabarty et al., 2012; Elersek et al., 2016; Sun et al., 2009). Two methodologies were suggested for evaluating the mixture effects of anticancer drugs: "the pharmacological approach" where drugs are selected based on the regimen administered; and "the environmental approach", where drugs are chosen based on the likelihood of their simultaneous presence in the aquatic environment (most consumed drugs) (Brezovšek et al., 2014).

From the included studies, 27 research investigated the adverse effects of drugs mixtures, where 12 studies examined the effect of anticancer drugs mixtures exclusively, and 15 studies examined the mixture effect of different classes of pharmaceuticals, including anticancer drugs. Several interpretations were extrapolated from the studies presented in **Table S2**. It was evident in some studies that mixture response was much higher than the response of the compounds alone, which is known as the synergism/potentiation effect. For instance, the mixture of cyclophosphamide, ifosfamide and their metabolites has caused higher growth inhibition of the cyanobacteria *S. leopoliensis* than the sum of the individual compounds (Česen et al., 2016). Also, tamoxifen and 4-hydroxy-tamoxifen affected the reproduction of *D. pulex*, whereas no effect was observed when tested alone at the same concentrations (Borgatta et al., 2016). Antagonism was another effect observed and expressed by the suppression of one compound to the harmful activities of the other/s. An antagonistic response was reported for the mixtures imatinib/cisplatin and imatinib/etoposide in *C. magna*; and for etoposide/cisplatin and etoposide/5-fluorouracil in *C. dubia* (Parrella et al., 2014a). Similarly, the toxicity response was lowered when vincristine and bleomycin were combined in the growth inhibition test of *L. minor* and *P. putida* and the mobility inhibition test with *D. magna* (Jureczko and Przystaś, 2019). Nonetheless, in these two cited studies, the antagonistic interaction mechanism was difficult to explain as these drugs have different modes of action and, hence, different molecular targets (Jureczko and Przystaś, 2019; Parrella et al., 2014a).

In fact, mixture effects can be predicted using two reference models: the concentration addition (CA) and the independent action (IA). The CA model is used for a mixture of compounds with the same mode of actions, and the IA model is used for compounds that behave by different pathways (Kundi et al., 2016; Parrella et al., 2014a). However, numerous

528 studies have proved that these models can underestimate or overestimate the toxicity of
529 mixtures when compared to the experimental toxicities (Elersek et al., 2018, 2016; Jureczko
530 and Przystaś, 2019; Kundi et al., 2016; Parrella et al., 2014a).

531 Another interesting point raised by these studies is that the responses obtained could also be
532 influenced by experimental parameters such as the type of species tested and the selected
533 endpoints. For instance, in Brezovšek *et al.* (2014) study, a synergistic response was reported
534 in *P. subcapitata* for the binary mixture of imatinib and 5-fluorouracil; however, antagonism
535 was observed when tested in *S. leopoliensis* (Brezovšek et al., 2014). In da Fonseca *et al.*
536 (2019) study, the mixture of tamoxifen, cyclophosphamide and cisplatin was evaluated on *N.*
537 *diversicolor* where an antagonist effect was observed on SOD activity (provoked by
538 tamoxifen), and a synergistic response was recorded for CAT activity (triggered by cisplatin
539 and tamoxifen) (da Fonseca et al., 2019).

540 Finally, regarding endocrine-disrupting chemicals (EDCs), several mixture studies were
541 conducted to prove that antagonistic EDCs can neutralise or decrease the harmful effects of
542 these compounds. In general, estrogenic and antiestrogenic activities were assessed against
543 each other, and it appeared that antiestrogenic chemicals were able to mask some signs of
544 estrogen exposure, but not entirely (Elias et al., 2007; Kuhl and Brouwer, 2006). For example,
545 tamoxifen can act as an estrogen agonist and antagonist when mixed with bisphenol A, 17α-
546 ethynylestradiol (EE2), nonylphenol or estradiol, depending on the parameter measured
547 (García-Hernández et al., 2016; Maradonna et al., 2009; Xia et al., 2016). Moreover, in Sun *et*
548 *al.* (2009; 2011b) studies, anti-estrogens were proved to work differently depending on
549 whether they act by inhibiting estrogens' binding to their receptors or inhibiting aromatase
550 activity (reducing E2 production). Therefore, different results could be obtained by different

551 anti-estrogens such as letrozole and tamoxifen. For instance, the induction of one vitellogenin
552 gene transcription by EE2 was blocked by letrozole but not by tamoxifen (Sun et al., 2011b,
553 2009).

554

555 ## 9. Risk assessment

556 With the growing interest in evaluating pharmaceuticals' adverse effects in the aquatic
557 environment, risk assessment guidelines were established to reduce animal testing when the
558 risks are low (Vestel et al., 2016). From the included studies, only twenty-five assessed the
559 environmental risk based on predictions and calculations (**Table S4**). Almost all the studies
560 included have implemented the US EPA guidelines or the European Union guidelines (EMA).
561 The major difference between the two procedures is that the first permits the use of acute
562 toxicity; however, EMA recommends using chronic toxicity to determine the predicted no-
563 effect concentration (PNEC) (Valcárcel et al., 2011). To express the risk, the risk quotient (RQ)
564 or hazard quotient (HQ) is calculated by dividing the predicted environmental concentration
565 (PEC) or the measured environmental concentration (MEC), if available, by the PNEC, which
566 is derived from toxicological data from fish, daphnids and algae's standard tests (Franquet-
567 Griell et al., 2015).

568 In case chronic data was not available, acute data was considered, and when both
569 toxicological information was missing, acute toxicity was predicted by using the developed
570 QSAR tool (Franquet-Griell et al., 2017; Valcárcel et al., 2011). However, this tool proved to
571 be not sufficiently precise as all the anticancer drugs tested in Madden *et al.* (2009) study fell
572 outside the model's applicability domain. Hence, less confidence would be provided with this
573 prediction, except for the compound 5-fluorouracil (Madden et al., 2009). Nevertheless, QSAR

predicted the toxicity of tamoxifen and imatinib's transformation products where only their chemical structures were obtainable (Negreira et al., 2015; Secrétan et al., 2019).

From the results obtained, most of the anticancer drugs studied achieved a risk quotient below one, suggesting that they do not pose any risk for the aquatic environment. The compounds that showed to have moderate to high (risk quotient higher than one) risk were the following: bleomycin, vincristine, imatinib, irinotecan, ifosfamide, 5-fluorouracil, cisplatin and tamoxifen (Ferrando-Climent et al., 2014; Jureczko and Przystaś, 2019; Mišík et al., 2019; Olalla et al., 2018; Orias et al., 2015a; Załeska-Radziwiłl et al., 2011; Załęska-Radziwiłł et al., 2017). In addition to that, it was proven in some studies that the risk assessment based on the measured environmental concentration was higher than the risk calculated from the predicted environmental concentration, which could potentially contribute to the accuracy of the results (Gouveia et al., 2019).

While risk assessment guidelines provide many advantages like reducing animal testing, limiting laboratory research and obtaining results in a shorter period, many limitations were identified, such as (1) the studies selected are only based on three trophic levels and considering one endpoint (mainly the mortality), which is not sufficient to predict the risk on the entire ecosystem (Grung et al., 2008; Jureczko and Przystaś, 2019); (2) chronic toxicity was derived by simple calculations based upon acute toxicity which was predominantly used in the included studies and consequently, results could underestimate the actual risk of anticancer drugs in the water environment (Fent et al., 2006).

10. Conclusion

A high number of studies have attempted to evaluate the risk of anticancer drugs on non-target organisms in the aquatic environment. However, complete toxicity data for all the drugs in use are still lacking, and more work needs to be done in order to understand the full impact on the aquatic biota. From the 152 studies included in this systematic review, several conclusions and recommendations were made:

- For the reason of differences in the sensitivity of different aquatic organisms, the ecotoxicity tests must be performed in organisms from different trophic levels.
- Multi-generational exposure is important to recognise the impact of anticancer drugs in the long-term.
- Chronic tests are more relevant than acute tests as they mimic the reality of aquatic exposure.
- Depending on the mode of action of the tested drug, suitable endpoints should be selected.
- It is important to link any genotoxic effects to physiological and behavioural responses.
- Hormesis, adaptation and bioaccumulation should be taken into consideration while assessing the results.
- Whenever metabolites and transformation products of anticancer drugs are commercially available, they should also be evaluated.
- It is essential to assess the mixture effect of anticancer drugs as it could reveal more significant responses.

- Current guidelines are not adequate for assessing the real impact of anticancer drugs in the aquatic environment and should be reviewed and updated to include more sensitive and relevant endpoints.

In conclusion, some tested anticancer drugs have affected the aquatic organisms in the long-term and at concentrations relatively close to those detected in the aquatic environment, in the range of ng/L – µg/L. Therefore, non-target organisms' exposure to trace concentrations of anticancer drugs could endanger certain species starting from causing genetic modifications and leading, in worse case scenarios, to their extinction. As a result, more attention should be paid to developing efficient water treatments to eliminate trace concentrations of anticancer drugs and their metabolites before discharging them into the aquatic environment. In addition to that, where wastewater treatment is not available or effective, stricter guidelines for cytotoxic waste should be implemented. Another important focus to put in another systematic review is whether exposure to environmental concentrations of anticancer drugs could also affect humans.

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