

RESEARCH ARTICLE



# Genotoxicity of selected pharmaceuticals, their binary mixtures, and varying environmental conditions – study with human adenocarcinoma cancer HT29 cell line

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## ABSTRACT

Pharmaceutical residues are present in the environment in mixtures and their adverse effects may also result from interactions that occur between compounds. Studies presented in this work focus on genotoxicity of pharmaceuticals from different therapeutic groups in mixtures and in individual solutions impacted with different environmental conditions assessed using comet assay (alkaline approach). Binary mixtures of pharmaceuticals (in different concentration ratios) and in individual solutions impacted with pH change (range from 5.5 to 8.5) or addition of inorganic ions, were incubated with HT29 cells and after 24 h time period cells were tested for the presence of DNA damage. To estimate whether mixtures act more (synergistic) or less (antagonistic) efficiently Concentrations Addition (CA) and Independent Action (IA) approaches were applied followed by a calculation of the Model Deviation Ratio (MDR) to determine deviation from the predicted values. Addition of inorganic ions mainly reduced their genotoxicity. Diclofenac s. was the most susceptible to potassium, fluoride, and bromide ions. Change of the pH of pharmaceutical solutions had significant impact on genotoxicity of diclofenac s. and fluoxetine h. Among mixtures, more commonly observed interactions were synergistic ones, exactly twenty-five cases (ten pairs containing chloramphenicol or oxytetracycline h.) and ten cases of antagonism (four for pairs containing chloramphenicol or fluoxetine h.). The results obtained indicate that interactions between tested compounds occur frequently and can lead to DNA damage. This topic especially concerning *in vitro* tests using cells is still rare, however, it should not be neglected.

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## KEYWORDS

Drugs residues; drugs mixtures; cytotoxicity; genotoxicity; MTT; comet assay




## 1. Introduction


Environmental samples may contain variety of harmful substances just to mention heavy metals, plasticizers, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), nanomaterials, and pharmaceuticals residues (Huang *et al.* 2014, Ávila and García 2015, Bocchi *et al.* 2016). The latter is introduced into the environment due to the increasing activity of pharmaceutical industry, hospitals and households, also as a result of using veterinary medicines, agriculture, aquaculture, and animal husbandry (Gaw *et al.* 2014, Li 2014, Sui *et al.* 2015).

Pharmaceutical residues in the environment create a threat of long-term exposure, in particular, because drugs rarely undergo full elimination in classic water treatment processes and are difficult to degrade, what contributes to their accumulation in ecosystems (Eggen *et al.* 2010). Many conducted studies have confirmed that drug residues can cause adverse effects in single and multicellular organisms for example non-steroidal anti-inflammatory drugs (NSAIDs) are particularly toxic to aquatic organisms such as algae and fish (Cuthbert *et al.* 2016, Zanuri *et al.* 2017). Presence of hormone-like compounds in the environment may lead to

endocrine disorders and reproductive issues in organisms, including mammals, birds or fish (Li 2014).

Until recently most of toxicological studies have concentrated mainly on determining the harmfulness of individual compounds, however, in reality, living organisms generally are exposed to mixtures of chemical compounds originating from e.g., environmental pollution, the effects of which (including effects at the DNA levels) are difficult to predict (Parasuraman 2011). In the literature, there are few examples of toxicity of drug and other compounds mixtures studies and such as those described in this publication regarding research on human cells are even rarer. Just to mention research conducted by Chen *et al.* (2015) (performed with mixtures of often used herbicides and insecticides) showed that they are much more toxic towards soil organisms than solutions of single compounds (Chen *et al.* 2015); also 17 $\alpha$ -ethinylestradiol commonly used in i.e., birth control pills in a mixture with androgen 17 $\beta$ -trenbolone (used in veterinary and illegally in doping) affects the process of differentiation of gonads in zebrafish and leads to sexual dysfunction in a significant way (Touber *et al.* 2007, Nasuhoglu *et al.* 2012, Örn *et al.* 2016). There are also few examples of studies,

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which show that drug mixtures present in the environment can be harmful to human cells, e.g., Pomati *et al.* (2006) observed in their studies that the drugs mixture of atenolol, bezafibrate, carbamazepine, ciprofloxacin, cyclophosphamide, furosemide hydrochlorothiazide, ibuprofen, lincomycin, ofloxacin, ranitidine salbutamol, and sulfamethoxazole (at the environmentally relevant exposure level) inhibited the growth of human embryonic cells (Pomati *et al.* 2006).

Determining the toxic impact of chemical mixtures in relation to individual compounds is a difficult, complex and often debatable subject, yet an accurate prediction of the overall toxicity is very important for environmental risk assessment and due to the fact that significant amounts of pharmaceuticals are now detected in drinking water, this knowledge also contributes to the estimation of the impact on human health (Caban *et al.* 2015).

In the literature phenomena of interactions between compounds in mixtures have been described long time ago; the definitions of synergy (interaction of two or more substances that mutually reinforce each other's actions) and antagonism (reduction of activity as a result of interactions between compounds) as well as models with which the type of these interactions can be determined are described (Fent *et al.* 2006, González-Pleiter *et al.* 2013, Wieczerzak *et al.* 2015, 2016b).

Concentration Addition (CA) and Independent Action (IA) models are commonly used in ecotoxicological studies, to calculate the predicted toxicity values of mixture based on the toxicity of individual pharmaceutical solutions. Both models are based on different assumptions about active sites and modes of actions (MOA), still, especially in ecotoxicological studies, this type of knowledge is frequently incomplete (Drescher and Boedeker 1995). CA models are more frequently applied than IA although the last one is more accurate (if all assumptions of the model are fulfilled, which sometimes is problematic), still, in most of the cases, differences are negligible (Belden *et al.* 2007).

Studies described in this paper aimed at estimation of genotoxicity of selected pharmaceuticals (which are present in environment at different concentration levels) towards HT29 cell line studied in binary mixtures of selected pharmaceuticals in individual solutions impacted with pH change or addition of inorganic ions (at environmentally stated levels). Information on physicochemical properties, medical applications, levels of concentrations found in the environment and toxicity of pharmaceuticals selected for studies are listed in Table 1.

In order to eliminate the risk of using cytotoxic concentration of pharmaceutical solutions in the genotoxicity studies, Thiazolyl Blue Tetrazolium Bromide (MTT) test was performed, which assesses cell metabolic activity. Concentrations determined in the MTT test were used in the next stage of genotoxic studies, which have been carried out with alkaline comet assay (to determine magnitude of DNA damage) (Koss-Mikołajczyk *et al.* 2015). In order to determine the nature of the phenomena observed the Model Deviation Ratio (MDR) was used, which compare predicted values (calculated using CA and IA) and experimental results (Wieczerzak *et al.* 2016a, 2016b, 2018b, Szczepańska *et al.* 2018).

Application of such research methodology gives not only information on the cytotoxic concentrations of the studied drugs but also allows for the assessment of observable interactions occurring in the mixture of selected drugs and the influence of environmental conditions such as coexistence of inorganic ions and pH changes in pharmaceutical solutions to their genotoxicity towards cells. Studies presented in this publication are a continuation of research on the impact of pharmaceutical mixtures and environmental conditions on single and multicellular organisms and supplement the knowledge obtained in earlier studies on *Vibrio fischeri*, *Sorghum bicolor* and *Saccharomyces cerevisiae* (Wieczerzak *et al.* 2016a, 2016b, 2018a, 2018b).

## 2. Experimental

### 2.1. Chemicals, reagents, and apparatus

#### 2.1.1. Model compounds and ions

Model substances selected for the study: diclofenac sodium salt (diclofenac s.) (CAS no. 153907-79-6), chloramphenicol (CAS no. 56-75-7), oxytetracycline hydrochloride (oxytetracycline h.) (CAS no. 2058-46-0), fluoxetine hydrochloride (fluoxetine h.) (CAS no. 56296-78-7), estrone (CAS no. 53-16-7), ketoprofen (CAS no. 22071-15-4), progesterone (CAS no. 57-83-0), gemfibrozil (CAS no. 25812-30-0), and androstenedione (CAS no. 63-05-8) were purchased from Sigma Aldrich (Germany) and were of analytical purity grade (>99%). Diazepam (CAS no. 439-14-5, purity grade >99%) was purchased from LGC Standards (UK). Inorganic ions in the salt form (KCl (CAS no. 7447-40-7), NH<sub>4</sub>Cl (CAS no. 12125-02-9), NaF (CAS no. 7681-49-4), NaBr (CAS no. 7647-15-6)) were purchased from Avantor Performance Materials S.A. (Poland) while NaCl (CAS no. 7647-14-5) from Sigma Aldrich (Germany). pH values were adjusted with concentrated solutions of NaOH (CAS no. 1310-73-2) or HCl (CAS no. 7647-01-0) purchased from Avantor Performance Materials S.A. (Poland). All purchased ions were analytical purity grade >99%.

#### 2.1.2. MTT test and comet assay reagents

MTT (CAS no. 298-93-1, biological grade), fetal bovine serum (FBS), penicillin, and streptomycin solution, McCoy's 5a (Modified) Medium, DMSO (CAS no. 67-68-5, biological grade), H<sub>2</sub>O<sub>2</sub> (CAS no. 21-67-63), N<sub>2</sub>EDTA (CAS no. 6381-92-6, biological grade), trypsin-EDTA solution (sterile-filtered), NaCl (CAS no. 7647-14-5), NaOH (CAS no. 1310-73-2), Trizma<sup>®</sup>-base (CAS no. 77-86-1, biological grade), Trizma<sup>®</sup> hydrochloride (CAS no.1185-53-1, biological grade), Triton<sup>™</sup> X-100 (CAS no. 92046-34-9, biological grade), low (LMP) and normal melting (NMP) points agarose, trypsin-EDTA and SYBR<sup>®</sup> Green I nucleic acid gel stain were purchased from Sigma-Aldrich (Germany). Sterile serological pipette (5, 10 and 25 ml), cell cultures bottles, UltraFine<sup>™</sup> tips, coverslips, microscope slides, 10-ml syringes, sterile centrifuge tubes, and filters were purchased from VWR (Poland). All reagents were of analytical grade purity or better, in the case of reagents for microbiological purposes. Water was purified with a Milli-Q<sup>®</sup> system from Millipore (Germany).

Table 1. List of pharmaceutical's properties selected for testing (Blystone et al. 2011, van Leeuwen et al. 2012, Chae et al. 2015, Huebner et al. 2015, Hurst et al. 2015, Liu et al. 2015, Petrie et al. 2015, Torres et al. 2015, pageorgiou et al. 2016, Syed et al. 2016, Tahrani et al. 2016, Wu et al. 2016, Wiczerzak et al. 2016a, Aydin2017, Barreto et al. 2017, Charles et al. 2017, Ebele et al. 2017, Fu et al. 2017, Ogueji et al. 2017, Ren et al. 2017, Warkus and Marikawa 2018).

Analyte/CAS no.	M <sub>w</sub> /logP	Medical application	Action at the cellular level	Concentrations detected in environment	Observed toxicity towards cells/ single or multicellular organisms
Diclofenac sodium/15307-76-6	318.13/0.6	Nonsteroidal anti-inflammatory drug.	A potent inhibitor of COX-2, which reduces the synthesis of prostaglandins, prostacyclin and thromboxane-compounds associated with inflammation.	2900 and 2100 ng/L lake and river water <sup>a</sup> 2100 ng/L effluent wastewater <sup>a</sup> 1200 surface water <sup>a</sup> 1200 surface water <sup>a</sup>	Teratogenic to <i>Xenopus</i> embryos <sup>a</sup> . Inhibition of oxidative phosphorylation (ATP synthesis) in rats <sup>a</sup> .
Ketoprofen/22071-15-4	254.28/3.12	Nonsteroidal anti-inflammatory drug.	Reversibly inhibits COX-1 and COX-2 enzymes, which reduces the production of prostaglandins.	128–184 ng/L influent wastewater <sup>a</sup> 7–57 ng/g sediment and sewage	Mitochondrial toxicity in yeast <i>Saccharomyces cerevisiae</i> Toxic for embryonic stages of zebrafish Carcinogenic in B6C3F1 mice.
Androstenedione/63-05-8	286.41/2.75	Male and female sex hormone precursor: testosterone, estrone and estradiol.	Acts as a weak androgen receptor agonist.	5 ng/L surface water 0.1 ng/g sediment	
Progesterone/57-83-0	314.46/3.87	Female steroid hormone, used to complement the decrease of naturally occurring progesterone to prevent miscarriage, it prevents menstrual cycle disorders.	Genomic pathway: Regulates expression of specific genes by binding to specific nuclear receptors (PRA and PRB) upon entry into the cell. Non-genomic pathway: Induces rapid growth of intracellular Ca <sup>2+</sup> concentrations, and regulates the flow of Na <sup>+</sup> and Cl <sup>-</sup> ions. Estron is synthesized by the aromatase complex that converts androstenedione to estrogens. It has been shown that rats in aortic tissues estrene induce rapid non-genomic stimulation of NOS and COX activity and inhibit platelet aggregation. Prevents chain lengthening by inhibiting peptidyltransferase activity of bacterial ribosomes	2.4 ng/g sediment 26 ng/L surface water	Apoptosis of insulin-secreting cells.
Estrone/53-16-7	270.37/3.13	Steroid estrogen with estradiol-like effects.		<1–5 ng/L surface water 65 ng/L river water 28 ng/L surface water	No data.
Chloramphenicol/56-75-7	323.13/1.14	Antibacterial and bacteriostatic agent.		5800–47400 ng/L sediment and sewage 3000 ng/L wastewater	Cytotoxic to myeloma cells.
Oxytetracycline h.779-57-2	460.43/-0.9	Natural antibiotic with bacteriostatic and antiallergic effect.	Inhibits protein synthesis by blocking the aminoacyl-tRNA binding at site A on the bacterial ribosome. This prevents the introduction of new amino acids into the growing peptide chain. Mammalian cells are less susceptible to tetracycline, despite the fact that tetracycline binds to prokaryotic and eukaryotic ribosomal subunits (30S and 40S sites respectively). Benzodiazepines are positive allosteric modulators of type A GABA receptors. GABA receptors are selective ion channels for Cl <sup>-</sup> ions. Binding of benzodiazepines to the GABA receptor complex increases the chloride ions' conductivity through the membrane of neuronal cells. Diazepam seems to work in the limbic, hippocampal and hypothalamic areas. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI).	200–5700 ng/L sediment and sewage <sup>c</sup> 564.30 ng/L effluent and 337.81 ng/L influent wastewater	Inhibition of expression of phase I and II detoxification genes in swimming crab ( <i>Portunus trituberculatus</i> ) larvae <sup>b</sup> . Acute toxicity to <i>Pseudokirchneriella subcapitata</i> <sup>b</sup> .
Diazepam/439-14-5	284.74/2.82	Psychotropic drug, anticonvulsant, sedative and anxiolytic.		335 ng/L surface water LOD=0.21 ng/L surface water	Inhibits proliferation and activation of T lymphocytes isolated from rat main lymphoid organ. Oxidative damage in the liver and gill tissues catfish <i>Clarias gariepinus</i> .
Fluoxetine h./56296-78-7	345.79/1.8	Used mainly in the treatment of depressive and obsessive-compulsive disorders.		1.7 to 1244 ng/L effluent waste water <sup>b</sup> LOD=2.7 ng/L surface water <sup>b</sup>	Induce an early necrosis in human peripheral blood lymphocytes and Jurkat cells <sup>c</sup> . Inhibition of canonical Wnt signaling and reduction of cellular proliferation <sup>c</sup> . Genotoxic to gilthead seabream ( <i>Sparus aurata</i> ).
Gemfibrozil/25812-30-0	250.33/4.77	It normalizes blood fats, lowers LDL cholesterol and triglycerides, improves HDL cholesterol.	An α-receptor (PPAR) activator, a nuclear receptor that participates in the metabolism of carbohydrates and fats, as well as the differentiation of adipose tissue.	6 ng/g sediment and sewage > 1000 ng/L STP effluents	

LOD: limit of detection; <sup>a</sup>results obtained for diclofenac; <sup>b</sup>results obtained for fluoxetine; <sup>c</sup>results obtained for oxytetracycline.

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**Table 2.** Main concentration values of pharmaceuticals solution used in MTT test and the concentrations selected for the comet test.

Analyte	Stock concentration of solution [mol/L]	Concentration (C2) of pharmaceutical solutions selected for further studies with comet assay [mol/L]
Diclofenac s.	0.0064	0.00066
Oxytetracycline h.	0.0047	0.00021
Chloramphenicol	0.00058	0.00006
Fluoxetine h.	0.0008	0.00045
Analyte	Stock concentration of solution [mol/L]	Maximal tested concentration of pharmaceutical solutions with effect $\pm$ SD
Ketoprofen	0.00295	Concentration could not be calculated (max. tested conc. 0.00074 mol/L tested with effect 99.0 $\pm$ 1.3%)
Gemfibrozil	0.0006	Concentration could not be calculated (max. tested conc. 0.00015 mol/L tested with effect 112.0 $\pm$ 1.3%)
Estrone	0.00094	Concentration could not be calculated (max. tested conc. 0.00024 mol/L tested with effect 78.0 $\pm$ 2.6%)
Diazepam	0.00088	Concentration could not be calculated (max. tested conc. 0.00022 mol/L tested with effect 94.4 $\pm$ 9.0%)
Androstenedione	0.0008	Concentration could not be calculated (max. tested conc. 0.0002 mol/L tested with effect 123.5 $\pm$ 1.0%)
Progesterone	0.0008	Concentration could not be calculated (max. tested conc. 0.0002 mol/L tested with effect 111.6 $\pm$ 1.0%)

### 2.1.3. Cell lines

HT29 (human colon adenocarcinoma, obtained from American Type Culture Collection, Manassas, USA) were grown in a monolayer culture at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in the Smart cell incubator (Heal Force, China) in McCoy's 5a (modified) medium, supplemented with 10% FBS and antibiotic (1% penicillin-streptomycin) in a culture flask. The medium was changed twice a week. Single cell suspensions were prepared with a trypsin-EDTA solution (diluted ten times) and finally re-suspended in McCoy's 5a (modified) medium, supplemented with serum and antibiotics (all activities took place in aseptic environment). Cellular studies were conducted due to courtesy of Professor Agnieszka Bartoszek from the Department of Chemistry, Technology, and Biotechnology of Food at Gdańsk University of Technology.

## 2.2. Methods

### 2.2.1. MTT

MTT test was performed for individual solution of pharmaceuticals mainly to avoid using concentration of pharmaceuticals which could prove to be cytotoxic during the comet assay. The cells were seeded in 96-well tissue culture plates (about  $20 \times 10^3$  cells/well in 150  $\mu$ L) and allowed to settle for 24 h at 37 °C after this period cells were treated for 24 h with 50  $\mu$ L of different concentrations of pharmaceutical solution in distilled waters. Due to the fact that some of the studied compounds were found to react with PBS, this buffer either in the MTT test or in the comet assay was not used, instead purified water was used as control or in dilutions. After 24 h of the incubation time, 50  $\mu$ L of MTT solution (4 g/L) was added to each well in the plate and cells were left for another 3 h at 37 °C (up to this point all operations were performed under aseptic conditions). Finally, the medium was carefully removed from wells, and formazan crystals formed

were dissolved in 50  $\mu$ L of DMSO added to each well. The absorption of the resultant solutions was determined at 540 nm wavelength with TECAN Infinite M200 plate reader (Tecan Group Ltd., Switzerland) (Koss-Mikołajczyk *et al.* 2015). The cytotoxicity was expressed as growth inhibition of cells exposed on tested plant samples compared to control of non-treated cells whose growth was regarded as 100%. Each dilution of pharmaceuticals was studied in triplicates on two plates in separate repetition performed at weekly intervals. Concentrations of pharmaceutical solutions studied in the MTT test ranged from 1 to 25% (v/v) of stock concentration listed in Table 2. Drugs concentration values to perform comet assay studies were selected as 30% cytotoxicity decrement in relation to the control if the cytotoxicity of studied solution was significant (refer to Table 2). The data were analyzed using Q-Dixon test to eliminate major errors followed by *t*-Student comparison test. The statistical significance was set to \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and are presented in the supplementary materials (Supplementary Figures 1–2).

### 2.2.2. Comet assay

Epithelial colon cancer cells were grown in a monolayer culture at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in McCoy's 5a (modified) medium, supplemented with 10% fetal bovine serum and antibiotic (1% penicillin-streptomycin) in a culture flask. The medium was changed twice a week. Single cell suspensions were prepared with a trypsin-EDTA solution (diluted 10 times) and finally re-suspended in McCoy's 5a (modified) medium, supplemented with serum and antibiotics.

The cells from the culture flask were seeded in 24-well tissue culture plates (about  $200 \times 10^3$  cells/well in 1800  $\mu$ L) and allowed to settle for 24 h at 37 °C, after this period cells were treated for 24 h with 200  $\mu$ L of different concentrations of pharmaceutical solutions diluted in deionized water (different concentrations of pharmaceuticals solution were used at

different stages of the study, which will be reported when discussing the individual results) each sample was performed in three repetitions.

After 24 h incubation period medium from cell surface was carefully removed and cells were trypsinized (200  $\mu$ L of trypsin was added to each well) in order to detach the cells from well wall. Afterward to the suspension of the cells in each well 1800  $\mu$ L of fresh McCoy's medium was added, then the cell suspension was pipetted and 1 mL was transferred to 1.5 mL volume vortex tubes (up to this point of procedure all operations were performed under aseptic conditions). Vortex tubes were centrifuged (1000 rpm, 5 min, 4  $^{\circ}$ C) the supernatant was removed, and then 1 mL of PBS was added to the tubes and centrifuged again. The supernatant was removed again in such a way to leave about 100  $\mu$ L of buffer. Cell suspensions were pipetted and 50  $\mu$ L of each suspension was mixed with 150  $\mu$ L of 0.5% low melting point (LMP) agarose in PBS maintained at 37  $^{\circ}$ C. 40  $\mu$ L agarose with cells was transferred two times on glass microscope slides pre-coated with 1% normal melting point (NMP) agarose. Gels were covered with a coverslip and kept on ice for 5 min. Two gels per glass slide were prepared. For the purpose of this study, protocol for the exposure of slides, which was previously described by (Lah *et al.* 2005) and later developed by (Durgo *et al.* 2009, Mihaljevic *et al.* 2011) was adopted. Following the treatment the cells subjected to lysis solution (2.5 mol/L NaCl, 0.1 mol/L Na<sub>2</sub>EDTA, 10 mmol/L Trizma<sup>®</sup>-base, pH 10 and 10% Triton X-100, min. 1 h of incubation, 4  $^{\circ}$ C, in the darkness). The comet assay was performed under alkaline conditions (pH >13.3) (Fairbairn *et al.* 1995), as described in (Kazimirova *et al.* 2012) with some modifications. After lysis, slides were placed in a horizontal electrophoresis tank and DNA was allowed to unwind for 20 min in electrophoretic buffer (0.3 mol/L NaOH, 1 mmol/L Na<sub>2</sub>EDTA, pH >13.3, 4  $^{\circ}$ C) before electrophoresis was performed for 20 min at 26 V (300 mA). After neutralization by washing gels three times, each for 5 min with 0.4 mol/L Trizma<sup>®</sup>-base (pH 7.5) at 4  $^{\circ}$ C, and then with water and dried at room temperature. After staining the slides with SYBR GREEN I solution, the comets were detected and quantified as described below.

For quantitative analysis of nuclear DNA damage, the slides were viewed at 50 $\times$  magnification with an epifluorescence microscope Zeiss Imager Z2. Microscopic images of comets were taken with digital camera (CoolCube1) connected to computer, and the comets were scored using Metafer 4 Software. The data were analyzed by Q-Dixon test to eliminate major errors and were expressed as the mean values with the standard deviations (means  $\pm$  SD) of the three independent experiments and were used as numeric values for model studies (Kudlak *et al.* 2016). In order to systematize the research sequence, the test procedure is schematically shown in Figure 1.

The concentration levels of the model compounds solutions (diclofenac s., oxytetracycline h., chloramphenicol, and fluoxetine h., refer to Figure 2) calculated based on results of the MTT test, are summarized in Table 2. Concentrations of inorganic ions selected for genotoxicity studies correspond to the environmental concentrations and were selected based on previous research on surface water samples collected from waters bodies receiving waters from sewage treatment plants in Poland i.e., Na<sup>+</sup> C = 1.09 mmol/L, K<sup>+</sup> C = 0.13 mmol/

L, NH<sub>4</sub><sup>+</sup> C = 0.042 mmol/L, Cl<sup>-</sup> C = 1.41 mmol/L, F<sup>-</sup> C = 0.022 mmol/L, Br<sup>-</sup> C = 0.013 mmol/L (Kudlak *et al.* 2016).

### 2.3. Calculation of MDR values

In order to determine whether the genotoxicity towards HT29 (if so: how and to what extent) depends on the concentration of compounds in the binary mixture, each of the compounds was tested at three concentrations levels. Concentrations (C2) determined during the MTT test (exactly 30% of the toxic concentration) was reduced by 50% (C1) and increased by 50% (C3). The binary mixtures were constructed in such a way that C1 concentration of first substance as mix with C1, C2 or C3 concentration of second substance and subsequently C2 concentration of first substance with C1, C2 or C3 concentration of second substances etc. All concentrations of studied compounds are summarized in Table 2.

In this study, the combined toxicological effect of a mixture of pharmaceuticals and the impact of environmental conditions toward HT29 cells was assessed both with the CA and IA models using Equations 1 and 2, respectively (Kienzler *et al.* 2016):

$$EC_{X_{mix}} = \left( \sum_{i=1}^n \frac{p_i}{EC_{X_i}} \right)^{-1} \quad (1)$$

where:

- $EC_{X_{mix}}$  is the  $x_{mix}$  effect cause by total concentration of the mixture of studied chemicals (components) (Expected value).
- $p_i$  indicates the fraction of component  $i$  in the mixture, calculated on the basis of the concentration of  $i$  component in the mixture;
- $n$  indicates the number of components in the mixture;
- $EC_{X_i}$  indicates the  $x_i$  effect caused by component  $i$  at given studied concentration in the mixture,

$$E(C_{mix}) = 1 - \prod_{i=1}^n (1 - E(c_i)) \quad (2)$$

where:

- $EC_{mix}$  is overall effect expressed as a fraction of maximal possible effect of a mixture of  $i$  chemical, (Expected value).
- $c_i$  indicates the concentration of component  $i$  in the mixture;
- $n$  indicates the number of components in the mixture;
- $E(c_i)$  indicates the effect of component  $i$ , applied separately.

In order to verify the difference between the predicted and observed effect, the Model Deviation Ratio (MDR) approach was applied, defined as shown in the Equation (3) (Kudlak *et al.* 2016, Wiczerzak *et al.* 2016b):

$$MDR = \frac{\text{expected}}{\text{Observed}} \quad (3)$$

where:

- Expected is the effective toxicity (raw values of genotoxicity calculated according to Equation (1) or (2) of the mixture predicted by the CA or IA model,

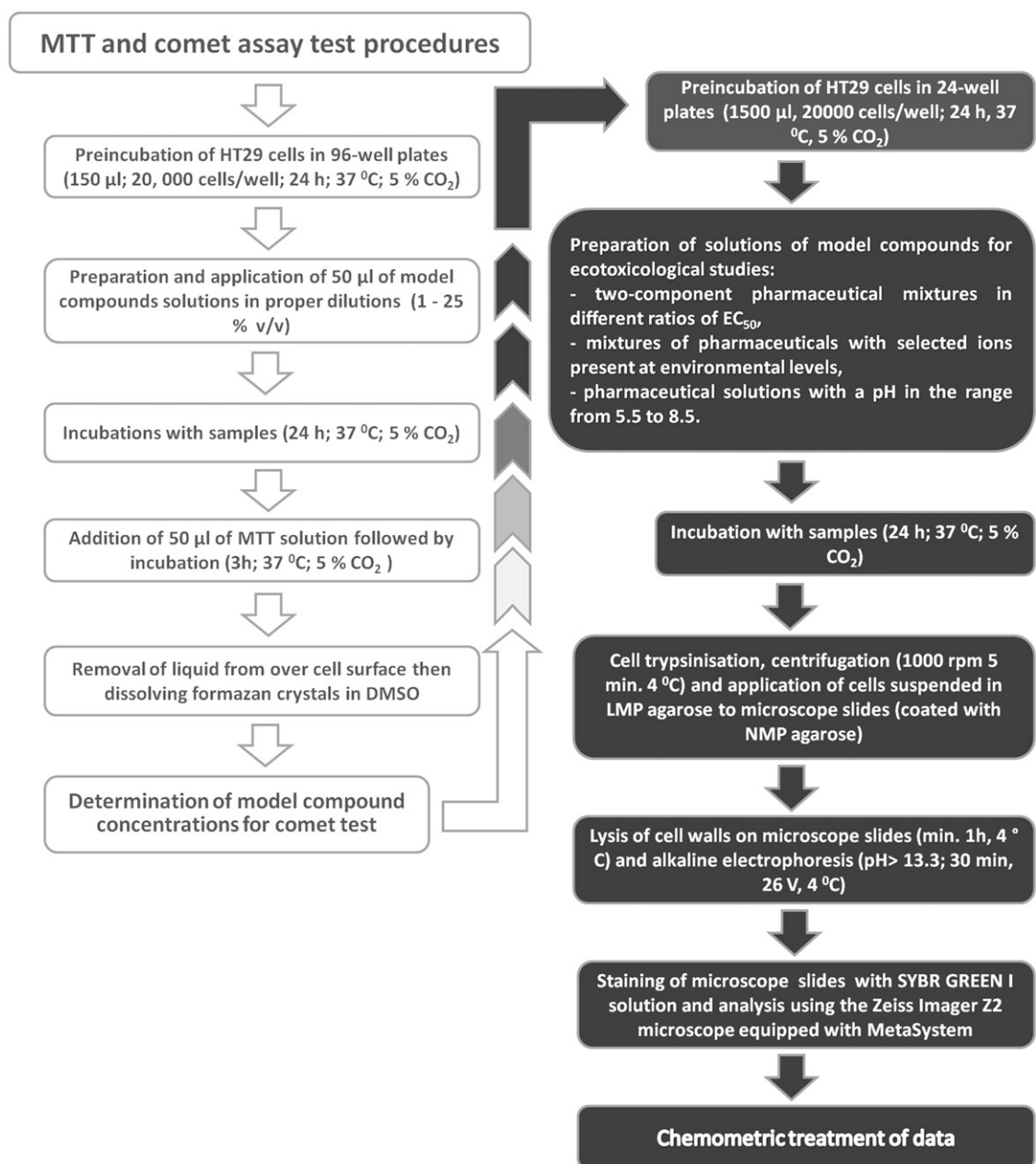


Figure 1. Schematic representation of test procedure.

- Observed is the effective toxicity (raw values of genotoxicity calculated) for the mixture obtained during the toxicity studies.

In the case of studies on impact of pH change pharmaceuticals solutions, use of CA and IA modeling methods was impossible due to the fact that these solutions cannot be treated as a mixture of chemicals. Therefore, in this case, a simple ratio was used (described by the Equation (4.)) to assess impact of pH change:

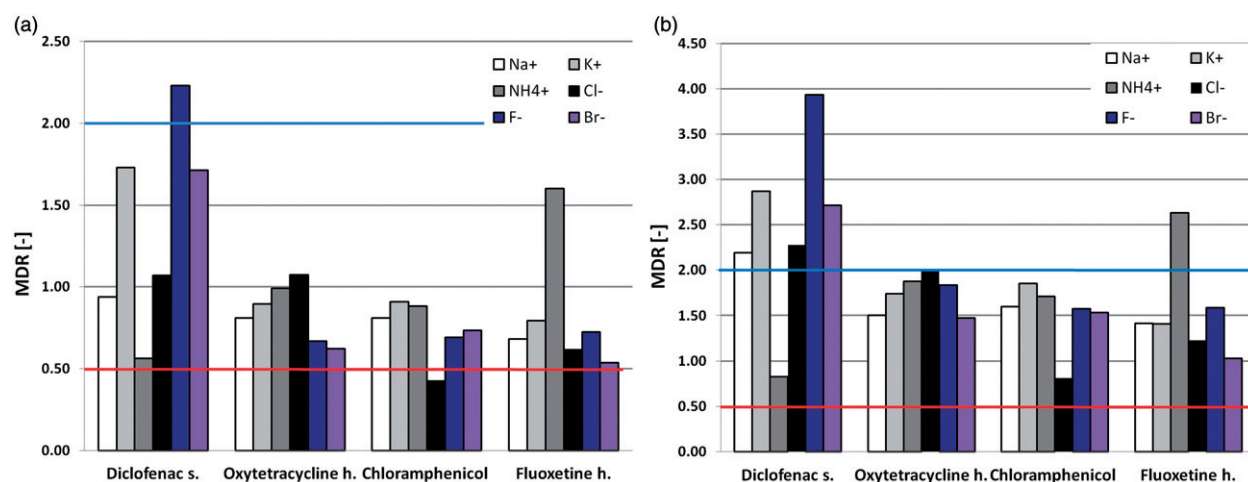
$$\text{Effect ratio} = \frac{\text{Effect observed for pharmaceuticals solutions with corrected pH}}{\text{Effect observed for pharmaceuticals solutions without pH adjustment}} \quad (4)$$

Solutions of pharmaceuticals with set pH for which the value of the ratio was lower than one were less toxic and those with values above one were more toxic toward HT29 cells.

### 3. Results and discussion

#### 3.1. Impact of pharmaceuticals solutions to cytotoxicity towards HT29 cell line

Diclofenac s., oxytetracycline h., chloramphenicol, and fluoxetine h. have been shown to be significantly cytotoxic to HT29 cells. The most cytotoxic turned out to be chloramphenicol, which decreased the mitochondrial activity of cells by half at 0.088 mmol/L concentration (concentrations selected for further genotoxicity studies are summarized in Table 2). Other studies carried out by Parolini *et al.* (2011) at different cell from the zebra mussel *Dreissena polymorpha* (haemocytes, gills and digestive gland cells) showed that among the tested drugs (diclofenac, atenolol, carbamazepine) – diclofenac turned out to be very cytotoxic in the Trypan Blue Exclusion test and only slightly cytotoxic in the MTT test



**Figure 2.** MDR values variations for CA 2a) and IA 2b) toxicity modeling for selected pharmaceuticals depending on co-presence of anions and cations at environmentally relevant levels.

(in the concentration range up to 10 mg/L), the second most toxic turned out to be gemfibrozil, which shows to be cytotoxic to HT29 cells (Parolini *et al.* 2011). In case of drugs: ketoprofen, gemfibrozil, estrone, diazepam, androstenedione, and progesterone no significant cytotoxic or genotoxic effect was observed at concentrations levels that exceed solubility equilibrium, therefore, no further studies with these substances were conducted. Maximum reduction of mitochondrial activity for described above substances was observed for estrone it was 78% at the concentration 0.24 mmol/L in relation to the control if the cytotoxicity of studied solution was significant (maximum effects obtained in the MTT test for the highest concentrations of discussed substances studied are summarized in Table 2). Raw results obtained for MTT cytotoxicity of solutions of pharmaceuticals are presented in supplementary materials: supplementary Figures 1–2.

### 3.2. Genotoxicity studies

In this article, mixtures of pharmaceuticals at various concentration levels were tested on HT29 cells for the first time and CA and IA modeling was used to evaluate the results obtained and compare them with controls with individual compounds solutions. The application of the CA and IA models alone does not allow for the determination of possible interactions between chemicals (in this case in pharmaceuticals) in mixture and define their character. The deviation from the predicted effect of CA and IA mode may be evidence for occurrence of synergistic or antagonistic action. The mixtures with  $MDR > 2.0$  exhibit a high probability of antagonism while those with values below 0.5 show synergistic character (Faust *et al.* 2000, Backhaus and Faust 2012, Kienzler *et al.* 2016, Wiczerzak *et al.* 2016b). In current research it was arbitrarily assumed that MDR falling within 0.50–0.71 and 1.40–2.00 justify the conclusion on, respectively, possible under- and overestimation of presented models.

#### 3.2.1. Impact of inorganic ions on genotoxicity towards HT29 cell line of selected compounds

In case of impact of ion addition on toxicity of diclofenac s. one can notice a reduction in genotoxicity practically for all

ions (see Figure 2 to compare), the highest MDR values were observed for bromide ions (for the CA model they were above 2.0 and for the IA model above 3.5 which indicates strong antagonism). The exceptions were ammonium ions, which clearly affected toxicity of diclofenac s., MDR values for this mixture were bit above 0.5. The MDR parameter for oxytetracycline h. varied depending on the model used, for the CA model only two values were in the overestimated range (fluorides and bromides), and for the IA model all values and MDR were in the underestimated range (chloride ions were closest to 2.0).

In the case of chloramphenicol, only chloride ions significantly influenced its genotoxicity, resulting in an increase in the CA model, the MDR value decreased below 0.5 which means synergistic activity was observed (see Table 2). Surprising behavior is observable in case of studies on fluoxetine h.; ammonium ions show antagonistic impact being, most probably, result of shifting ion equilibrium. It is proven by fact that all anions seem to show synergistic impact on toxicity of fluoxetine h. (with CA model studies).

In earlier conducted by the authors test on *Vibrio fischeri* bacteria, the opposite values of the MDR parameter were observed, the addition of ions rather contributed to the increase of acute toxicity of the studied drugs, it may result from various cell structure, and other defense and repair mechanisms, however without additional testing it is only conjecture (Wiczerzak *et al.* 2018a). In the case of previously tested yeast *Saccharomyces cerevisiae* (also by the authors), ions had no significant effect on the endocrine potential tested in the XenoScreen YES/YAS test, the only compound susceptible to both anions and cations was fluoxetine h., ions present in its solution contributed to the increase of its agonistic properties against estrogen receptors (Wiczerzak *et al.* 2018a). Raw results obtained for genotoxicity of solutions of pharmaceuticals impacted with ions addition are presented (as % of DNA in the comet tail  $\pm$  SD) in supplementary materials: Supplementary Table 1.

#### 3.2.2. Impact of pH change on genotoxicity of solution of selected pharmaceuticals towards HT29 cell line

Synergic impact of pH change is noticeable in case of diclofenac, significant (>90%) increase of this drug's toxicity

took place at pH of 6.50 (refer to Figure 3). In case of oxytetracycline, the synergy was observed in low and high pH values, namely at 5.50, 8.00, and 8.50 (increment by over 24, 46 and 77%, respectively). Chloramphenicol is not synergistically impacted by pH variations, only antagonistic influence was detected at 6.00–7.00 pH range. Low pH values act in a synergic way on genotoxicity fluoxetine (84 and 116% increase of DNA damage at 5.50 and 6.00, respectively), some tendency of antagonistic behavior is observed at pH  $\geq 8.00$ . Raw results of genotoxicity of pharmaceuticals solution with ions and pharmaceuticals solution with pH adjustment (presented as % of DNA in the comet tail  $\pm$  SD) are summarized Supplementary Table 1.

Studies on the pH change of pharmaceutical solutions regarding both *Vibrio fischeri* and *Saccharomyces cerevisiae* did not show any effect on the test organisms, only oxytetracycline h. has a slight antagonistic effect on estrogen receptors under the influence of pH change to 8.5. HT29 cells seem to be more sensitive to pharmaceutical solutions that have changed pH (Wieczerzak *et al.* 2018a). Raw results obtained for genotoxicity of solutions of pharmaceuticals impacted with pH change are presented (as % of DNA in the comet tail  $\pm$  SD) in supplementary materials: Supplementary Table 1.

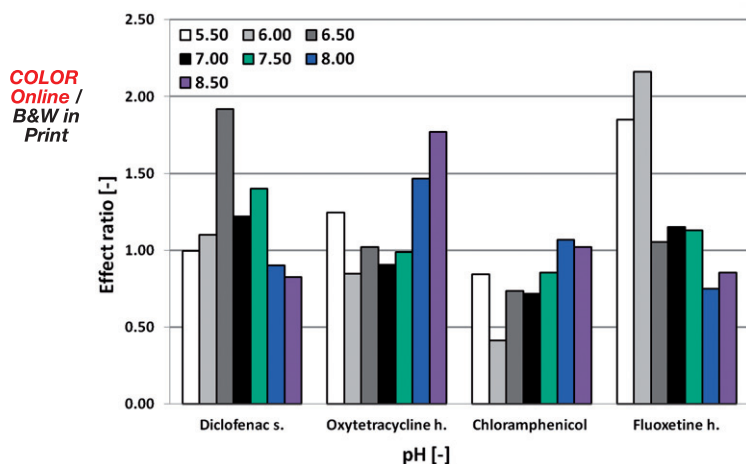


Figure 3. Toxicity modeling for selected pharmaceuticals depending on pH change.

Table 3. MDR values variations for CA and IA for mixtures of selected drugs at three concentration levels for each tested compounds (color coding: red: synergy; blue: antagonism; green: overestimation; yellow: underestimation).

	C [mmol/L]	Diclofenac s.						Oxytetracycline h.						Chloramphenicol						Fluoxetine h.					
		CA		IA		CA		IA		CA		IA		CA		IA		CA		IA					
		C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2				
Oxytetracycline h.	C1	0.96	1.79	0.35	0.60	0.72	1.47	0.09	0.26	0.24	0.55	1.07	1.92	0.91	1.94	0.67	1.71	0.33	0.82						
	C2	0.79	1.51	0.54	0.97	0.98	2.06	0.10	0.27	0.14	0.30	0.81	1.43	0.85	1.62	0.38	1.49	2.46	1.09	2.07	0.65	1.24			
	C3	1.01	1.97	0.28	0.52	0.80	1.53	0.31	1.06	0.27	0.76	0.27	0.58	0.91	1.94	0.67	1.71	0.33	0.82						
Chloramphenicol	C1	0.65	1.37	1.29	2.28	1.11	1.34	0.09	0.26	0.24	0.55	1.07	1.92	0.91	1.94	0.67	1.71	0.33	0.82						
	C2	0.55	1.87	0.42	1.17	0.73	1.11	0.10	0.27	0.14	0.30	0.81	1.43	0.85	1.62	0.38	1.49	2.46	1.09	2.07	0.65	1.24			
	C3	1.77	6.31	0.30	0.87	0.68	1.19	0.31	1.06	0.27	0.76	0.27	0.58	0.91	1.94	0.67	1.71	0.33	0.82						
Fluoxetine h.	C1	0.19	0.36	1.68	3.02	1.67	3.21	0.65	1.39	1.34	2.74	0.85	1.62	0.91	1.94	0.67	1.71	0.33	0.82						
	C2	0.43	0.81	0.35	0.66	0.48	1.01	0.85	1.52	0.91	1.71	0.20	0.38	1.49	2.46	1.09	2.07	0.65	1.24						
	C3	0.42	0.88	0.44	1.20	0.95	1.64	0.74	1.45	0.26	0.52	0.60	1.13	2.10	3.20	0.64	1.12	0.72	1.28						

Diclofenac s. C1: 0.330 mmol/L; C2: 0.660 mmol/L; C3: 0.990 mmol/L; Oxytetracycline h. C1: 0.105 mmol/L; C2: 0.210 mmol/L; C3: 0.315 mmol/L; Chloramphenicol C1: 0.029 mmol/L; C2: 0.059 mmol/L; C3: 0.088 mmol/L; Fluoxetine h. C1: 0.225 mmol/L; C2: 0.450 mmol/L; C3: 0.675 mmol/L.

### 3.2.3. Modeling of results of selected pharmaceuticals binary mixtures genotoxicity studies

Diclofenac s. impacts oxytetracycline h. in synergic way at medium concentration level of diclofenac h., some overestimation/synergy cases are detected at lowest and highest concentration levels of diclofenac (refer to Table 3 for details). The situation is different when diclofenac is present in mixture with chloramphenicol – more cases of underestimation/synergy are detected at medium, highest and lowest concentrations when CA model was applied. Similar situation is confirmed in case of diclofenac-fluoxetine although the lowest concentration of fluoxetine has antagonistic impact on genotoxicity of medium and highest concentration levels of diclofenac. Oxytetracycline h. is influenced by diclofenac at medium level concentration in a synergic manner what is confirmed with both models used. Lowest and highest levels of diclofenac affect oxytetracycline h. genotoxicity in antagonistic way. Interestingly mixture of chloramphenicol with oxytetracycline shows synergy in most cases (despite situation when oxytetracycline is present in mixture at highest concentration level with low and medium content of chloramphenicol). Situation of fluoxetine-oxytetracycline mixture is complex in its nature; one can say that antibiotic affect activity of fluoxetine at lowest concentration level in antagonistic manner. Other cases show most often synergic behavior. Chloramphenicol in mixture with oxytetracycline shows synergic impact in most cases with both models used. In case of mixture with fluoxetine synergy is observed only at lowest concentration level of fluoxetine and highest content of chloramphenicol – other cases seem to exhibit either antagonistic or overestimated behavior. Diclofenac at medium concentration level impact chloramphenicol from antagonistic to synergy with increasing content of chloramphenicol. Low content of diclofenac shows fully antagonistic impact on chloramphenicol at its highest concentration level. Fluoxetine is impacted by low concentration levels of diclofenac in synergic manner, only in case of lowest fluoxetine content with medium and high levels of diclofenac some overestimation and antagonism takes place. Medium and highest levels of oxytetracycline affect fluoxetine (at medium and highest concentration levels) in a synergic way.

Interactions observed between substances studied were mainly of synergistic character. Most cases of synergistic



**Table 4.** Percentile values for model deviation ratios (MDR) and the number of cases for each group of CA and IA experiments.

		Synergism	Under-estimation	Over-estimation	Antagonism	Percentile			
						80%	90%	95%	99%
Ions	CA	1	7	3	1	1.339	1.794	1.893	2.097
	IA	0	0	14	6	1.071	1.68	1.727	2.116
Mixtures	CA	20	9	4	1	2.226	2.688	2.848	3.691
	IA	5	6	15	9	0.994	1.325	1.674	1.945
						1.925	2.41	3.084	4.855

interactions (ten) were observed for the mixture chloramphenicol with oxytetracycline h. and confirmed applicability of CA and IA as prediction models. The number of interaction cases increased with the decreasing amount of oxytetracycline h. in the mixture, these mixtures also had the lowest values of MDR. In the mixture of diclofenac s. and fluoxetine h., seven cases of synergism were observed with the lowest MDR value (0.2 – CA and 0.4 – IA) for the mixture of lowest concentrations of these compounds. In all cases of mixtures tested, some synergistic effects have been observed.

There were several (four) examples of antagonistic impact, in most cases for fluoxetine h. and chloramphenicol mixture. In all cases, there were ten antagonistic interactions observed. Raw results of genotoxicity of mixtures tested (presented as % of DNA in the comet tail  $\pm$ SD) are summarized in [Supplementary Table 2](#).

In previous studies of mixtures made on *Vibrio fischeri*, no pairs, compounds that behaved synergistically or antagonistically were detected all values of the MDR parameter were between 0.5 and 2.0 for both models. Similarly, in the case of earlier testing of yeast, there was no significant effect on one of the test substances on the other, using CA and IA models and MDR parameters. It seems that this time the HT29 cell is more sensitive, it may be related to the fact that the comet test is a very sensitive test, thanks to which even a small influence of tested compounds can be pinched (Olive and Banáth 2006, Wiczerzak *et al.* 2016b).

#### 4. Conclusions

Mixtures of chemicals may be genotoxic despite the fact that the same compounds do not possess such properties when act separately, additionally, under environmental conditions, they may undergo various chemical or physical transformations, which can affect the toxicity of chemical compounds. Residues of drugs always occur in mixtures with other pollutants in the environment, impact of these environmental mixtures on human and animal organisms is a huge gap in knowledge as far as the environmental fate of these compounds is in stake. The lack of cheap and green technology that eliminates this type of chemical pollution from sewage causes the human and animal organisms to be constantly exposed to residues of pharmaceuticals. In our studies, we determined that environmental conditions such as a change in the pH of the solution resulted in increased toxicity of diclofenac, oxytetracycline, and fluoxetine, but only in a limited range of pH, chloramphenicol showed lower genotoxicity at low pH ranges. All ions, in particular, potassium, fluoride, and bromide, contributed to the reduction of toxicity of the pharmaceuticals tested, however, only in the case of diclofenac

and fluoxetine antagonism was observed. The results indicate that synergism was mainly observed for mixtures of fluoxetine h. with diclofenac s. and chloramphenicol with oxytetracycline h. with fluoxetine h., chloramphenicol showed antagonistic interactions depending on the concentrations used.

The results obtained indicate that the environmental conditions can contribute to increase or decrease of drug's genotoxicity, which is worth considering when conducting ecotoxicological studies, because the results obtained in the laboratory may differ from phenomena actually occurring in the media due to the presence of other substances or additional factors. [Table 4](#) specifies how many cases of synergism and antagonism were observed for both CA and IA models and the percentiles for the whole of the research stages that were counted.

Comet assay showed high sensitivity to 4 pharmaceuticals (out of ten studied) and the research was carried out on cell culture, organisms from higher organization level have defensive and repair mechanisms start when exposure to genotoxic compounds is detected, however, it does not mean that this type of chemicals can be ignored. This study provides an important basis for further investigation into examination of mixtures of chemical compounds and characterization of genotoxic mechanism of drug residues and they can be an introduction to more complex multi-component mixtures.

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#### References

Ávila, C., and García, J., 2015. Pharmaceuticals and personal care products (PPCPs) in the environment and their removal from wastewater

- through constructed wetlands. *Comprehensive Analytical Chemistry*, 67, 195–244.
- Aydin, S., et al., 2017. Antidepressants in urban wastewater treatment plant: occurrence, removal and risk assessment. *Global Nest Journal*, 19, 100–106.
- Backhaus, T., and Faust, M., 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. *Environmental Science and Technology*, 46 (5), 2564–2573.
- Barreto, A., et al., 2017. Genotoxicity of gemfibrozil in the gilthead seabream (*Sparus aurata*). *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 821, 36–42.
- Belden, J.B., Gilliom, R.J., and Lydy, M.J., 2007. How well can we predict the toxicity of pesticide mixtures to aquatic life? *Integrated Environmental Assessment and Management*, 3 (3), 364–372.
- Blystone, C.R., et al., 2011. Toxicity and carcinogenicity of androstenedione in F344/N rats and B6C3F1 mice. *Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association*, 49 (9), 2116–2124.
- Bocchi, C., et al., 2016. Characterization of urban aerosol: seasonal variation of mutagenicity and genotoxicity of PM 2.5, PM 1 and semi-volatile organic compounds. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 809, 16–23.
- Caban, M., et al., 2015. Determination of pharmaceutical residues in drinking water in Poland using a new SPE-GC-MS (SIM) method based on Speedisk extraction disks and DIMETRIS derivatization. *Science of the Total Environment*, 538, 402–411.
- Chae, J.P., et al., 2015. Evaluation of developmental toxicity and teratogenicity of diclofenac using *Xenopus* embryos. *Chemosphere*, 120, 52–58.
- Charles, E., et al., 2017. The antidepressant fluoxetine induces necrosis by energy depletion and mitochondrial calcium overload. *Oncotarget*, 8, 3181–3196.
- Chen, C., et al., 2015. The synergistic toxicity of the multiple chemical mixtures: implications for risk assessment in the terrestrial environment. *Environment International*, 77, 95–105.
- Cuthbert, R.J., et al., 2016. Continuing mortality of vultures in India associated with illegal veterinary use of diclofenac and a potential threat from nimesulide. *Oryx*, 50 (01), 104–112.
- Drescher, K., and Boedeker, W., 1995. Assessment of the combined effects of substances: the relationship between concentration addition and independent action. *Biometrics*, 51 (2), 716–730.
- Durgo, K., et al., 2009. The assessment of genotoxic effects of wastewater from a fertilizer factory. *Journal of Applied Toxicology*, 29 (1), 42–51.
- Ebele, A.J., Abdallah, M.A.E., and Harrad, S., 2017. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. *Emerging Contaminants*, 3 (1), 1–16.
- Eggen, T., Moeder, M., and Arukwe, A., 2010. Municipal landfill leachates: a significant source for new and emerging pollutants. *Environmental Science and Technology*, 408 (21), 5147–5157.
- Fairbairn, D.W., Olive, P.L., and O'Neill, K.L., 1995. The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology*, 339 (1), 37–59.
- Faust, M., et al., 2000. Predictive assessment of the aquatic toxicity of multiple chemical mixtures. *Journal of Environment Quality*, 29 (4), 1063–1068.
- Fent, K., Weston, A.A., and Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology (Amsterdam, Netherlands)*, 76 (2), 122–159.
- Fu, L., et al., 2017. Toxicity of 13 different antibiotics towards freshwater green algae *Pseudokirchneriella subcapitata* and their modes of action. *Chemosphere*, 168, 217–222.
- Gaw, S., Thomas, K.V., and Hutchinson, T.H., 2014. Sources, impacts and trends of pharmaceuticals in the marine and coastal environment. *Philosophical Transactions of the Royal Society B*, 369, 1–11.
- González-Pleiter, M., et al., 2013. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: implications for environmental risk assessment. *Water Research*, 47 (6), 2050–2064.
- Huang, B., et al., 2014. Chlorinated volatile organic compounds (Cl-VOCs) in environment—sources, potential human health impacts, and current remediation technologies. *Environment International*, 71, 118–138.
- Huebner, M., et al., 2015. Rapid analysis of diclofenac in freshwater and wastewater by a monoclonal antibody-based highly sensitive ELISA. *Analytical and Bioanalytical Chemistry*, 407 (29), 8873–8882.
- Hurst, N.F., Bibolini, M.J., and Roth, G.A., 2015. Diazepam inhibits proliferation of lymph node cells isolated from rats with experimental autoimmune encephalomyelitis. *Neuroimmunomodulation*, 22 (5), 293–302.
- Kazimirova, A., et al., 2012. Genotoxicity testing of PLGA-PEO nanoparticles in TK6 cells by the comet assay and the cytokinesis-block micronucleus assay. *Mutation Research*, 748 (1–2), 42–47.
- Kienzler, A., et al., 2016. Regulatory assessment of chemical mixtures: requirements, current approaches and future perspectives. *Regulatory Toxicology and Pharmacology*, 80, 321–334.
- Koss-Mikołajczyk, I., et al., 2015. Juices from non-typical edible fruits as health-promoting acidity regulators for food industry. *LWT- Food Science and Technology*, 64 (2), 845–852.
- Kudlak, B., et al., 2016. Environmental risk assessment of Polish wastewater treatment plant activity. *Chemosphere*, 160, 181–188.
- Lah, B., et al., 2005. Monitoring of genotoxicity in drinking water using *in vitro* comet assay and Ames test. *Food Technology and Biotechnology*, 43, 139–146.
- Li, W.C., 2014. Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil. *Environmental Pollution (Barking, Essex: 1987)*, 187, 193–201.
- Liu, S., et al., 2015. Steroids in marine aquaculture farms surrounding Hailing Island, South China: occurrence, bioconcentration, and human dietary exposure. *Science of the Total Environment*, 502, 400–407.
- Mihaljevic, Z., et al., 2011. Assessment of genotoxic potency of sulfate-rich surface waters on medicinal leech and human leukocytes using different versions of the Comet assay. *Ecotoxicology and Environmental Safety*, 74 (5), 1416–1426.
- Nasuhoglu, D., Berk, D., and Yargeau, V., 2012. Photocatalytic removal of 17 $\alpha$ -ethinylestradiol (EE2) and levonorgestrel (LNG) from contraceptive pill manufacturing plant wastewater under UVC radiation. *Chemical Engineering Journal*, 185–186, 52–60.
- Ogueji, E.O., et al., 2017. Toxicity of diazepam on lipid peroxidation, biochemical and oxidative stress indicators on liver and gill tissues of African catfish *Clarias gariepinus*. *International Journal of Fisheries and Aquatic Studies*, 5, 114–123.
- Olive, P.L., and Banáth, J.P., 2006. The comet assay: a method to measure DNA damage in individual cells. *Nature Protocols*, 1 (1), 23.
- Örn, S., Holbech, H., and Norrgren, L., 2016. Sexual disruption in zebrafish (*Danio rerio*) exposed to mixtures of 17 $\alpha$ -ethinylestradiol and 17 $\beta$ -trenbolone. *Environmental Toxicology and Pharmacology*, 41, 225–231.
- Papageorgiou, M., Kosma, C., and Lambropoulou, D., 2016. Seasonal occurrence, removal, mass loading and environmental risk assessment of 55 pharmaceuticals and personal care products in a municipal wastewater treatment plant in Central Greece. *Science of the Total Environment*, 543, 547–569.
- Parasuraman, S., 2011. Toxicological screening. *J Pharmacol Pharmacother*, 2 (2), 74.
- Parolini, M., et al., 2011. Cytotoxicity assessment of four pharmaceutical compounds on the zebra mussel (*Dreissena polymorpha*) haemocytes, gill and digestive gland primary cell cultures. *Chemosphere*, 84 (1), 91–100.
- Petrie, B., Barden, R., and Kasprzyk-Hordern, B., 2015. A review on emerging contaminants in wastewaters and the environment: current knowledge, understudied areas and recommendations for future monitoring. *Water Research*, 72, 3–27.
- Pomati, F., et al., 2006. Effects of a complex mixture of therapeutic drugs at environmental levels on human embryonic cells. *Environmental Science and Technology*, 40 (7), 2442–2447.
- Ren, X., et al., 2017. Toxic responses of swimming crab (*Portunus trituberculatus*) larvae exposed to environmentally realistic concentrations of oxytetracycline. *Chemosphere*, 173, 563–571.
- Sui, Q., et al., 2015. Occurrence, sources and fate of pharmaceuticals and personal care products in the groundwater: a review. *Emerging Contaminants*, 1 (1), 14–24.
- Syed, M., Skonberg, C., and Hansen, S.H., 2016. Mitochondrial toxicity of diclofenac and its metabolites via inhibition of oxidative

- phosphorylation (ATP synthesis) in rat liver mitochondria: Possible role in drug induced liver injury (DILI). *Toxicology in Vitro*, 31, 93–102.
- Szczepańska, N., Kudlak, B., and Namieśnik, J., 2018. Assessing ecotoxicity and the endocrine potential of selected phthalates, BADGE and BFDGE derivatives in relation to environmentally detectable levels. *Science of Total Environment*, 610–611, 854–866.
- Tahrani, L., et al., 2016. Occurrence of antibiotics in pharmaceutical industrial wastewater, wastewater treatment plant and sea waters in Tunisia. *Journal of Water and Health*, 14 (2), 208–213.
- Tian, F., et al., 2016. The antibiotic chloramphenicol may be an effective new agent for inhibiting the growth of multiple myeloma. *Oncotarget*, 7, 51934.
- Torres, N.H., et al., 2015. Detection of hormones in surface and drinking water in Brazil by LC-ESI-MS/MS and ecotoxicological assessment with *Daphnia magna*. *Environmental Monitoring and Assessment*, 187, 379.
- Touber, M.E., et al., 2007. Multi-detection of corticosteroids in sports doping and veterinary control using high-resolution liquid chromatography/time-of-flight mass spectrometry. *Analytica Chimica Acta*, 586 (1–2), 137–146.
- van Leeuwen, J.S., et al., 2012. Differential involvement of mitochondrial dysfunction, cytochrome P450 activity, and active transport in the toxicity of structurally related NSAIDs. *Toxicology in Vitro*, 26 (2), 197–205.
- Warkus, E.L., and Marikawa, Y., 2018. Fluoxetine inhibits canonical Wnt signaling to impair embryoid body morphogenesis: potential teratogenic mechanisms of a commonly used antidepressant. *Toxicological Sciences*, doi: [10.1093/toxsci/kfy14](https://doi.org/10.1093/toxsci/kfy14).
- Wieczerek, M., Kudlak, B., and Namieśnik, J., 2015. Environmentally oriented models and methods for the evaluation of drug × drug interaction effects. *Critical Review in Analytical Chemistry*, 45 (2), 131–155.
- Wieczerek, M., Kudlak, B., and Namieśnik, J., 2016. Study of the effect of residues of pharmaceuticals on the environment on the example of bioassay Microtox®. *Monatshfte Für Chemie - Chemical Monthly*, 147 (8), 1455–1460.
- Wieczerek, M., Kudlak, B., and Namieśnik, J., 2018. Impact of selected drugs and their binary mixtures on the germination of *Sorghum bicolor* (sorgo) seeds. *Environmental Science and Pollution Research*, 25 (19), 18717–18727.
- Wieczerek, M., et al., 2016. Modeling of pharmaceuticals mixtures toxicity with deviation ratio and best-fit functions models. *Science of Total Environment*, 571, 259–268.
- Wieczerek, M., et al., 2018. Impact of inorganic ions and pH variations on toxicity and endocrine potential of selected environmentally relevant pharmaceuticals. *Environmental Pollution*, 237, 549–558.
- Wu, M., et al., 2016. Distribution, fate, and risk assessment of antibiotics in five wastewater treatment plants in Shanghai, China. *Environmental Science and Pollution Research*, 23 (18), 18055–18063.
- Zanuri, N.B.M., Bentley, M.G., and Caldwell, G.S., 2017. Assessing the impact of diclofenac, ibuprofen and sildenafil citrate (Viagra®) on the fertilisation biology of broadcast spawning marine invertebrates. *Marine Environmental Research*, 127, 126–136.



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