Combined toxicity of therapeutic pharmaceuticals to duckweed, *Lemna minor*

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Pharmaceuticals, which are designed to be biologically active at low concentrations, are found in surface waters, meaning aquatic organisms can be exposed to complex mixtures of pharmaceuticals. In this study, the adverse effects of four pharmaceuticals, 17α-ethynylestradiol (synthetic estrogen), methotrexate (anticancer drug), diclofenac (nonsteroidal anti-inflammatory drug) and fluoxetine (antidepressant), and their binary mixtures at mg/L concentrations were assessed using the 7-day *Lemna minor* test, with both apical and biochemical markers evaluated. The studied biochemical markers included chlorophyll a, chlorophyll b, carotenoids and oxidative stress enzymes catalase, glutathione-S-transferase and glutathione reductase, with effects compared to solvent controls. The adverse effects on *Lemna minor* were dose-dependent for frond number, surface area, relative chlorophyll content and activity of glutathione S-transferase for both individual pharmaceuticals and binary mixtures. According to the individual toxicity values, all tested pharmaceuticals can be considered as toxic or harmful to aquatic organisms, with methotrexate considered highly toxic. The most sensitive endpoints for the binary mixtures were photosynthetic pigments and frond surface area, with effects observed in the low mg/L concentration range. The concentration addition model and toxic unit approach gave similar mixture toxicity predictions, with binary mixtures of methotrexate and fluoxetine or methotrexate and 17α-ethynylestradiol exhibiting synergistic effects. In contrast, mixtures of diclofenac with fluoxetine, 17α-ethynylestradiol or methotrexate mostly showed additive effects. While low concentrations of methotrexate are expected in surface water, chronic ecotoxicological data for invertebrates and fish are lacking, but this is required to better assess the environmental risk of methotrexate.

1. Introduction

Over the last decade, the annual consumption of many “over the counter” drugs (e.g. common analgesics) and prescription medicines (e.g. antidepressants and anticancer drugs) has increased (e.g. Noordam et al., 2015; Wertli et al., 2017), raising concerns about their occurrence and potential adverse effects in the environment. For example, diclofenac (DCF), 17α-ethynylestradiol (EE2), fluoxetine (FLX) and methotrexate (MTX) have all been classified as high priority pharmaceuticals in Switzerland, meaning they should be monitored closely due to their high use and potential to cause harm to the aquatic environment (Perazzolo et al., 2010). Further, a national survey of Australian rivers found that pharmaceuticals were the most common trace organic contaminants present (Scott et al., 2014). Wastewater treatment plants (WWTPs) are considered the main pathway of therapeutic pharmaceuticals to the aquatic environment (Kay et al., 2017). Many WWTPs primarily treat municipal wastewater, but can also receive hospital wastewater (Ferrando-Climent et al., 2013; Cardenas et al., 2016). Due to the low degradation efficiency and poor removal in WWTPs, effluent discharged to the environment can contain both parent pharmaceuticals and metabolites (Fent et al., 2006).

Reported levels of DCF, a common nonsteroidal anti-inflammatory drug (NSAID), range from ng/L to μg/L levels in the aquatic environment (Sathishkumar et al., 2020). Similar levels have been observed over the last 10–30 years in sewage, wastewater effluent and rivers for synthetic estrogen EE2 (Ternes et al., 1999; Almeida et al., 2020), which has led to well-characterised adverse effects on fish and amphibian populations (Kidd et al., 2007; Tamschick et al., 2016; Matthiessen et al.,...
2. Materials and methods

2.1. Chemicals

The four pharmaceuticals selected in this study were of high purity grade (>90%) and supplied by Sigma-Aldrich Pty. Ltd. (Castle Hill, Australia). The physicochemical properties of the selected pharmaceuticals are given in Table S1 in the Supplementary Material. Other chemicals used for the preparation of stock solutions, culture media and biochemical marker assays were of analytical grade.

2.2. Lemma minor toxicity assays

Cultures of L. minor were obtained from a laboratory colony cultured at CSIRO facilities. L. minor was grown in Swedish Standard (SIS) Lemma spp growth medium adapted from the OECD (2006). Phytotoxicity was assessed using standard ecotoxicological endpoints, such as frond count and frond surface area, determined with the aid of ImageJ software (https://imagej.nih.gov/ij/). Additionally, biochemical markers, such as the amount of Chl a, Chl b and carotenoids, as well as the oxidative stress enzymes CAT, GST, and GR, were analysed (Section 2.3).

Assays were initially performed during 7-day exposures for single pharmaceuticals and then for binary mixtures of pharmaceuticals according to standardised protocols for Lemma spp. (OECD, 2006). Experiments were conducted under semi-static conditions with the test solution renewed on days 3 and 5 to compensate for the potential decrease in exposure concentrations as degradation of MTX and EE2 by photocatalysis and photolysis has been reported (Zuo et al., 2013; Bialk-Bielińska et al., 2017). Briefly, tests were performed at 24 ± 1 °C under continuous light (60–80 μmol photons s⁻¹ m⁻²) in 100 mL of test solution based on the OECD protocol (OECD, 2006). The initial frond number in each test vessel was 12 (one week old). The treatments included either single pharmaceuticals or binary mixtures of pharmaceuticals, with six replicates per test concentration, and a solvent control. The SIS growth medium was prepared 24 h before conducting the tests. The number of fronds was determined at days 0, 3, 5 and 7, while frond surface area, photosynthetic pigment content and oxidative stress enzymes were determined on day 7. The solvent control met the OECD test validity criteria, with a relative growth rate (RGR) of 0.275/d over the 7-day exposure. The pH, conductivity and dissolved oxygen were measured at the beginning of each test and on day 7. The health conditions (chlorosis, necrosis and yellowing) of the plants were also recorded regularly (data not shown). The morphological analysis included signs of necrosis, chlorosis or gibbosity, colony break-up or loss of buoyancy, and changes in root length and appearance.

To refine the concentrations used for the definitive tests, range finding tests were conducted for DCF, EE2, FLX and MTX. Stock solutions of all pharmaceuticals were prepared in methanol and the necessary volume of stock solution was added to the test vessels to obtain the desired concentrations. The solvent was left to evaporate, then SIS growth medium was added to the test beakers and mixed thoroughly using a Pasteur pipette. The nominal concentrations of single pharmaceuticals used in the definitive tests were 1, 2, 4, 8, 16 and 32 mg/L for DCF and EE2, 0.63, 1.25, 2.5, 5, 7.5 and 10 mg/L for FLX, and 2, 4, 8, 16, 32, 64 and 128 μg/L for MTX. The tested concentrations were not environmentally relevant concentrations as they were around one order of magnitude above the measured environmental concentrations (Scott et al., 2014; Roberts et al., 2016), but were chosen to obtain reliable dose-response curves for both apical endpoints and biochemical markers.

Binary mixtures of DCF + EE2, DCF + FLX, DCF + MTX, EE2 + FLX, EE2 + MTX and FLX + MTX were prepared using a fixed-ratio approach based on the growth inhibition (frond number) EC₅₀ values of the individual pharmaceuticals to L. minor measured in the current study. Concentrations selected for the binary mixture assay were 2 × EC₅₀, 1 × EC₅₀, 0.5 × EC₅₀, 0.25 × EC₅₀, 0.125 × EC₅₀ and 0.0625 ×
EC50 (n = 6) for all endpoints (Table S2). The concentrations were converted to molar units for mixture toxicity modelling.

2.3. Biochemical markers of effect

2.3.1. Photosynthetic pigments

Chlorophylls and carotenoids were extracted from L. minor fronds using 5 mL of 80% acetone in cold and dark conditions. The absorbance was measured 48 h later at 470 nm for carotenoids and 646 nm and 663 nm for Chl a and Chl b, with absorbance at 750 nm also measured as a correction factor (Lichtenhaler, 1987; Porra et al., 1989). Chlorophyll and carotenoids for each treatment were measured in triplicate using a Multiskan Ascent microplate reader (Thermo Scientific™) and expressed as a percentage relative to the controls.

2.3.2. Oxidative stress enzyme activity

The activity of three enzymes, CAT, GST and GR, considered as antioxidant enzymes, was determined. L. minor fronds were frozen immediately after the test was completed. They were subsequently homogenised in cold phosphate buffer (0.1 M, pH 7.4) at a ratio of 1:10 (tissue:buffer, w/v). The mixture was centrifuged at 10,000 g for 20 min at 4 °C to obtain the supernatant for enzyme analyses. The total protein content of the plant tissue was determined using the Bradford protein fitting software previously used by Kumar et al. (2010) and developed by Barnes et al. (2003). All statistical analyses were performed in SIGmaplot version 13 (Systat Software) unless stated otherwise. Prior to analysis, data were subjected to a Shapiro-Wilk test for normality and to a Bartlett’s test for homogeneity. Differences between treatments were evaluated using an ANOVA with a Tukey’s multiple comparisons test. All significant differences were determined at p < 0.05.

2.4. Data analysis

Effective concentrations (ECx) for individual pharmaceuticals and binary mixtures and their associated 95% confidence intervals were determined for growth inhibition (frond number and surface area), relative chlorophyll content and oxidative enzyme activity using model-fitting software previously used by Kumar et al. (2010) and developed by Barnes et al. (2003). All statistical analyses were performed in Sigmaplot version 13 (Systat Software) unless stated otherwise. Prior to analysis, data were subjected to a Shapiro–Wilk test for normality and to Bartlett’s test for homogeneity. Differences between treatments were evaluated using an ANOVA with a Tukey’s multiple comparisons test. All significant differences were determined at p < 0.05.

2.5. Mixture toxicity assessment

2.5.1. Toxic unit model

The TU model for assessing mixture toxicity is a robust and useful approach where TU is the sum of the adverse effects of each component in the mixture (Forget et al., 1999; Woods et al., 2002). The TU for a binary mixture is given in Eq. 1:

\[
TU = \frac{ECx_{mixture \ A}}{ECx_A} + \frac{ECx_{mixture \ B}}{ECx_B}
\]

where A and B are different components (pharmaceuticals), ECx mixture (A or B) is the concentration of each pharmaceutical in the binary mixture that exhibits either 20 or 50% effect and ECX (A or B) is the EC50 or EC90 of each pharmaceutical applied individually. While EC50 is commonly reported in the literature, we were also interested in low-level effects, so EC20 was also considered. The effect of the mixture is considered additive if the TU = 1. However, the effect of the mixture is interpreted as antagonistic (less than additive) if the TU > 1.2, while the effect is assumed to be synergistic (more than additive) if the TU < 0.8 (Broderius et al., 1995).

2.5.2. Concentration addition and independent action models

Two well-established models for predicting the toxicity of mixtures where the components do not interact are the CA (Loewe and Muischnek, 1926; Berenbaum, 1985) and IA models (Bliss, 1939). We applied the CA model, which assumes the mixture components are acting by the same mode of action, as described by Altenburger et al. (2004) (Eq. 2):

\[
ECx_{CA} = \sum_{i=1}^{n} p_i \frac{ECx_i}{ECx_i^{1-1}}
\]

where ECxCA is the effective concentration of the binary mixture predicted by the CA model resulting in an effect of x%, ECx is the concentration of the particular pharmaceutical i resulting in an effect of x% when tested alone, and pi is the proportion of component i (i = 1,...,n) in the mixture. The ECx values used in the CA model were determined from RGRs calculated according to OECD 221 Guideline (OECD, 2006) using the frond number as it is the primary measurement variable.

The accuracy of the CA model was evaluated using the model deviation ratio (MDR) approach (Belden et al., 2007). The MDR was derived by dividing the predicted effective concentration (EC20 and EC50) by the observed effective concentration of the mixture from the experiment for the same x% effect. MDR values less than 0.7 indicate an antagonistic interaction, while values greater than 1.3 indicate that the toxicity of the mixture complies with synergism (Phyu et al., 2011). Mixtures with MDR values between 0.7 and 1.3 are considered additive.

The IA model was also applied in the current study and assumes that the mixture components are acting by different modes of action (Bartels et al., 2012) (Eq. 3):

\[
E_{IA} = 1 - \prod_{i=1}^{n} (1 - E_i)
\]

where EIA is the effect predicted based on independent action and Ei is
the effect of each component in the binary mixture. Like CA, IA was lower ECx values in comparison to ECx values of single pharmaceuticals, and thus is expected to inhibit the division of new fronds. MTX is known to cause growth inhibition ECx values for the binary mixtures in molar concentration of MTX detected in surface water is 5 ng/L (Gouveia et al., 2019). Based on the EC50 value of MTX, which falls under chronic category 1, indicating it is very toxic to aquatic life. (Table 1). The lowest EC50 value was derived for MTX (0.02 mg/L), which falls under chronic category 1, indicating it is very toxic to aquatic life.

The detected values of MTX in hospital effluent can range from 2 to 4756 ng/L (Yin et al., 2010; Olalla et al., 2018), while the maximum concentration of MTX detected in surface water is 5 ng/L (Gouveia et al., 2019). Based on the EC20 value of 0.008 mg/L, MTX concentrations in surface water are unlikely to pose a risk to L. minor growth, though concentrations in hospital effluent are only 1.7 times lower than the EC20. Hospital effluents and WWTP effluents containing anticancer drugs (both parent compounds and transformation products) were found to inhibit reproduction at concentrations as low as 1.4% (v/v) raw wastewater in water flea Ceriodaphnia dubia (Isidori et al., 2016). The ECOTOX knowledgebase (US EPA, 2020) contains MTX toxicity data for fish (Danio rerio) and molluscs (Elliptio complanate), leaving a significant gap for the environmental risk assessment by regulators.

Białk-Bielińska et al. (2017) previously reported MTX EC50 values of 0.08–0.16 mg/L for L. minor, which is higher than the current study. The EC50 values reported in the study by Białk-Bielińska et al. (2017) were dependent on the frequency of renewal during the exposure, with the EC50 value two times lower under daily renewal conditions (0.08 mg/L) compared to static exposure (0.16 mg/L). The value reported in the current study was determined using a semi-static renewal test, with the test solution renewed on days 3 and 5. Białk-Bielińska et al. (2017) emphasised the importance of studying the toxicity of MTX degradation products due to its instability in test solutions. In this study, the degradation of MTX was not assessed, but rather tests were done under standardised conditions to support general comparisons of ECx values and establish mixture effects of pharmaceuticals with MTX.

The growth inhibition ECx values for the binary mixtures in molar units are provided in Table 2, with the ECx values in units of mg/L provided in Table S3. In general, binary mixtures with MTX exhibited lower ECx values in comparison to ECx values of single pharmaceuticals, excluding MTX (Tables 1 and 2). The intended therapeutic effect of MTX is to inhibit cellular proliferation, with a non-selective mode of action (Allwood et al., 2002), and to interfere with DNA synthesis, so it may be expected to inhibit the division of new fronds. MTX is known to cause strong phytotoxic and cytotoxic effects in other plants by inducing cell apoptosis (Lutterbeck et al., 2015).

The observed adverse effects on L. minor growth were similar when exposed to DCF or a mixture with DCF (DCF+EE2 and DCF+FLX) (Tables 1 and 2). However, growth inhibition in the presence either EE2 or FLX combined with DCF was reduced in comparison to the individual effects of EE2 or FLX alone, which may be caused by antagonistic interactions between these pharmaceuticals. This was investigated further using the TU approach.

### 3. Results and discussion

#### 3.1. Single pharmaceuticals and binary mixtures effects on growth inhibition

Based on their EC50 values, substances can be classified according to their toxicity to aquatic organisms as chronic category 1 (<1 mg/L), chronic category 2 (>1 to ≤10 mg/L) or chronic category 3 (>10 to ≤100 mg/L) (EC, 2008). With the exception of MTX, the individual pharmaceuticals measured in this study can be considered chronic category 2 or chronic category 3, meaning they are toxic or harmful to aquatic life (Table 1). The lowest EC50 value was derived for MTX (0.02 mg/L), which falls under chronic category 1, indicating it is very toxic to aquatic life.

### Table 2

<table>
<thead>
<tr>
<th>Mixtures</th>
<th>EC20 (M)</th>
<th>EC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCF + EE2</td>
<td>3.55 × 10^-5</td>
<td>5.03 × 10^-5</td>
</tr>
<tr>
<td>DCF + FLX</td>
<td>2.48 × 10^-3</td>
<td>4.80 × 10^-3</td>
</tr>
<tr>
<td>DCF + MTX</td>
<td>1.61 × 10^-5</td>
<td>3.43 × 10^-5</td>
</tr>
<tr>
<td>EE2 + FLX</td>
<td>7.94 × 10^-6</td>
<td>1.92 × 10^-5</td>
</tr>
<tr>
<td>EE2 + MTX</td>
<td>2.46 × 10^-6</td>
<td>6.61 × 10^-6</td>
</tr>
<tr>
<td>FLX + MTX</td>
<td>2.78 × 10^-6</td>
<td>6.23 × 10^-6</td>
</tr>
</tbody>
</table>

DCF, Diclofenac sodium; EE2-17α, ethynylestradiol; FLX, fluoxetine; MTX, methotrexate.

### Table 3

<table>
<thead>
<tr>
<th>Measurement variable</th>
<th>TU for EC20</th>
<th>TU for EC50</th>
<th>Type of joint action</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCF + EE2</td>
<td>2.48</td>
<td>1.39</td>
<td>antagonistic</td>
</tr>
<tr>
<td>DCF + FLX</td>
<td>1.89</td>
<td>1.32</td>
<td>antagonistic</td>
</tr>
<tr>
<td>DCF + MTX</td>
<td>1.57</td>
<td>1.29</td>
<td>antagonistic</td>
</tr>
<tr>
<td>EE2 + FLX</td>
<td>1.22</td>
<td>0.87</td>
<td>antagonistic</td>
</tr>
<tr>
<td>EE2 + MTX</td>
<td>0.64</td>
<td>0.54</td>
<td>synergistic</td>
</tr>
<tr>
<td>FLX + MTX</td>
<td>0.72</td>
<td>0.63</td>
<td>synergistic</td>
</tr>
</tbody>
</table>

DCF, diclofenac sodium; EE2-17α, ethynylestradiol; FLX, fluoxetine; MTX, methotrexate.

### Fig. 1

TU at EC50, for all binary mixtures for frond number, frond surface area and carotenoids. Green shading indicates the binary mixtures that were antagonistic, blue shading indicates binary mixtures showing additive effects and red shading indicates the binary mixtures that were synergistic. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

#### 3.2. Toxic unit approach for growth inhibition

The toxicological interactions of the four pharmaceuticals were...
The TU approach indicated a synergistic joint action for both frond number and frond surface area for the EE2 + MTX and FLX + MTX mixtures at EC50 (Fig. 1). While binary mixtures with MTX generally exhibited synergistic interactions for both frond number and frond surface area, this was not the case for DCF + MTX. The differing sensitivity of the frond number and surface area endpoints may explain the difference in the estimated joint action effect (e.g. antagonistic based on frond number and additive (EC50)/synergistic (EC50) based on surface area for the DCF + MTX mixture). The mixture toxicity ECx values for frond surface area (Table S4) were lower than the ECx values for frond number (Table S5). Therefore, the more sensitive ecologically relevant endpoint for mixtures with MTX was frond surface area (EC20 from 0.50 to 1.71 mg/L or 1.61 × 10⁻⁶ to 5.37 × 10⁻⁶ M). The single pharmaceutical ECx values for frond surface area are provided in Table S5.

The nature of the effect interactions also depended on the concentration of both pharmaceuticals, with the interaction found to change for some mixtures. For example, the EE2 + FLX mixture was antagonistic at EC20 (TU 1.35) and synergistic at EC50 (TU 0.74) for surface area. Further, the DCF + FLX mixture was antagonistic at EC20 (TU 1.27) and additive at EC50 (TU 0.92) for surface area (Table 3). The difference in mixture effects at EC20 and EC50 may be related to the slope of the dose-response curve as the TU model is based on point data, rather than the full dose-response curve.

### 3.3. Toxic unit approach for biochemical markers

Photosynthetic pigments Chl a, Chl b and carotenoids were the most sensitive endpoints for the EE2 + MTX mixture, with estimated EC50 values of 0.51, 0.52 and 0.52 mg/L, respectively (1.72 × 10⁻⁶, 1.75 × 10⁻⁶ and 1.75 × 10⁻⁶ M, respectively) (Table S6). A decrease in the relative content of Chl a, Chl b and carotenoids may be caused by the reduction in the biosynthesis of chlorophyll due to exposure to either of the pharmaceuticals in the mixture. The single pharmaceutical ECx values for Chl a, Chl b and carotenoids are provided in Table S7.

The activities of oxidative stress enzymes GST, GR and CAT were determined and a concentration-dependent increase in activity was observed only for GST when single pharmaceuticals (Table S8) and mixtures were tested (Tables S9 and S10). For both single pharmaceuticals and mixtures, the increase in activity was found to be statistically significant (p < 0.05) in most of the treatments compared to the controls. The highest recorded activity of GST was observed in response to MTX tested alone (0.11 ± 0.02 U/mg protein). GST helps protect plants exposed to toxic xenobiotics from oxidative injury by catalysing the formation of glutathione (GSH)-toxin conjugates (Cummins et al., 1999). GST also has a stress metabolism mechanism where it acts as a glutathione peroxidase that helps to reduce cytotoxic DNA and hydroperoxides (Dixon et al., 2002). A significant difference in CAT activity (p < 0.05) was observed between the control and treated plants at higher concentrations for the pharmaceuticals, both tested individually (Table S9) and as mixtures (Table S9). The highest activity of the CAT was recorded for mixtures EE2 + MTX (1078 ± 21 U/mg protein) and DCF+MTX (1432 ± 10 U/mg protein). MTX is known to be an inhibitor of the stomata opening (Aroca et al., 2006), and can hinder photosynthesis by an excess of produced electrons and reactive oxygen species in chloroplasts (Sofo et al., 2005). DCF alone has also been shown to increase CAT activity at 100 µg/L (Alkinim et al., 2019). Therefore, the observed changes in the GST and CAT activity can be associated with oxidative stress due to exposure to pharmaceuticals (Kummerova et al., 2016; Alkinim et al., 2020).

The TU approach based on biochemical markers established synergistic interactions for binary mixtures with MTX for Chl a, Chl b and carotenoids (Table 4, Fig. 1), with GST activity showing additive or antagonistic joint action. For example, the TUs for the FLX + MTX mixture at EC50 for Chl a, Chl b and carotenoids ranged from 0.44 to 0.48, while the TU for GST activity was 0.85. With an increase in the concentration of both pharmaceuticals, the type of joint action changed for all mixtures without MTX for Chl a, Chl b and carotenoids.

Comparison of binary mixture EC50 values based on growth inhibition (frond number) to EC50 values for other endpoints (surface area, chlorophyll content and oxidative stress) showed that the lowest toxicity values for each endpoint were observed when L. minor was exposed to mixtures containing MTX (Tables S3, S4, S6 and S10). When the EC20 values were compared, the sensitivity of all endpoints decreased in the order: surface area > Chl a, Chl b and carotenoids > frond number > GST activity. However, when the concentration of both pharmaceuticals in the mixture increased (EC50), the order of sensitivity changed: Chl a, Chl b and carotenoids > surface area > frond number > GST activity. Thus, photosynthesis is among the most sensitive endpoint for binary mixtures with the anticancer drug.

### 3.4. Predicted versus the observed toxicity values based on the relative growth rate

The experimentally determined ECx values of the binary mixtures based on RGR were compared with ECx toxicity values predicted by the CA and IA models (Fig. 2). The compliance with the CA model was...
assessed using the MDR approach (Table 5). Experimental ECx values in units of mg/L are also provided in Table S11.

CA was found to be the best fit for the DCF + EE2 and EE2 + FLX binary mixtures but slightly overestimated the toxicity of the DCF + FLX and DCF + MTX mixtures (Fig. 2). In the case of DCF + FLX and DCF + MTX, the IA model predictions were a closer fit to the experimental results. However, the MDR for these two binary mixtures were greater than 0.7, suggesting that the CA model is still appropriate. Neither CA or IA could predict the toxicity of the EE2 + MTX and FLX + MTX binary mixtures, with both models underestimating the effect. The MDR for both mixtures were greater than 1.3, suggesting synergistic effects. This also fits with the findings from the TU approach for frond number, surface area and the photosynthetic pigments.

Despite the pharmaceuticals having different modes of action, the MDR analysis indicates that CA was the most suitable model in most cases. CA is considered as a more conservative approach regardless of the pharmaceutical mode of action in the mixture (Backhaus and Faust, 2012) and is typically the first approach used to evaluate mixture toxicity (Belden et al., 2007; Evans et al., 2016). The CA model has also been reported as a model of higher predictive power in comparison to the IA model (Escher et al., 2017). Further, while true synergism is observed rarely for binary mixtures (Cedergreen, 2014), it is not expected to be relevant for environmentally relevant mixtures containing many chemicals at low concentrations.

4. Conclusions

The presence of complex mixtures of pharmaceuticals in surface water is a cause of concern for the aquatic environment. MTX is reported to be the second most abundant cytostatic in hospital effluent waters (4.8 μg/L) (Olalla et al., 2018) and is one of the pharmaceuticals of high priority for monitoring (Perazzolo et al., 2010) due to its potential harm to aquatic organisms. Based on the current study using L. minor, MTX can be considered as a highly toxic chemical. The most sensitive endpoint for MTX was the relative content of photosynthetic pigments, with the lowest toxicity values recorded for the EE2 + MTX binary mixture (EC50 values from 1.16 to 1.19 mg/L or 3.91 × 10^{-6} to 4.01 × 10^{-6} M). Both the EE2 + MTX and FLX + MTX binary mixtures showed synergistic interactions for most endpoints tested. However, it should be noted that the MDR analysis indicated additive joint toxicity for most mixtures despite the pharmaceuticals having different modes of action. Expected environmental concentrations for all tested pharmaceuticals are currently below the determined EC20 values, though MTX concentrations reported in hospital effluent are only around 2 times lower than the reported EC20 value. Consequently, the effects of mixtures and sensitivity of biochemical markers, such as photosynthesis, are important aspects to consider in future ecological risk assessments of pharmaceuticals, including anticancer drugs.

CRediT authorship contribution statement

Marijana Markovic: Formal analysis, Writing - original draft, Visualization. Peta Neale: Formal analysis, Writing - review & editing.
Visualization. Bhanu Nidumolu: Investigation, Writing - review & editing. Anu Kumar: Conceptualization, Writing - review & editing, Visualization, Supervision, Project administration.

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.2020.111428.

References
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