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Modeling of pharmaceuticals mixtures toxicity with deviation ratio and best-fit functions models

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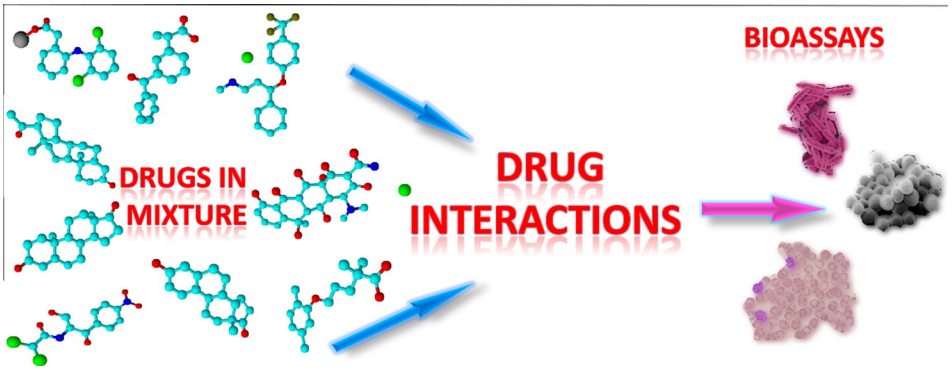
GRAPHICAL ABSTRACT



HIGHLIGHTS

- Drug synergism or antagonism is carte blanche in modern environmental sci-ence.
- Quantitative assessment of drug mix-tures toxicological parameters is given.
- CI, IA and SI by model deviation ratio (MDR) studies were carried out.
- Best-fit function modeling was also ap-plied as an assessment option.
- Independent action was stated for most of drug mixtures studied with bioas-says.

abstract

The present study deals with assessment of ecotoxicological parameters of 9 drugs (diclofenac (sodium salt), oxytetracycline hydrochloride, fluoxetine hydrochloride, chloramphenicol, ketoprofen, progesterone, estrone, androstenedione and gemfibrozil), present in the environmental compartments at specific concentration levels, and their mutual combinations by couples against Microtox® and XenoScreen YES/YAS® bioassays. As the quan-titative assessment of ecotoxicity of drug mixtures is an complex and sophisticated topic in the present study we have used two major approaches to gain specific information on the mutual impact of two separate drugs present in a mixture. The first approach is well documented in many toxicological studies and follows the procedure for assessing three types of models, namely concentration addition (CA), independent action (IA) and simple inter-action (SI) by calculation of a model deviation ratio (MDR) for each one of the experiments carried out. The sec-ond approach used was based on the assumption that the mutual impact in each mixture of two drugs could be described by a best-fit model function with calculation of weight (regression coefficient or other model param-eter) for each of the participants in the mixture or by correlation analysis. It was shown that the sign and the ab-solute value of the weight or the correlation coefficient could be a reliable measure for the impact of either drug A on drug B or, vice versa, of B on A. Results of studies justify the statement, that both of the approaches show

Keywords: Pharmaceuticals, Hormones, Interactions modeling, Microtox®, XenoScreen YES/YAS®

Abbreviations: CA, concentration addition; MDR, independent action (IA) and simple interaction (SI) by calculation of a model deviation ratio; YES+, YES agonist; YES−, YES antagonist; YAS+, YAS agonist; YAS−, YAS antagonist.

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similar assessment of the mode of mutual interaction of the drugs studied. It was found that most of the drug mixtures exhibit independent action and quite few of the mixtures show synergic or dependent action.

1. Introduction

Numerous biologically active compounds are produced by humans and are present in the environment. Main sources of drugs' residues in the environment are considered to be products of pharmaceutical and veterinary industry, hospital facilities and agriculture, minor sources include municipal wastewaters and poorly sealed medical waste landfills. Despite the constantly increasing knowledge on environmental fate of pharmaceuticals we still do not fully understand all the processes that can occur between drug residues in the environment and their effects on living organisms (Kudłak et al., 2011; Halling-Sørensen et al., 1998). The risk of environmental exposure to residues of pharmaceuticals becomes greater due to the fact that they are biologically active substances, and often are not subjected to proper biodegradation in sewage treatment plants (Fatta-Kassinos et al., 2011). The literature provides some information in order to establish a uniform definition of drug interactions, namely: synergism, antagonism and additivity or to predict risk assessment of chemical mixtures (U.S. EPA, 1986; Wieczerzak et al., 2015; Backhaus and Faust, 2012; Vasquez et al., 2014; Watanabe et al., 2016). Within the environmental research on fate of pharmaceuticals it should not be forgotten that the residues of pharmaceuticals are present in the ecosystem in a mixture with other drugs and various stressors. Although numerous treatment methodologies involve the use of drug mixtures to achieve adequate therapeutic effect, this type of drugs co-presence is undesirable for the environment. As presented in Supplementary Table 1 studies on mutual impact (as well as in mechanistic response of drug in living body) of drugs when present in complex matrices were conducted mostly for health studies and in case of higher animals what does not reflect processes that occur in different environmental compartments. Environmental ecotoxicological studies in this area are still scarce and are conducted by few scientific centres (Watanabe et al., 2016, Altenburger et al., 2004; Backhaus, 2014, Silva E, Rajapakse N, Kortenkamp, 2002, Escher and Hermens, 2002, Dubiella-Jackowska et al., 2010).

The clarification of the toxic impact of different chemicals is a difficult, complex and, often, disputable problem. This holds true for assessing the effect of a single compound (e.g. a certain drug) and the task is much more complex in assessing the toxicity of drug mixtures. The traditional experimentation in studies of this type relies on the use of the responses of laboratory animals (usually rats) to the administration of drug combinations (Jakovljevic et al., 2009, Gan, 2010). In cases of this type the interaction between the administered drugs is considered to be “independent”, “dependent” or “synergetic” if the drug impact is neutral, negative or additive with respect to the influence on the general toxicity.

The problem is becoming even more complicated if the drugs mixture impact is regarded with respect to the ecotoxicity response of environmental compartments to the administration of drugs as wastes to environmental phases like surface waters, soils or sediments. On one hand the environmental systems are very different when compared to animal ones (including human), so it becomes a problem to interpret the mechanism of possible drug interactions offered for biota and to apply it the environmental samples. Different ecotoxicological tests (for acute ecotoxicity, chronic ecotoxicity, endocrine potential or DNA disruptors) require specific experimental design and assessment of results obtained, on the other hand. Therefore, the organization and performance of model experiments using different ecotoxicity test could be of significant importance in detection of any possible kind of interaction between drugs in waste water samples (independent, dependent or synergetic). The model experiment output should be considered as response of a black box system where the input is the concentration of each drug in a mixture and the output – the ecotoxicity measure for a certain type of bioassay (EC50, mortality, inhibition of luminescence etc.). Using the best fit function approach or MDR method as modeling procedures an adequate modeling of the mutual impact of the drugs in a mixture is possible although without exact description of the possible interaction mechanism.

It is the aim of the present study to assess by model experiments in a semi-quantitative way the combined ecotoxicological and endocrine impact of two drugs in a mixture at levels stated in environmental samples and to determine the possible independent, dependent and synergetic behavior of the separate drugs.

2. Materials and methods

In the present work, the influence of mixtures of 9 pharmaceuticals against 2 organisms from different trophic levels was assessed. The selected organisms were: bacterium *Vibrio fischeri* (Microtox®) and genetically modified yeast (XenoScreen YES/YAS®).

Vibrio fischeri is a G(−) bacteria found in salt and brackish waters. Any change in the bioluminescence of bacterial suspension after a period of incubation with the test sample is the basis of the Microtox® calculations used in this study (Marugán et al., 2012). In the XenoScreen YES/YAS® test genetically modified yeasts are used which, due to genetically introduced the androgenic (YAS) and estrogenic (YES) receptors, are sensitive to presence of substances with hormonal properties. The test allows the assessment of the agonistic and the antagonistic properties of chemicals present in the sample. Stained by β-galactosidase growth medium with CPRG is measured using a spectrophotometer.

Tested pharmaceuticals, namely: diclofenac (sodium salt), oxytetracycline hydrochloride, fluoxetine hydrochloride, chloramphenicol, ketoprofen, progesterone, estrone, androstenedione and gemfibrozil, are widely used in various therapeutic treatments. Their presence in the environment at different concentration levels has been confirmed in numerous studies (Vulliet et al., 2011; Kasprzyk-Hordern et al., 2008; Kim et al., 2007; Lin and Tsai, 2009). In Table 1 information on concentration levels of select group of pharmaceuticals in the environment is summarized. The data indicates that there is a risk of adverse effects of those compounds' presence. The compounds selected for studies are those prescribed in the highest quantitates and representing chemicals of different mode of action to human beings and for this reason determining toxicity of such mixtures is environmentally relevant.

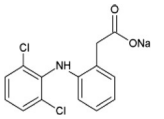
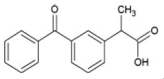
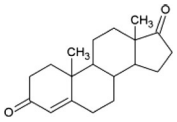
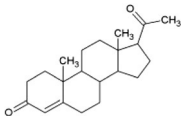
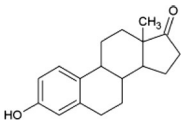
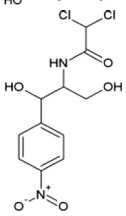
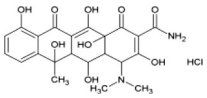
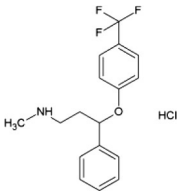
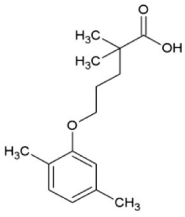
2.1. Microtox® bioassay protocol

The Microtox® test acute reagent (lyophilized *Vibrio fischeri*), osmotic adjustment solution (OAS, 22% solution of sodium chloride), reconstitution solution (RS), and diluent (2% solution of sodium chloride) were purchased from Modern Waters (USA). The study was conducted using Microtox® Analyzer M500 model. Apparatus is equipped with 30 incubation wells as well as reagent (bacterial suspensions) and read wells. Temperatures are assigned to the corresponding type of performed test (in this case acute toxicity test) and the internally maintained at 5.5 ± 1 °C for reagent well and 15 ± 0.5 °C for both the incubator part and the read well.

2.2. XenoScreen YES/YAS® bioassay protocol

A set of XenoScreen YES/YAS® was purchased in Xenometrix AG (Switzerland), namely: hERα yeasts (to determine estrogenic activity)

Table 1
Literature study on occurrence and concentration levels of selected pharmaceuticals in the environment.

Analyte	Structural formula	Application/action	Concentration in environment (ng/L)	Sample matrix/sample location
Diclofenac (sodium salt)		Anti-inflammatory, analgesic and antipyretic drug (NSAIDs) mainly used to treat musculoskeletal pain and chronic pain (Ryshetti et al., 2015).	10–55	Surface water samples (Vantaa River), Finland (Vieno et al., 2007).
Ketoprofen		Inflammatory and degenerative changes in rheumatic diseases, and sometimes used to relieve some of the pain syndromes (Karaman et al., 2006).	0–180	Surface water, wastewater (effluents) samples, Switzerland (Tixier et al., 2003).
Androstenedione		Male and female hormone, testosterone, estrone and estradiol precursor,is produced by the ovaries (Makris and Ryan, 1975; Lee and Migeon, 1975).	>100	River water samples (Fenholloway River), USA (Durhan et al., 2002)
Progesterone		Female steroid hormone. Used to supplement the decrease in the naturally occurring progesterone, to prevent miscarriages, prevents the disturbance of the menstrual cycle. Is partly produced by luteal cells of the corpus luteum and mostly by placenta especially during pregnancy (Makris and Ryan, 1975).	66 ± 36	Wastewater samples (influents and effluents), Beijing, China (Chang et al., 2011).
Estrone		Estrogen with a steroid-like estradiol is produced by the ovaries (Makris and Ryan, 1975; Lee and Migeon, 1975).	1.7–36	Surface, drinking, and waste waters samples (effluent), Jeolla province, Jeju Island, (Youngsan River), South Korean (Kim et al., 2007).
Chloramphenicol		Drug with antibacterial and bacteriostatic, properties against the G(+) and G(−), but due to the undesirable side effects is used only in case of life-threatening infections such as tuberculosis. Chloramphenicol is used in small amounts in the form of ointments or suspensions can be applied local to the skin, eye or ear (Cho et al., 2013)	<2–15	River water samples (Taff and Ely rivers), South Wales, UK (Kasprzyk-Hordern et al., 2008).
Oxytetracycline hydrochloride		Natural antibiotic with a bacteriostatic effect and antiallergic properties against both G(−) and G(+) bacteria, is used as in veterinary and aquaculture industry (Gao et al., 2013).	377,000 ± 142,000	Waste water, China (Li et al., 2008)
Fluoxetine hydrochloride		Mainly used in the treatment of depressive disorders and obsessive-compulsive. Fluoxetine belongs to the group of selective serotonin reuptake inhibitors (SSRI) (Stokes, 1992).	0.5–43.2	Stream water samples, USA (Schultz et al., 2010).
Gemfibrozil		The drug normalizes the levels of fat in the blood plasma, lowers "bad" LDL cholesterol and triglyceride levels, increases levels of "good" HDL cholesterol (Frick et al., 1987).	<0.09–19.8	Seawater samples, Singapore (Bayen et al., 2013).

and hAR (to determine androgenic activity) settled on the filtration paper, basal medium, vitamin solution, L-aspartic acid solution, L-threonine solution, $CuSO_4$ solution, 17β-estradiol (E2, YES+ ("+" describes agonistic effects) control), 5α-dihydrotestosterone (DHT, YAS+ control), 4-hydroxytamoxifen (HT, YES− ("−" describes antagonistic effects) control), flutamide (FL, YAS− control), DMSO. CPRG (chlorophenol red-β-D-galactopyranoside) was purchased from Sigma Aldrich (Germany). Measurement of cell density (wavelength 690 [nm]) and of the intensity of the CPRG transformation product (at 570 [nm] wavelength) was performed with a TECAN Infinite M 200 spectrophotometer.

2.3. Model compounds

Compounds selected to study are co-present in the environment, some even in significant quantities e.g., oxytetracycline (377 ± 142 μg/L), ketoprofen (0–180 ng/L) therefore they became the subject of interest of the authors of this article. Model substances selected for the study were: diclofenac (sodium salt) (CAS no. 15307-79-6), chloramphenicol (CAS no. 56-75-7), oxytetracycline hydrochloride (CAS no. 2058-46-0), fluoxetine hydrochloride (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). The concentration ranges (mM) studied in order to calculate EC_{50} together with $EC_{50} \pm SD$ are given in Table 2 below.

2.4. Microtox® methodology

Determination of EC_{50} parameter (median effective concentration) was held by two protocols, one for water-soluble substances and the other for insoluble chemicals. The EC_{50} parameter for water-soluble pharmaceuticals (namely diclofenac (sodium salt), chloramphenicol, oxytetracycline, fluoxetine) was determined by standard Acute Toxicity Test Basic 81.9% protocol using the Model 500 Analyzer Microtox® and serial dilutions. The range-screening test was performed to narrow the range of tested concentrations of pharmaceuticals, followed by additional tests in selected ranges of concentrations (each compound in triplicates in four serial dilutions). Lyophilized reagent with *Vibrio fischeri* bacterium was hydrated with 1 mL of RS and maintained at 5.5 ± 1.0 °C temperature, subsequently 100 μL of bacterial solution and a pre-made samples of standard dissolved in distilled water were added into the vials. To produce a suitable osmotic pressure (above 2%) OAS was added to the vial with the highest concentration and serial dilutions were prepared in diluent. The incubation time was 30 min. To determine EC_{50} of insoluble compounds modification of the assay protocol was necessary as the samples were treated as solid ones - the appropriate volume of sample was added directly to the vials of bacterial suspension, OAS and diluent. As in the case of soluble substances, range-screening test for insoluble substance was also performed to narrow the range of concentrations tested, afterwards proper tests were performed in triplicates to determine range of linearity and calculate EC_{50}.

In order to determine whether the addition of one substance to solution of another one would change the toxic effect, concentrated solutions of the compounds were prepared. Test mixtures were prepared in such a way that the compounds were present in an appropriate ratio respectively 100% of first model substance and the second substance with a reduced effect to 33% and 66% of EC_{50}. Incubation time of samples with bacteria for all of the tests was 30 min according to the test protocol.

Table 2
The concentration ranges (mM) studied in order to calculate EC_{50} together with SD.

Microtox®		
Analyte	Concentration ranges tested [mM]	$EC_{50} \pm SD$ [mM]
Oxyteracycline hydrochloride	0.054–0.44	0.113 ± 0.042
Fluoxetine hydrochloride	0.011–0.084	0.0337 ± 0.0072
Chloramphenicol	0.6–5	0.937 ± 0.092
Diclofenac (sodium salt)	0.057–0.46	0.0727 ± 0.0022
Ketoprofen	0.43–0.57	0.465 ± 0.021
Gemfibrozil	0.090–0.11	0.0995 ± 0.0016
Progesterone	0.12–0.16	0.1518 ± 0.0031
Androstenedione	0.10–0.13	0.1121 ± 0.0019
Estrone	0.36–0.45	0.392 ± 0.010
XenoScreen YES/YAS®		
Analyte	Concentration ranges tested [mM]	Medium effect [mM]
Oxyteracycline hydrochloride	0.0058–0.023	0.014
Fluoxetine hydrochloride	0.0083–0.033	0.021
Chloramphenicol	0.0087–0.35	0.022
Diclofenac (sodium salt)	0.0092–0.037	0.023
Ketoprofen	0.0069–0.027	0.017
Gemfibrozil	0.0037–0.015	0.0093
Progesterone	0.0095–0.038	0.024
Androstenedione	0.0065–0.026	0.016
Estrone	0.0085–0.034	0.021

2.5. XenoScreen YES/YAS® methodology

To investigate endocrine potential of mixture of drugs slightly modified protocol of XenoScreen YES/YAS® was utilized, which uses genetically modified yeast cells of *Saccharomyces cerevisiae*. For this purpose the DNA sequence of human estrogen hERα or androgen hAR receptors was stably integrated into the main chromosome of the yeast cells. Yeasts exposed to compounds that act endocranially produce β-galactosidase, which oxidizes the dye CPRG in growth medium. The interpretation occurs by measuring the density of the cell suspension and the color saturation of the oxidized dye. Furthermore, the cells also contain an expression plasmid carrying the *lacZ* reporter gene encoding the enzyme β-galactosidase and means responsive to estrogens (YES) or androgen (YAS) (Routledge and Sumpter, 1996). The yeast cells were cultured from the filter papers in growth medium (basic medium with a vitamin solution, solution of L-threonine, L-aspartic acid and copper sulfate (VI)). 5 mL of growth medium was transferred to a labeled culture bottles with caps with a gas permeable filter, afterwards the yeast disks were sterilely transferred and placed on an orbital shaker set at 32 °C temperature and 100 rpm for 48 h. 100 μL of DMSO was added to each control vial containing standards: E2 (17β-estradiol control of YES agonist), DHT (5α-dihydrotestosterone control of YAS agonist), HT (4-hydroxytamoxifen control of YES antagonist), FL (flutamide control of YAS antagonist). Test plates were prepared in such a way that, the controls were in duplicate in eight serial dilutions respectively:

- YES Agonist plate E2 (min. concentration 1×10^{-11} M max. concentration 1×10^{-8} M),
- YES Antagonist plate HT (min. con. 1×10^{-8} M max. con. 1×10^{-5} M, additionally in the entire plate E2 was present at constant concentration of 1×10^{-9} M),
- YAS Agonist plate DHT (min. concentration 1×10^{-9} M max. con. 1×10^{-6} M),
- YAS Antagonist plate FL (min. con. 1×10^{-7} M max. con. 1×10^{-4} M, additionally in the entire plate DHT was present at constant concentration of 3×10^{-8} M).

Addition of E2 or DHT present at the same concentration to the entire YES or YAS antagonist plate, respectively, is intended to examine (confirm/deny) andro- and estrogenic antagonistic activity of samples. A substance with the antagonist properties competes with E2 or DHT present on the plate and binds to the receptor without inducing the expression of β-galactosidase. Without the enzyme substrate staining does not occur, however, if the test sample does not contain antagonistic substances, then E2 and DHT the present in the wells bind with the receptor expressing β-galactosidase and the staining of the substrate occurs.

To each assay well 60 μL of CRPG dye was added at 6 mM concentration. Pharmaceuticals were mixed in three concentration ratios in such a way to detect a broad range of possible interactions. All of the studies on mixtures were performed in triplicates, furthermore controls were made for pure substances in duplicates. Into agonist and antagonist YES and YAS plates, 100 μL of YES and YAS suspension of yeast culture (yeast cells density > 0.3 OD_{690}) was added, respectively. Assay plates were sealed with semi-permeable membranes and placed in the bag zipper moistened with watered gauze on an orbital shaker for 48 h at 32 °C 100 rpm. After 48 h of incubation, a cell density by OD was read at a wavelength of 690 nm and color intensity at a wavelength of 570 nm was determined. Afterwards the activity of β-galactosidase was calculated as ratio of $[(OD_{570} - OD_{690}) / OD_{690}]$.

2.6. Modeling by best-fit functions

Model selection is one of the fundamental scientific approaches to an existing experimental set of data. The goal of this approach is to find out the principles that explain the series of observations and, if possible, to

predict by a mathematical way these observations. Since the mechanisms, especially in a field like ecotoxicity testing, leading to the data collection could be countless in number, the best choice of a model is related to the choice of best-fit function ensuring sufficient explanation of the data and having a significant validation power. Important condition in choosing an appropriate model is the possible simple scientific correspondence with phenomenological processes or mechanisms.

Usually, the modeling starts with simple regression and polynomial models but, at the end of the best-fit function modeling they could be of more sophisticated in nature (Gaussian, exponential, smoothing etc.) (Burnham and Anderson, 2002).

Very often the meaning of "best" in the modeling approach could be controversial and a reliable solution is to search for compromise between some metrics for goodness of fit and simplicity in data interpretation. More complex models will be better able to adapt their shape to fit the data but the additional parameters may not represent anything useful. Goodness of fit is generally determined using a likelihood ratio approach, or an approximation of this, leading to a chi-squared test. The complexity is generally measured by counting the number of parameters in the model. Model selection techniques can be considered as estimators of some physical quantity, such as the probability of the model producing the given data. The bias and variance are both important measures of the quality of this estimator; efficiency is also often considered.

In the present study we have used mainly exponential Gaussian best-fit functions for the data with Microtox® test keeping in mind the impact of the pharmaceuticals on the test organisms. The parameter describing the height of the function (a) served as a measure for the impact of one pharmaceutical on the other in the drug mixtures. Comparison of these parameters makes it possible, according to our assumptions, to distinguish between independent, antagonistic or synergetic effects in the drug mixture. Correlation coefficient was used as estimator of goodness of fit. In the study of (Dawson et al., 2012) the importance of modeling of toxicity by best-fit functions is convincingly shown.

For the second test Xenoscreen YES/YAS® we have used correlation analysis of the experimental data for each of the drug mixture assuming that value and sign of the correlation coefficient could be a relative measure for independent or dependent action.

Additionally, cluster analysis was used to find similarities and dissimilarities between the drug mixtures described by the correlation analysis.

2.7. Calculation of model deviation ratio (MDR)

When a biosystem is exposed to chemicals mixtures, different types of joint action may occur. The substances in the mixture could act in several modes: similar (jointly) and dissimilar (independently) on the one hand and interactive or without interaction on the other hand. Compounds which act similar behave like dilutions of each other while these which act dissimilar reveal independent responses (Plackett and Hewlett, 1952). If there is interaction between the chemicals in the mixture it could be synergistic or antagonistic. In case of no interaction - the combined effect can be predicted by the expected effect of each component (Belden et al., 2007) according to the mode of action of substances in the mixture.

The concentration addition (CA) model is used to test pharmaceuticals in a mixture for a similar mode of action. The concept is that the similarly acting substances, after normalizing for potency, act jointly in an additive manner (Altenburger et al., 2003). According to the CA model, the total concentration of toxicity mixture can be calculated using the following equation (Faust et al., 2000):

$$ECx_{mix} = \left(\sum_{i-1}^{n} \frac{pi}{ECx_i}\right)^{-1} \quad (1)$$

where ECx_{mix} is the total concentration of the mixture that causes x effect; p_i indicates the proportion of component i in the mixture; n indicates the number of components in the mixture; ECx_i indicates the concentration of component i that would cause x effect.

Frequently CA experiments are expressed by toxic unit scale (TU) because it allows addition of concentrations of each compound (Belden et al., 2007).

TU can be calculated by the equation:

$$TU = \sum_{i=1}^{n} \frac{c_i}{ECx_i} \quad (2)$$

where C_i is the concentration of the ith chemical in the mixture; ECx_{mix} is the total concentration of the mixture that causes x effect.

The independent action (IA) model is used to test toxicants in a mixture for a dissimilar mode of action. The concept is that they act independently. In fact the IA model is a statistical approach to predict the chance that one of multiple events will occur (Altenburger et al., 2003). The total mixture concentration is calculated using the following equation (Faust et al., 2000):

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \quad (3)$$

where $E(c_{mix})$ is the total effect of the mixture; $E(c_i)$ is the effect expected from component i.

For model accuracy assessment of both models model deviation ratio (MDR) is used. MDR is defined as:

$$\mathrm{MDR} = \frac{Expected}{Observed} \quad (4)$$

where *Expected* is the effective concentration of the mixture predicted by any one of the models and *Observed* is the effective concentration for the mixture obtained from toxicity testing. The *Observed* values in this study are calculated as a mean of toxicity mixtures values at Microtox® and XenoScreen YES/YAS® experiments for each pair of pharmaceuticals. The MDR values for each test obtained by CA and IA models are presented on cumulative distribution plots (Belden et al., 2007). Pairs of pharmaceuticals with MDR values falling outside MDR range from 0.5 to 2.0 will be discussed for possible synergistic or antagonistic interaction between substances. Only mixtures containing toxic pharmaceuticals will undergo abovementioned modeling procedure.

3. Results and discussion

3.1. Microtox®

3.1.1. Modeling by best-fit functions of drug mixtures for Microtox® assay

The major idea of the modeling of the results (effects) from the simultaneous action of two drugs in Microtox® test for ecotoxicity is as follows:

- to find a best-fit-function describing adequately the experimental points (in concentration relations 100/0; 100/33.3 and 100/66.7) for each couple of drugs (A and B) checking both the impact of B on A and, vice versa, A on B;
- to determine the specific parameters of the best-fit function;
- to compare the models from the best-fit function assuming that independent action (IA) for both members of the drug mixture is better if the model was one and the same and if the specific coefficients of the model are statistically equal for A/B and B/A impact;
- if the models for the couples A/B and B/A are different or the specific coefficients from the best fit function are statistically non-equal, then we conclude that at hand is dependent action (DA) which could be either synergetic (SA) or antagonistic (AA).

3.1.1.1. Results from the best-fit functions modeling. Altogether 72 models were calculated (9 drugs in combination with the rest of them). The

most common best-fit function for 54 models was a Gaussian function of the type:

$$Y = a \cdot e^{\left[\frac{(x-b)^2}{2c^2}\right]} \quad (5)$$

a – height of the Gaussian function (our measure for impact, effect),
b – position of the middle point,
c – width of the Gaussian function (measure for standard deviation).

In Supplementary Table 2 the a-parameter values with respective standard error (SE) for all 54 mixtures following the Gaussian model are presented. For the rest of the models we have mixtures where one of the couple (A/B) could be described by Gaussian model but the opposite (B/A) requires different best-fit functions like Power Law function, Saturation Growth Rate function, and Exponential Association function. Most of these functions have also parameter "a" which could be compared with that of the Gaussian both representing the sensitivity of the system in consideration.

The couples fluoxetine – estrone, estrone – fluoxetine, estrone – oxyteracycline and oxyteracycline – estrone are cases where no adequate models for any of the couple could be obtained and no decision about the kind of interaction is possible.

For couples oxytetracycline – progesterone and progesterone – oxytetracycline the "a" parameters (Gaussian) and for *Power Law Family/Modified Hoerl*

$$y = a \cdot b^{\left(\frac{1}{x}\right)} \cdot x^c \quad (6)$$

reached 66.3 and 35.12, respectively. The a-coefficient for the Gaussian model is bigger than that of the modified Hoerl one. It might mean that the decrease of the effect with respect to the standard model (Gaussian) is probably due to the dependent action with antagonistic effect between the pharmaceuticals.

For couples diclofenac - progesterone and progesterone – diclofenac the "a" parameters reached 78.8 (Gaussian) and 140285 (*Growth Models/Exponential Association 3*):

$$y = a \cdot \left(b - e^{(-cx)}\right) \quad (7)$$

The a-coefficient for the Gaussian model is much lower than that of the exponential model (four orders of magnitude). It might mean the strong increase of the effect caused with respect to the standard model (Gaussian). Probably the one faces dependent action with synergetic effect.

$$y = a \cdot \frac{x}{b + x} \quad (8)$$

For couples estrone – diclofenac (*Growth Models/Saturation Growth Rate*) and diclofenac – estrone (Gaussian) the "a" parameters reached 43.4 and 81.1, respectively. The a-coefficient for the Gaussian model is bigger than that of the Saturation Growth Model. It might mean that the decrease of the effect with respect to the standard model (Gaussian) is caused probably by dependent action with antagonistic effect.

For couples ketoprofen – androstenesione (Gaussian) and androstenedione – ketoprofen (*Growth Models/Exponential Association 3* (Eq. 7)) the "a" parameters reached 68.0 and 4.56, respectively. The a-coefficient for the Gaussian model is much lower than that of the exponential model (three orders of magnitude). It might mean the strong increase of the effect caused by the standard model (Gaussian). Probably the one faces dependent action with synergetic effect.

For couples gemfibrozil – progesterone (Gaussian) and progesterone – gemfibrozil (*Growth Models/Exponential Association 3* (Eq. 7)) the "a" parameters reached 49.3 and 75.3, respectively. The a-coefficient for the Gaussian model is much lower than that of the exponential model (three orders of magnitude). It might mean the strong increase of the effect caused with respect to the standard model (Gaussian). Probably the one faces dependent action with synergetic effect.

For couples gemfibrozil – estrone (Gaussian) and estrone – gemfibrozil (*Growth Models/Saturation Growth Rate* (Eq. 8)) the "a" parameters reached 47.4 and 9.7, respectively. The "a" coefficient for the Gaussian model is five times bigger than that of the Saturation growth model. It might mean that the decrease of the effect with respect to the standard model (Gaussian) is caused probably by dependent action with antagonistic effect.

For couples estrone – ketoprofen (*Growth Models/Exponential Association 3* (7)) and ketoprofen – estrone (Gaussian) the "a" parameters reached 648,255 and 34.8, respectively. The "a" coefficient for the Gaussian model is much lower than that of the exponential model (four orders of magnitude). It might mean the strong increase of the effect caused with respect to the standard model (Gaussian). Probably the one faces dependent action with synergetic effect.

It could be assumed that in the couples A/B and B/A where one of the models is subject to Gaussian model, the interaction (the deviations from the Independent Action we call "Dependent Action" which could be either synergetic or antagonistic) is rather synergic one (increase of "a" parameter value with respect to value of the Gaussian model) since in the rest of the models antagonistic action could be expected.

For the couples of models fluoxetine – estrone/estrone – fluoxetine and estrone – oxyteracycline/oxyteracycline – estrone one cannot apply these empiric rules since the models for the couples are rather different and also the experimental data are quite specific.

It can be noticed that estrone is very often in "Dependent Action" mode (10 combinations). In cases of progesterone (6 combinations), diclofenac (4 combinations), ketoprofen (4 combinations), gemfibrozil (4 combinations), oxytetracycline (4 combinations), fluoxetine (2 combinations), androstenedione (2 combinations) they also exhibit dependent mode of action. Chloramphenicol (all of the combinations) show only "independent action" mode.

3.1.2. Calculation of model deviation ration (MDR) for Microtox® assay

Model deviation ratios for CA and IA experiments with Microtox® test are summarized in Fig. 1. Median MDR values for CA and IA models are very close to one, exactly 1.04 and 0.92, respectively. There is a good agreement between predicted and observed effective concentrations for both models. All MDR values are within range from 0.5 to 1.5 and there is no evidence for synergistic or antagonistic interaction between pharmaceuticals.

3.2. XenoScreen YES/YAS®

3.2.1. Modeling by correlation analysis of drug mixtures for XenoScreen YES/YAS® assay

Correlation coefficients (based on correlation between the series of input experimental data) for each drug mixture was calculated for each one of the four ecotoxicity tests marked as YES+, YES−, YAS+ and YAS−. The four different types of tests indicated quite different type of correlation for even one and the same drug combination. For instance, the combination "oxyteracycline – fluoxetine" is defined as "with independent action" by YES+ test and YAS+ test and as "with dependent action" by YES− and YAS− test (in both cases – antagonistic). Thus, for each of the mixtures and for each test a respective assumption could be made.

It has to be pointed out that the separation of the values of the correlation coefficient into categories (significant/non-significant) is widely used in statistical analysis. There are even more detailed ranking schemes with "lack of correlation at $r < 0.2$", "low correlation for $0.2 < r < 0.4$", "moderate correlation for $0.4 < r < 0.7$", "strong correlation for $r > 0.7$–0.8.

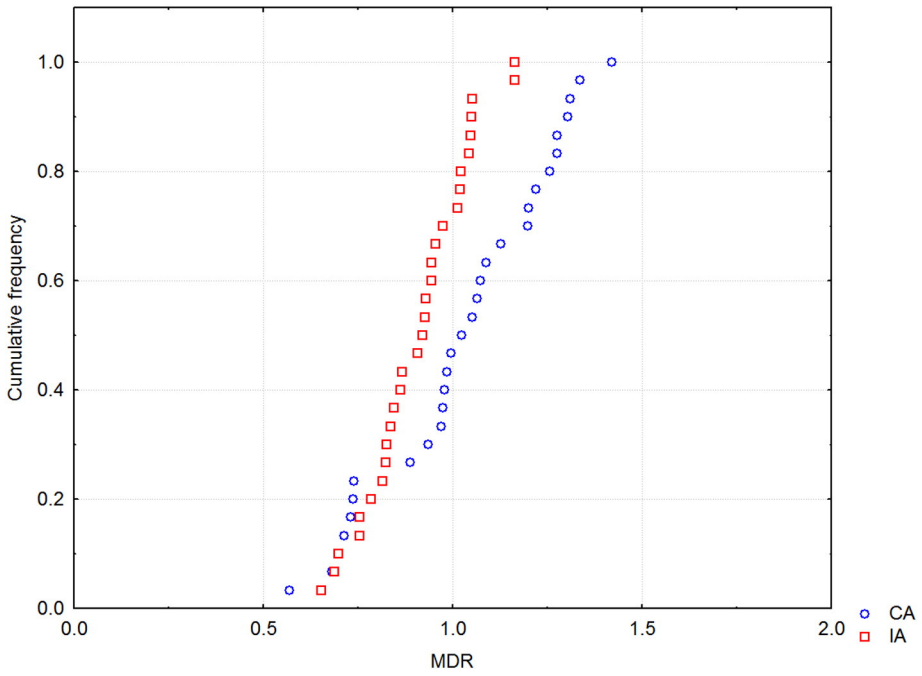


Fig. 1. The cumulative distribution of model deviation ratios (MDR) for CA and IA models of Microtox® experiments.

In order to improve the interpretation by correlation analysis from the experimental data cluster analysis of the correlation data table above was carried out. The goal is to detect specific patterns of similarity in the data structure both for drug mixtures and ecotoxicity tests (see Supplementary Table 2 for details).

The hierarchical cluster analysis was performed using Ward's linkage method and squared Euclidean distance as similarity measure. Cluster significance is checked by Sneath's criterion with 1/3 D_{max} and 2/3 D_{max} value.

In Fig. 2 the hierarchical dendrogram for linkage of variables (ecotoxicity tests) is presented.

It could be seen that the four test are divided into two groups (YES+, YAS+) and (YES−, YAS−), i.e. there is separation between "+" and "−" tests (the similarity between "−" tests is not very high).

In Fig. 3 the linkage between drug mixtures is shown (the same method of clustering). Four well separated clusters are formed as presented in Supplementary Table 3. Each member of the cluster is characterized by respective correlation coefficients in order to improve the interpretation.

Cluster 1 is formed by drug mixtures having dominantly synergetic action (by YES+ and to certain extent by YES−). There are several mixtures with synergetic indication by YAS+ (3 out of 11) and by YAS− (4 out of 11). Thus, formally this cluster could be determined as "*mixtures with dominantly synergetic behavior*".

The second cluster includes mixtures indicating dominantly antagonistic behavior if checked by YES+ and YAS+ and synergetic action if diagnosed by YES− and YAS−. Therefore, this cluster could be conditionally named "*separation of dependent action by '+' and '−' tests*".

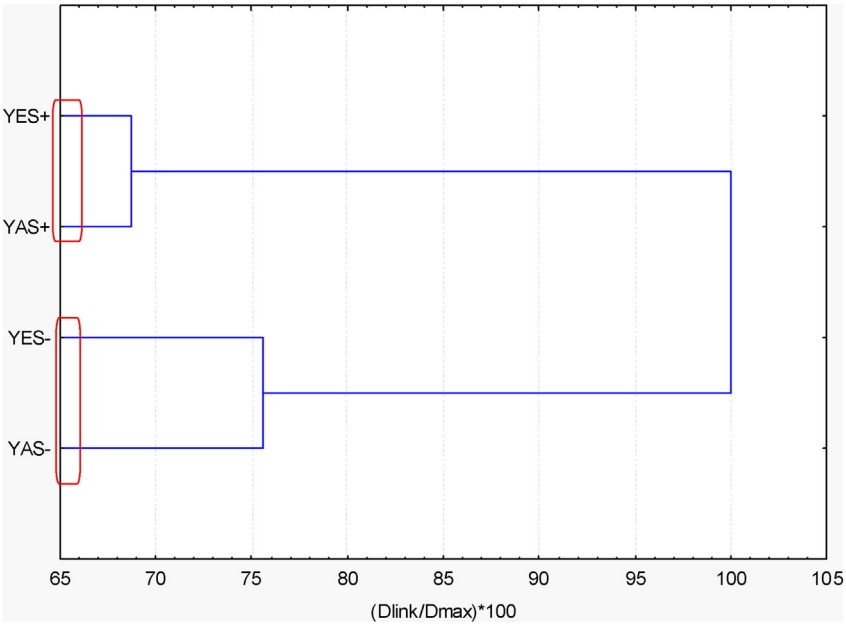


Fig. 2. Hierarchical dendrogram for 4 variables for XenoScreen YES/YAS® assay.

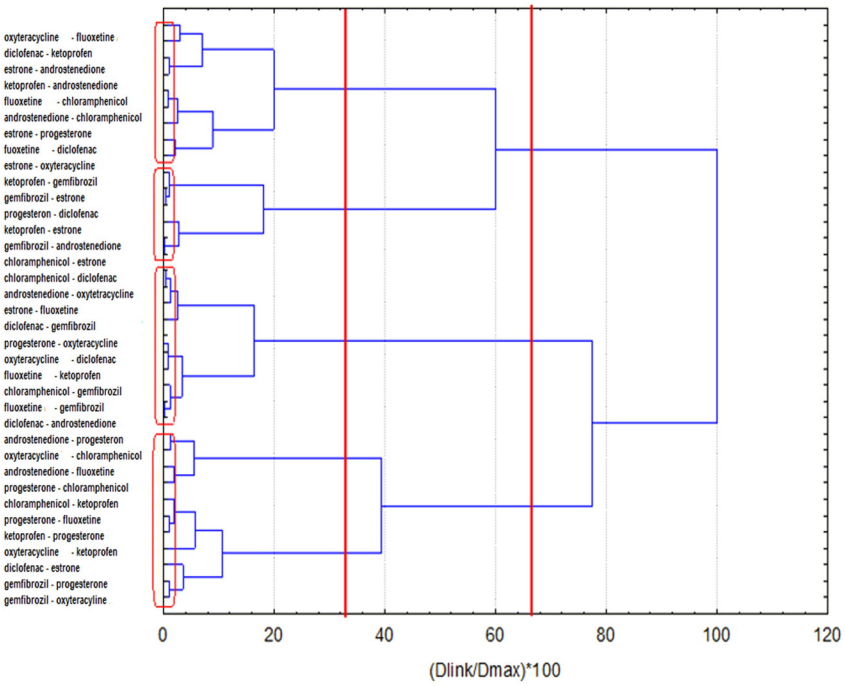


Fig. 3. Hierarchical dendrogram for 36 drug mixtures for XenoScreen YES/YAS® assay.

The third cluster is the smallest one. It includes drug mixture for which the YES tests (both + and -) indicate antagonistic action and the YAS tests – dominantly synergetic action. Its conditional name could be "*separation of dependent action by XenoScreen YES/YAS®*".

The final fourth cluster is quite specific one. If we accept that $r < 0.7$ should be considered as "no correlation" then 7 out of 10 mixtures tested by YES + and 6 out of 10 mixtures tested by YES — indicate independent action. For YAS tests 3 out of 10 mixtures are also ranked as mixtures with independent action. Conditionally, we could determine this cluster as "*dominantly mixtures with independent action*".

In Supplementary Table 4 the results for the behavior of the different drug mixtures with respect to the different tests is indicated. The comparison is performed using the correlation coefficients for the linear regression models for each combination of drugs.

3.2.2. Calculation of model deviation ration (MDR) for XenoScreen YES/YAS® assay

The 80% of obtained MDR values for CA and IA models with YES + test are higher than 1 (Fig. 4a) with medians 2.02 and 1.88, respectively. Such a results indicate overprediction of toxic concentrations for both models. The MDR values of 11 pairs of pharmaceuticals are higher than 2 for both models and could be considered as mixtures where antagonistic interaction between substances present. In 6 out of 11 mixtures one of the pharmaceuticals is diclofenac and the second is androstenedione, fluoxetine, oxyteracycline, progesterone, ketoprofen or chloramphenicol. In 2, out of 11, gemfibrozil is in combination with chloramphenicol and androstenedione. The other 3 mixtures are oxyteracycline - chloramphenicol, fluoxetine - estrone and ketoprofen - progesterone.

The MDR values for YES — test show bigger difference between both models (Fig. 4b). Median of MDR values for IA is 0.94 since median equal to 2.08 CA model overpredict toxicity of mixtures. Three mixtures fluoxetine - estrone, androstenedione - progesterone and ketoprofene - progesterone have MDR values higher than 2 for both models. For couples fluoxetine – estrone and ketoprofen - progesterone similar behavior indicating antagonistic interaction at YES + test was also observed.

The MDR values for YAS + test also exhibit difference between CA and IA results as both models slightly overpredict effective concentrations (Fig. 4c). Medians of MDR are 1.14 for IA and 1.40 for CA models. One of the mixtures gemfibrozil – diclofenac possesses MDR values for both models higher than 2 which is an indication for possible antagonistic interaction between pharmaceuticals. The MDR value of IA model of another mixture of gemfibrozil with chloramphenicol is close to 2 (1.97) and also could be considered for mixture antagonistic interaction between pharmaceuticals which already was shown at YES + test.

The MDR values for YAS- test clearly display the biggest difference between CA and IA predictions among all toxicity tests (Fig. 4d). The 90% of MDR values obtained by IA model are lower than 1 while all MDR values obtained by CA model are higher than 1.5. Medians of MDR values for both models, 1.97 for CA and 0.69 for IA, also indicate for underprediction of the toxicity of mixtures by IA and overprediction by CA model. Here only two pairs of pharmaceuticals ketoprofen - androstendione and progesterone – estrone could be considered antagonistic mixtures nevertheless that their MDR values for IA experiments are 1.99 and 1.82, respectively.

4. Conclusions

Hormones and pharmaceuticals known as believed to be newly emerging contaminants in the environment. Next to it, there is a problem of plausible changing ecosystems stability due to presence of pharmaceuticals at different concentration levels and their biotransformation products. Interactions occurring between them under environmental conditions are almost *carte blanche* in modern environmental science. The determination of the mixed effect of toxicity for combination of two drugs proved to be a complicated task. In the present study an effort was undertaken to assess the toxicity interaction of drug mixtures using statistical approach of modeling of the toxicity effects with respect to possible independent, antagonistic or synergistic action of both components of the mixture. One of the assessment approaches was based on model deviation ratio calculation. Most of the drug mixtures studied show independent mode of action and quite few of them are exceptions with antagonistic or synergistic action.

For data (obtained with the Microtox® test) modeling by best fit models we found that drugs in mixture with hormones have shown mainly antagonistic or synergistic actions *e.g.* ketoprofen in mixture

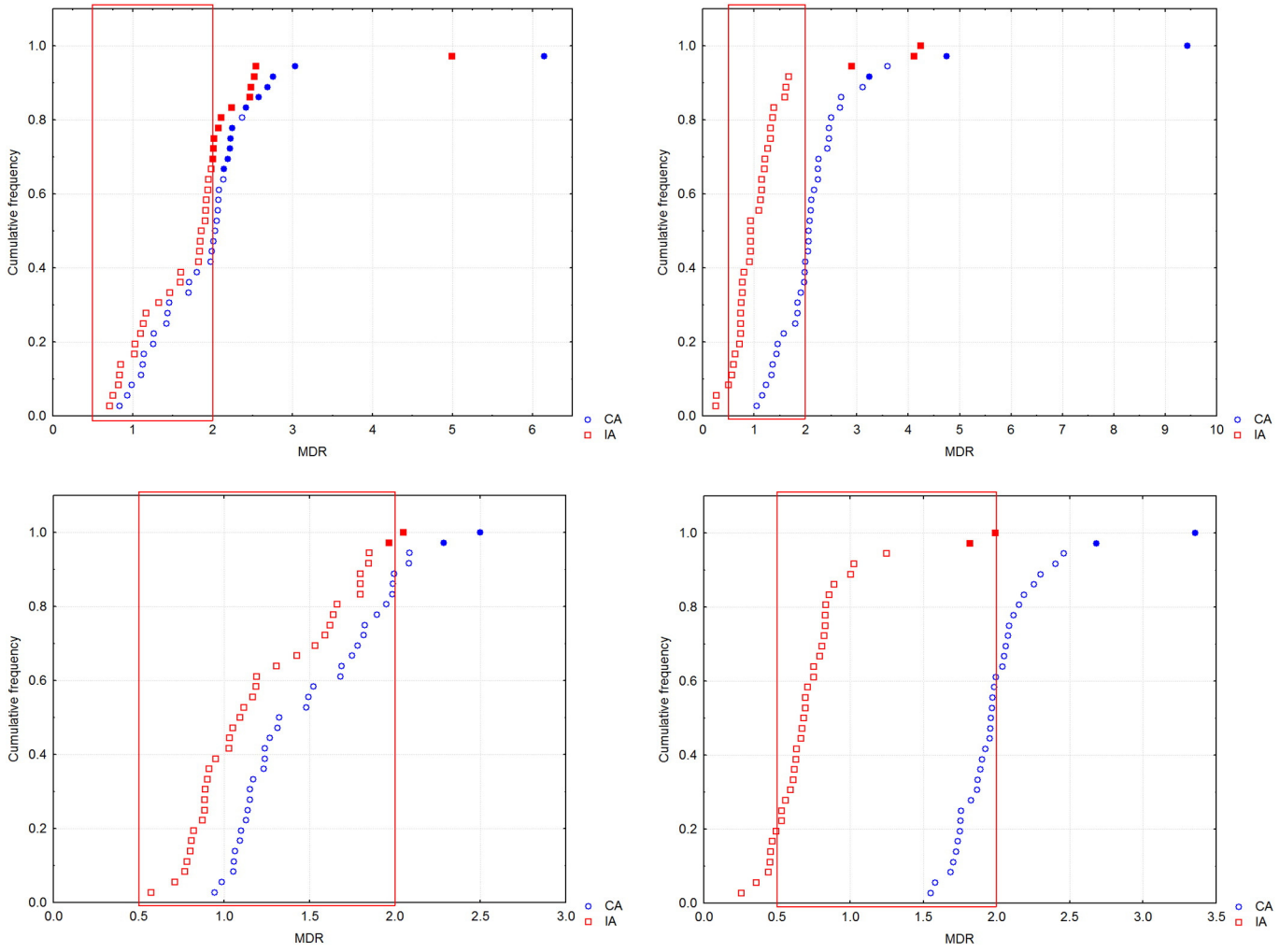


Fig. 4. The cumulative distribution of model deviation ratios (MDR) for CA and IA models of a) YES +, b) YES −, c) YAS +, d) YAS − test results (results of mixtures with MDR values outside range from 0.5 to 2 for both models are marked).

with androstenedione and estrone proved to be synergic in its nature, gemfibrozil with progesterone exhibited synergism while antagonism with estrone, diclofenac with progesterone exhibited synergic properties and antagonistic ones with estrone.

XenoScreen YES/YAS® data processed using the best fit model showed that oxytetracycline in the mixture with chloramphenicol, gemfibrozil and fluoxetine has synergic properties, while with chloramphenicol and gemfibrozil the antagonistic ones. Data compiled with MDR indicate the antagonist action in most cases of mixtures of diclofenac, gemfibrozil, fluoxetine, oxyteracycline, progesterone, ketoprofen and chloramphenicol.

Although very different in its nature, the best-fit function modeling approach found, in general, the same results – 75% of the tested mixtures indicate possible independent action and the rest 25% - possible antagonistic or synergistic action. Another important result is that different toxicity tests could indicate different mode of action for one and the same couple (XenoScreen YES/YAS®). The present study does not pretend to find answers about the mechanism of combined toxicity of drug mixture from the environment (such mechanisms are mostly studied in cases of toxicological studies on living organisms) but could serve as useful empirical manual for selecting ecotoxicity tests for assessment of possible additive, synergetic or independent action of binary drug mixtures.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.07.186.

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