Embryotoxicity of five cytostatics in fathead minnow (*Pimephales promelas*) larvae

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Abstract:

Cytostatics are compounds used in chemotherapy, known to be genotoxic, mutagenic, and teratogenic at low concentrations. The amount of cytostatic drugs prescribed increases every year as does their release into the aquatic ecosystems, which possibly is a major concern for the health of aquatic organisms. This study aimed to evaluate the putative toxicity of five cytostatics to fathead minnow (*Pimephales promelas*) larvae: tamoxifen, capecitabine, methotrexate, cyclophosphamide, and ifosfamide. Eggs collected post-fertilization were exposed for 6 days to a range of concentrations, including one above environmental level. At all environmental concentrations, no significant difference in mortality, hatching time, length, heart rate, and presence of malformations were found. Altogether, these cytostatics seems not embryotoxic. Although, an increased proportion of complete swim bladder were found after ifosfamide's exposure, suggesting an interaction with the thyroid axis, involved in swim bladder development. Complementary work should address other endpoints, such as behavioral changes, reproductive success, and transgenerational effects.

Keywords: cytostatics, anticancer, fish, embryotoxicity

Introduction:

Cancer is one of the leading causes of death throughout the world (Nussbaumer and al. 2011), and the number one in Canada (Statistics Canada, 2020). For decades, the incidence of cancerous diseases in the human population has been increasing. Cytostatics, also called anti-neoplastic drugs or anticancer drugs, are compounds commonly used in chemotherapy. Since the number of cancer incidences is increasing, this suggests that the administration of cytostatics is also in constant augmentation. From less than 5% a decade ago, the number of oral cancer agents in use has increased to approximately 17% by 2007, and it is now estimated that at least 25% of the existing antineoplastic agents are planned to be used as oral agents (Tadic et al. 2015). Most of these compounds prevent uncontrolled proliferation of cancers cells via DNA interaction and cell signaling (Novak et al. 2017). Due to their mode of action and overall, they are classified as cytotoxic, genotoxic, mutagenic, and teratogenic agents, and potentially endocrine disruptors (Novak et al. 2017; Kosjek et Heath 2011). Moreover, cytostatics act unselectively on cancer cells and noncancer cells, which often cause undesirable side effects during treatments (Novak et al. 2017; Kosjek and Heath 2011).

Residues of these compounds are excreted after administration to patients into domestic and hospital wastewater (Negreira et al. 2014; Johnson et al. 2013; Zhang et al. 2013; Kosjek and Heath 2011). These residues are a mixture of parent compounds and their metabolites (Novak et al. 2017; Zhang et al. 2013). However, several studies have shown their poor elimination efficiency in conventional wastewater treatment plants (Franquet-Griell et al. 2017; Negreira et

al. 2014; Zhang et al. 2013). Consequently, wastewater treatments plants are considered to be an important point source of drug contamination into the environment (Novak et al. 2017; Zhang et al. 2013; Brun et al. 2006). Cytostatics are usually present at low concentrations in the environment (sub ng/L to few µg/L; reviewed by CEAEQ (not published); Novak et al. 2017; Zhang et al. 2013; Kosjek and Heath 2011; Brun et al. 2006). However, as their use is in constant augmentation, their environmental concentrations will likely increase in the future. Moreover, due to their unselectively toxic properties, these compounds could affect countless organisms' cells, which is a major preoccupation for environmental safety.

The aim of this study was to evaluate fish embryotoxicity of the five above cytostatics found in Canadian surface waters and elsewhere, according to the need of knowledge on these substances. Five cytostatics were assessed in this study, according to their constant presence in the environment. Tamoxifen (TX) is used as an anti-estrogenic in breast cancer therapy (Zhang et al. 2013) as it inhibits the estrogen receptor binding. Methotrexate (MX) is used as an antifolic to treat several types of cancers, like non-Hodgkin's lymphoma (Nussbaumer et al. 2011) and inhibits folic acid synthesis, which is essential for DNA synthesis. Capecitabine (CAP) is used as an antipyrimidique in metastatic colorectal cancer therapy (Nussbaumer et al. 2011) and inhibits the thymidylate synthase, which blocks DNA replication. Finally, cyclophosphamide (CP) and its analogue, ifosfamide (IF), are two nitrogen mustards used to treat several types of cancers like solid tumours (Nussbaumer et al. 2011). These two nitrogen mustards are alkylating agents that form DNA adducts, which also blocks DNA synthesis. Fathead minnow (*Pimephales promelas*) present throughout North America's aquatic environments was chosen to conduct this work, as one of the species usually used in ecotoxicology assessment.

Methods and Materials:

TX (CAS No: 10540-29-1, 99 %), CAP (CAS No: 158798-73-3, ≥ 99 %), MX (CAS No: 59-05-2, 99.5 %), CP (CAS No: 50-18-0, ≥ 98 %), IF (CAS No: 3778-73-2, ≥ 98 %), dimethyl sulfoxide (DMSO), and 3–aminobenzoic acid ester methanesulfonate (MS – 222) were purchased from Sigma. Reconstituted water (deionized water with 0.3 mM $CaSO_4$, 0.2 mM $MgSO_4$, 0.05 mM KCl, and 2.4 mM $NaHCO_3$) was prepared and stored in a regulated experimental room (25 °C, 70% humidity, and 16/8h light/dark cycle) for all experiments. Temperature, pH, and conductivity were verified in daily to avoid undesired stress to the animals and readings were consistent.

The experimental design and measured endpoints as described follow OECD's guidelines for acute toxicity assessment (1998). CAP, CP, and IF were dissolved in reconstituted water, TX and MX were dissolved in DMSO due to their insolubility in water, the final solvent concentration was 0,01% v/v. Nominal concentrations of each cytostatic were 0.001 µg/L (or 1 ng/L), 0.1 µg/L (or 100 ng/L) and 10 µg/L as these concentrations are found within the environment (reviewed by CEAEQ). In addition, one treatment of 1 000 µg/L (or 1 mg/L) was added to the experimental design to test for a high-end concentration.

Fathead minnow eggs were obtained from a colony established at the Institut National de la Recherche Scientifique (INRS; Quebec City, QC, Canada). Breeding substrate made of cut sections of polyvinyl chloride tubing (4 po diameter) were placed in culture tanks containing reproductively mature males and females in the evening prior to test initiation. The following morning, the breeding substrates were removed from the culture tanks and placed in clean reconstituted water with aeration. The eggs were removed from the tiles 4 h later. Prior to exposure, eggs were observed under a microscope to select stage 13 (or one-quarter epiboly), which appears around 10 h post-fertilization according to Delvin and colleagues (1996). Then, three replicates of 30 eggs each were placed in 500-mL glass-beakers with 200 mL of exposure

mixture, reconstituted water or solvent controls water (0.01% DMSO). All exposure and control solutions were changed daily by transferring eggs/hatched larvae into freshly prepared solutions. All beakers were covered with Petri dishes to minimize evaporation. All treatments were analyzed by the Centre d'Expertise en Analyse Environnementale du Québec (CEAEQ; Quebec City, QC, Canada), except the lowest concentration of each treatment as the nominal concentration values were below the detection limit (15 ng/L to 28 ng/L) of available analytical equipment. All treatments were analyzed in duplicate, from stock solution, at time 0 (T0) and time 24 h (T24), by liquid chromatography coupled with mass spectrometry (LCMS, Xevo TQ-S, Waters® - LOD 2 ng/L to 3.2 ng/l, LOQ 7 ng/L to 16 ng/L) (Borgatta et al. 2016). Water sampled at T0 was stored at 4 °C for 24 h prior to being transported for chemical analyses at CEAEQ following the collection of the T24 samples. Mortality and hatching data were daily recorded and all dead egg/larva were removed daily. Then, only final mortality (over 6 days) and average hatching time were analyzed. The number of solutions that could be analyzed was limited due to budget restriction and limit of detection. For example, the 1 ng/L solution was considerably below the detection limit, so it was decided to not analyzed it. We prioritized treatment solutions rather than controls, since we considered it was more important to measure the degradation of compounds over 24 h, which is the maximum amount of time we used the solution.

After 6 days of exposure, all larvae were transferred individually to a 96–well plate. Heart rate was measured with an inverted microscope (Labomed® TCM 400). Temperature could not have affected fish heart rate over different exposure, while it remains constant for all experiments. The sampling was made over a day (always between 8 am and 6 pm) and replicates were taken for analysis randomly, so time of day seems an unlikely bias. These five experiments were conducted over three weeks by the same staff so seasonality and staff change should also not be the issue. Larvae were then anaesthetized with 100 µL of MS-222, freshly prepared at 100 mg/L buffered with $NaHCO_3$ at 200 mg/L after removing the remaining exposure solution. Then, they were observed with a compound microscope (Nikon® SMZ18) and photographed (Nikon® digital sight DS-L3).The presence of malformations (e.g., edema, hemorrhage, tube heart, scoliosis, craniofacial deformation, and cardiac deformation), the development of swim bladder (i.e., complete, incomplete/absence), and the number of malformed larvae in each replicate were recorded (Madison et al. 2020). Pictures were analyzed with ImageJ software to measure the length.

Data analysis was performed with GraphPad Prism 8. Mortality, hatching time, heart rate, length, malformation rate, presence and number of malformations, and presence/partial development/absence of swim bladder were assessed for each treatment using analysis of variance (one-way ANOVA) after reviewing conclusive normality (Shapiro-Wilk test) and homoscedasticity (Brown-Forsythe test). Dunnett's post-hoc tests were performed to identify significant differences between treatment groups.

Results:

For all cytostatics, measured concentrations remained consistent before water change and in agreement with the nominal concentrations, except for TX and CAP at 0.1 µg/L (Table 1). Measured concentrations for TX were twice lower than nominal concentration at T0 and T24, as describe by Borgatta et al. (2016), these differences were probably due to adsorption of the molecule on glass surface. All of the following results were reported with the nominal concentration.

There were no significant differences in survival of larvae or in hatching time (Table 1) with the exposure of CAP, MX, CP and IF. For TX, 100% mortality was measured in all replicates at 1,000 µg/L ($F = 43.16$; $p < 0.0001$, resp). Significant differences were measured in hatching

of eggs at 1,000 µg/L (F = 7.341; p = 0.0023), but this result is not robust because only 3 eggs hatched during the first 2 days of the exposure, in all 1,000 µg/L replicates, then all these larvae died before the end of the exposure.

There were no significant differences in heart rate and length between treated larvae (Table 1) in the exposure of all of the molecules. Of note, no measurements of length were analyzed for the CP exposition due to a malfunction of a data storage drive.

There were no significant differences in the number of malformed larvae (Table 1) for TX, CAP, MX, CP and IF. In addition, malformation data was also analyzed per type of malformation, and number of malformations per larvae in each treatment group. No significant differences were found for any treatments (data not showed).

137 **Table 1.** Measured endpoints after exposure to five cytostatics. RW: reconstitute water, ND: not determined. ANOVA * p<0.05.

Treatments	Nominal levels	Measured levels T0	Measured levels T24	Mortality (%)	Hatching (d)	Heart rate (/min)	Length (mm)	Malformation (%)	Uninflated swim bladder (%)
Tamoxifen	RW	ND	ND	4.4 ± 4.7	4.8 ± 0.04	184 ± 3	5.5 ±0.2	8.4 ± 4.2	17.6 ± 13.3
	DMSO	ND	ND	1.2 ± 2.1	4.9 ± 0.09	181 ± 3	5.4 ± 0.2	17.1 ± 6.6	22.9 ± 4.5
	0.001 µg/L	ND	ND	5.6 ± 2	5.0 ± 0.2	184 ± 2	5.4 ± 0.2	17.7 ± 9.2	29.4 ± 11.4
	0.1 µg/L	41 ± 14[a]	49 ± 5.6[a]	3.2 ± 5.6	5.0 ± 0.09	180 ± 5	5.4 ± 0.2	12.8 ± 3.9	28.0 ± 15.3
	10 µg/l	3.5 ± 0.7[a]	2.8 ± 0.1[a]	3.2 ± 3.2	4.9 ± 0.1	181 ± 3	5.4 ± 0.2	18.4 ± 12.1	28.7 ± 14.4
	1,000 µg/l	0.6 ± 0.6a	0.48 ± 0.1[a]	**100 ± 0 ***	**1.7 ± 2.1 ***	-	-	-	-
Capecitabine	RW	ND	ND	2.3 ± 4	4.9 ± 0.04	185 ± 6	5.4 ± 0.3	15.1 ± 4.5	19.7 ± 6.3
	0.001 µg/L	ND	ND	8.9 ± 7	4.6 ± 0.2	191 ± 1	5.5 ± 0.2	3.4 ± 6	7.1 ± 5.8
	0.1 µg/L	165 ± 21	170 ± 14	4.5 ± 2	4.6 ± 0.3	191 ± 3	5.5 ± 0.3	17.8 ± 9.6	11.9 ± 9.0
	10 µg/l	12.5 ± 2.1	13 ± 1.4	8.7 ± 8	4.8 ± 0.2	189 ± 2	5.4 ± 0.3	9.8 ± 4.4	13.9 ± 11.4
	1,000 µg/l	1.3 ± 0.7	1.5 ± 0.1	3.3 ± 6	4.7 ± 0.09	192 ± 3	5.4 ± 0.4	21.5 ± 7.5	16.3 ± 11.6
Methotrexate	RW	ND	ND	6.7 ± 6.7	4.8 ± 0.1	187 ± 6	5.5 ± 0.2	8.5 ± 7.8	13.5 ± 8.4
	DMSO	ND	ND	5.5 ± 6.9	4.8 ± 0.2	193 ± 5	5.4 ± 0.3	9.5 ± 2.5	14.4 ± 9.6
	0.001 µg/L	ND	ND	12 ± 7	4.7 ± 0.07	194 ± 5	5.5 ± 0.2	4.0 ± 4.2	7.8 ± 4.4
	0.1 µg/L	115 ± 7.1	102.5 ± 39	11.3 ± 7.3	4.7 ± 0.2	192 ± 5	5.4 ± 0.2	4.7 ± 1.2	7.1 ± 2.8
	10 µg/l	11.5 ± 0.7	8.4 ± 0.1	3.2 ± 0.1	4.7 ± 0.06	196 ± 2	5.4 ± 0.3	6.8 ± 9.2	15.9 ± 10.1
	1,000 µg/l	1.1 ± 0.2	1.15 ± 0.1	7.5 ± 7.4	4.7 ± 0.3	189 ± 4	5.3 ± 0.4	12.7 ± 4.8	16.5 ± 6.2
Cyclophosphamide	RW	ND	ND	2.6 ± 2.2	4.9 ± 0.1	191 ± 5	ND	14.9 ± 10.7	12.0 ± 8.4
	0.001 µg/L	ND	ND	2.5 ± 2.2	4.9 ± 0.08	189 ± 8	ND	11.9 ± 4.4	14.7 ± 5.4
	0.1 µg/L	120 ± 1.1	125 ± 21.2	4 ± 4.1	4.9 ± 0.1	192 ± 7	ND	11.5 ± 10	15.9 ± 9.6
	10 µg/l	13.5 ± 2.1	12 ± 1.4	9.9 ± 2.1	4.8 ± 0.1	193 ± 7	ND	6.8 ± 2.3	15.2 ± 9.7
	1,000 µg/l	1.3 ± 0.1	1.4 ± 0.1	6.2 ± 2.3	4.9 ± 0.09	196 ± 3	ND	13.1 ± 1.5	15.7 ± 3.4
Ifosfamide	RW	ND	ND	2.2 ± 1.9	4.8 ± 0.06	195 ± 4	5.6 ± 0.2	9.2 ± 10.5	27.3 ± 15.0
	0.001 µg/L	ND	ND	3.4 ± 3.5	4.7 ± 0.08	191 ± 3	5.3 ± 0.3	10.4 ± .2	**8.1 ± 2.3 ***
	0.1 µg/L	100.5 ± 13.4	115 ± 7.1	2.2 ± 1.9	4.7 ± 0.2	200 ± 3	5.3 ± 0.3	6.9 ± 3.4	**5.7 ± 5.3 ***
	10 µg/l	11 ± 0	10.3 ± 1	2.2 ± 3.9	4.9 ± 0.3	196 ± 11	5.3 ± 0.3	4.9 ± 4.0	**9.1 ± 8.4 ***
	1,000 µg/l	1.2 ± 0	1.14 ± 0.2	2.1 ± 1.8	4.9 ± 0.1	198 ± 5	5.6 ± 0.2	3.3 ± 3.3	**3.3 ± 3.3 ***

[a]: unreliability of the measured values

138

Finally, there were no significant differences in the development of swim bladders for TX, CAP, MX, and CP. However, a significant decrease of the proportion of fish with not fully developed swim bladders was observed for all the concentrations of IF ($F = 3.921$; $p = 0.0412$; Table 1).

Discussion:

The aim of this study was to investigate the acute toxicity of five cytostatics during fathead minnows' early development. At high concentration (1 mg/L; level not found in the environment), only the TX exposure led to complete mortality, while all the other cytostatics' treatments were not toxic for the endpoints measure on the developing fish. This result for TX is in agreement with previous studies with other species. DellaGreca and colleagues (2007) measured 50 % lethal concentration (LC_{50}) in several organisms: *Thamnocephalus platyurus*, $LC_{50;24h}$= 0.40 mg/L; *Brachionus Calyciflorus*, $LC_{50;24h}$ = 0.97 mg/L; *Daphnia magna*, $LC_{50;24h}$ =1.53 mg/L. The FASS (2011) also report $LC_{50;96h}$ for fish: *Lepomis macrochirus*, $LC_{50;96h}$ = 0.15 mg/L; *Oncorhynchus mykiss*, $LC_{50;96h}$ = 0.27/0.21 mg/L. In chronic study in zebrafish (*Danio rerio*), high mortality (≥ 88%) was observed for larvae exposed to 1 mg/L of TX and for juveniles exposed to 0.1 mg/L (Van der Ven et al. 2007). Although, exposure of medaka's eggs (*Oryzias latipes*) to TX showed in 100% mortality at concentrations above 3,125 µg/L, while 40% mortality was observed at 625 µg/L (Sun et al. 2007).

Also, some authors showed some effects in the range of the toxicology endpoints measured in the current study (NOEC of 10 µg/L nominal, LOEC of 1,000 µg/L nominal). Hatching rate and hatching time were altered at 125 and 625 µg/L, but no morphological deformation was observed (Sun et al. 2007). Wester et al. (2003) report that spawning, fertilization, hatching, survival and growth of adult zebrafish exposed to 10 to 320 µg/L of TX-citrate were reduced. Based on growth, the Lowest Observed Effect Concentration and the 63-day NOEC were 10 and 3.2 µg/L, respectively. Morphological changes were also observed in both the ovaries and testes starting at 10 µg/L. However, when testing for fish embryotoxicity at environmental levels of cytotatics (i.e., from 0.001 µg/L to 1,000 µg/L), no toxicity was observed, and this, for all five of the cytostatics tested. For MX, CAP, CP and IF, LC_{50}, EC_{50} and No Observed Effect Concentration (NOEC) reported in the literature are generally between mg/L to g/L. Henschel and colleagues (1997) showed $LC_{50;48h}$ of 85 mg/L in *Danio rerio* embryo exposed to MX. Straub (2010) measured a $LC_{50;48h}$ in *Daphnia magna* of 850 mg/L and $NOEC_{96h}$ of 867 mg/L in *Oncorhynchus mykiss* for CAP. Weigt and colleagues (2011) measured $LC_{50;72h}$ of 2,200 mg/L in *Danio rerio* for CP and 836 mg/L for IF. In fact, embryos were mostly exposed during pre-hatch development, so the selectivity of the chorionic barrier could therefore explain the absence of observed effects.

Noteworthy, only the individuals of the IF treatment were showing a fully developed and inflated swim bladder at the end of the experiment. Swim bladder is a fish organ separated in two chambers. It begins as a posterior chamber, which inflates at 5-6 days post-fertilization (dpf) and the second chamber is formed anterior to the first one, around 14 dpf (Cavallin et al. 2017; Nelson et al. 2016). Development of swim bladder is under thyroid axis control. Inhibition of the thyroid axis could lead to a decreased inflation and/or size of swim bladder and a decline of the surfactant protein production that prevents it from collapsing (Godfrey et al. 2017; Cavallin et al. 2017; Nelson et al. 2016). The presence of all fully inflated posterior swim bladder chambers in the fish exposed to IF could be a marker of a developmental acceleration. Impairment of swim bladder inflation was observed in fish exposed to dilbit for example (Alsaadi et al. 2017; Madison et al. 2017). In addition, stress response is characterized by an increase of cortisol plasma level, and moreover, cortisol acts on target tissues by binding to glucocorticoid receptors (Alsop and Vijayan 2009) and is known to work synergistically or

additively with thyroid hormones (Stephen et al. 1997). Therefore, it is possible that an exposure to IF as a stress factor, even at low concentrations, increase thyroid hormone/cortisol levels in fish larvae. Also, treatment with IF in human is known to be potentially involve in development of secondary tumors as thyroid cancer (FDA, 2012).

In conclusions, data suggest that the five cytostatics do not induce embryotoxicity in developing fish at environmental levels. However, because cytostatics were engineered to kill cells using molecular mechanisms of action shared among living organisms, one needs to investigate complementary biological endpoints (e.g., DNA damage, genotoxic effect) in other species and development stage to complete previous studies and ensure entire ecosystem safety. Moreover, most of these compounds are known to be persistent in the environment. Consequently, chronic exposure to cytostatics should also be assessed to further explore for behavior, teratogenic, and/or reprotoxic effects. Finally, cytostatics are also found in mixture within the environment and could potentially act synergically and/or additively; therefore, future studies should also address these research questions.

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Bulletin of Environmental Contamination and Toxicology

Embryotoxicity of five cytostatics in fathead minnow (Pimephales promelas) larvae.

--Manuscript Draft--

Manuscript Number:	BECT-D-20-01091R2
Full Title:	Embryotoxicity of five cytostatics in fathead minnow (Pimephales promelas) larvae.
Article Type:	Original Research
Keywords:	Chemotherapy drugs; Cytostatics; Fish embryos; Fathead minnows
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Abstract:	Cytostatics are compounds used in chemotherapy, known to be genotoxic, mutagenic, and teratogenic at low concentrations. The amount of cytostatic drugs prescribed increases every year as does their release into the aquatic ecosystems, which possibly is a major concern for the health of aquatic organisms. This study aimed to evaluate the putative toxicity of five cytostatics to fathead minnow (Pimephales promelas) larvae: tamoxifen, capecitabine, methotrexate, cyclophosphamide, and ifosfamide. Eggs collected post-fertilization were exposed for 6 days to a range of concentrations, including one above environmental level. At all environmental concentrations, no significant difference in mortality, hatching time, length, heart rate, and presence of malformations were found. Altogether, these cytostatics seems not embryotoxic. Although, an increased proportion of complete swim bladder were found after ifosfamide's exposure, suggesting an interaction with the thyroid axis, involved in swim bladder development. Complementary work should address other endpoints, such as behavioral changes, reproductive success, and transgenerational effects.

Institut national de la recherche scientifique

February 1st, 2021

Dr Erin Bennett
Editor-in-Chief
Bulletin of Environmental Contamination and Toxicology

Dear Dr Erin Bennet,

This letter accompanies our revised manuscript entitled "Embryotoxicity of five cytostatics in fathead minnow (*Pimephales promelas*) larvae" (ID: BECT-D-20-01091R1*)* written and revised by Molly Lefebvre-Raine and colleagues.

We would like to thank all the Associate Editor for her/his valuable comments for improving this new version of the manuscript. We have addressed her/his last set of suggestions and have thoroughly reviewed this new version by highlighting in yellow the changes. We have addressed all of the comments raised the Associate Editor in a Response to Reviews document attached.

We have thoroughly reviewed the author's checklist and made the corresponding edits to the manuscript. We thank you for considering this manuscript for publication in the *Bulletin of Environmental Contamination and Toxicology.*

Sincerely,

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ID : BECT-D-20-01091R1

Title: Embryotoxicity of five cytostatics in fathead minnow (Pimephales promelas) larvae

Response to the Associate Editor:

Lines 88-89 Here it says that the all exposure solutions were analyzed except the 1 ng/L solutions. However, the table also indicates that the control solutions were not analyzed ("ND"). Why not analyze the controls and even 1 ng/L? It is common practice to analyze all exposure solutions even controls as this can serve as a good check of the method. Additionally, please indicate the detection limit of the methods used.
R: This was added to the main text (lines 105-110): "The number of solutions that could be analyzed was limited due to budget restriction and limit of detection. For example, the 1 ng/L solution was considerably below the detection limit, so it was decided to not analyzed it. We prioritized treatment solutions rather than controls, since we considered it was more important to measure the degradation of compounds over 24 h, which is the maximum amount of time we used the solution".

Line 92 to 93 were Quality Control samples prepared at the same time as the T0 samples to evaluate stability? Or was stability already known?
R: The protocol used within (cytostatics sampling, preserving, and analyzing) was optimized by the Ministry of the Environment of Quebec (Canada). The Quality Control samples were prepared at time for the analysis only (by the one of the Ministry of the Environment of Quebec's laboratories). We followed their protocol for the preparation of the solutions, which included to store samples (T0 and T24) at 4 °C after collection and send them as soon as possible to be analyzed within seven days of collection. Therefore, we brought the sample immediately after the T24 collection (after 24 h). If their protocol requires chemical analysis to be performed within 7 days (at 4C) of sampling, this must be because they already evaluated the stability of all of these cytostatics of interest and know this is acceptable.

Missing from methods: was temperature maintained? What was the target temperature? Was temperature measured in some way? What about other water quality parameters such as pH?
R: This was added to the main text (lines 75-76): "Temperature, pH, and conductivity were verified in all times to avoid undesired stress to the animals and were consistent."

Lines 113-117 The comparison of disappearance of tamoxifen in the exposure system to the half-life in the blood of mammals is not valid. These are two completely different things and it is probably a coincidence that there appears to be a relationship between the two. Such a relationship doesn't hold for the other compounds; for example, capecitabine does not disappear in the aquatic exposure system, yet the half-life in humans is short: 0.55 to 0.89 hours. Rather than citing mammalian pharmacokinetics, it could be more enlightening to review what happened to tamoxifen in other aqueous exposure systems in the literature.
R: This was added to the main text (lines 157-164): "Also, some authors showed some effects in the range of the toxicology endpoints measured in the current study (NOEC of 10 µg/L nominal, LOEC of 1,000 µg/L nominal). Hatching rate and hatching time were altered at 125 and 625 µg/L, but no morphological deformation was observed (Sun et al. 2007). Wester et al. (2003) report that spawning, fertilization, hatching, survival and growth of adult zebrafish exposed to 10 to 320 µg/L of TX-citrate were reduced. Based on growth, the Lowest

Observed Effect Concentration and the 63-day NOEC were 10 and 3.2 µg/L, respectively. Morphological changes were also observed in both the ovaries and testes starting at 10 µg/L."

Lines 116-117 Therefore, the actual concentrations of tamoxifen to which the eggs and larvae were exposed is unknown, all you know is that the concentration was somewhere between the nominal and measured concentration.
R: The experiments were carried as soon as the tamoxifen was delivered. We do not have measurements of the tamoxifen during storing condition.

Lines 126-128 Do the authors have any hypotheses on why the heart rate appears to increase over the studies? In the tamoxifen experiment the average heart rate ranges from 180 to 184 while for ifosfamide the range is 191 to 200. What things affect this endpoint in these fish: temperature, time of day, etc?
R: This was added to the main text (lines 112-117): "Temperature could not have affected fish heart rate over different exposure, while it remains constant for all experiments. The sampling was made over a day (always between 8 am and 6 pm) and replicates were taken for analysis randomly, so time of day seems an unlikely bias. These five experiments were conducted over three weeks by the same staff so seasonality and staff change should also not be the issue."

Missing from results: Were any water quality parameters measured: temperature, pH? Reference for the analytical method used that contains details of the validity of the method.
R: This was added to the main text (lines 75-76): "Temperature, pH, and conductivity were verified in daily to avoid undesired stress to the animals and readings were consistent".

Lines 194-196 Since the authors added a concentration that was 100 times greater than the highest environmentally relevant concentrations, and still saw no toxicity or evidence of DNA damage, it seems to be a stretch to suggest that the presence in mixtures could result in effects.
R: The authors agree with the Associate Editor and have removed this statement.

In the manuscript, the authors presented the results of embrytoxicity testing of six cytotstatic anticancer drugs in fathead minnov larvae. Five commonly used cytostatics with different mode of action were tested. The study is interesting and highly relevant. The study was well performed and results are well presented.
An interesting and unusual finding was accelerated swim bladder development and inflation after the exposure to IF. Is in the literature any study of any compound or condition that would show such effect?
R: This was added to the main text (lines 183-184): "Impairment of swim bladder inflation was observed in fish exposed to dilbit for example (Alsaadi et al. 2017, Madison et al. 2017)". The references were also added to the reference list.