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Synergistic interaction of diclofenac and its metabolites with selected antibiotics and amygdalin in wastewaters

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ABSTRACT

Diclofenac (DCF), a non-steroidal anti-inflammatory drug (NSAID) belongs to one of the most frequently detected pharmaceutical residues in the environment. Little is known on the interactions of DCF as well as its major biodegradation metabolites 4′-OHDCF and 5-OHDCF with chemical compounds found in wastewater, including antibiotics such as ampicillin and kanamycin. In the present work we examined the potential interactions between DCF, its metabolites 4′-OHDCF and 5-OHDCF and ampicyllin and kanamycin. We also measured the effect of the mixture of DCF with natural compound – amygdalin. We evaluated the following parameters: *E. coli* K-12 cells viability, growth inhibition of *E. coli* K-12 culture, genotoxicity, oxidative stress parameters: *sodA* promoter induction and ROS generation. The reactivity of *E. coli* SM *recA:luxCDABE* biosensor strain in wastewaters matrices contaminated with DCF and kanamycin was also monitored. Obtained results indicated that used antibiotics (ampicyllin, kanamycin) enhanced the toxic effect of DCF used individually and in the mixtures with its metabolites 4′-OHDCF and 5-OHDCF toward *E. coli*. Similar effect was also obtained in genotoxicity assay. The oxidative stress assays revealed that the highest level of ROS generation and *sodA* promoter induction were obtained also for the mixtures of DCF, its metabolites with antibiotics. It was also showed that amygdalin influenced the activity of DCF and its biodegradation metabolites. The strongest luminescence response of *E. coli* SM biosensor strain with *recA:luxCDABE* genetic construct in filtered treated wastewaters, comparable to control sample was noticed. Obtained results showed that DCF and its biodegradation metabolites 4′-OHDCF and 5-OHDCF can interact with tested antibiotics and compounds of natural origin, i.e. amygdalin to form mixtures showing stronger antimicrobial activity against *E. coli* than parent chemicals. Moreover the assays in wastewater matrices revealed that *E. coli* SM *recA:luxCDABE* biosensor strains is a good tool for bacteria monitoring in wastewater environments.

1. Introduction

Diclofenac (DCF) 2-(2,6-dichloroanilino)phenylacetic acid belongs to the group of non-steroidal, anti-inflammatory (NSAID) and pain relieving drugs and it is one of the most frequently detected anthropogenic micropollutant in environmental matrices (Sochacki et al., 2018; Sathishkumar et al., 2019). The maximum detection limit of DCF in municipal wastewaters ranges around 7 μg/L (Vieno and Sillanpää, 2014; Lonappan et al., 2016). The DCF concentrations vary between countries with higher concentrations in hospital and manufacturers wastewaters (Felis et al., 2009; Huber et al., 2016). Several ecotoxicological studies revealed that exposure to DCF affects biochemical processes of living organisms such as aquatic organisms, duckweed plants and freshwater fish (Lonappan et al., 2016; Cunha et al., 2017; Moreira et al., 2018). DCF has been suggested to be added into the list of priority substances in EU's Water Framework Directive (Directive, 2013/39/EU) (Lonappan et al., 2016). The main products of DCF metabolism are hydroxylated metabolites: 4′-OHDCF, 5-OHDCF, 3-OHDCF and 4,5-diOHDCF (Lonappan et al., 2016; Stylianou et al., 2018; Palyzová et al., 2019). Most of the works concerns the assessment of toxicity and the environmental impact of APIs. However, the impact of API mixtures and their metabolites with other chemical compounds occurring in wastewater and surface water, both of anthropogenic and natural origin is still not completely understood (Liu and Wong, 2013;

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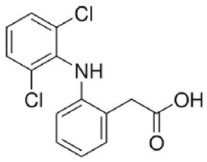
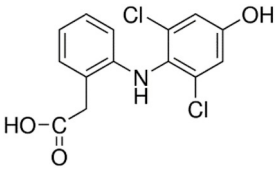
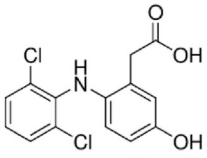
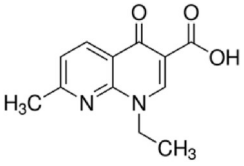
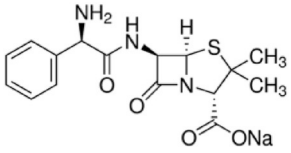
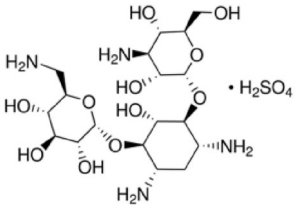
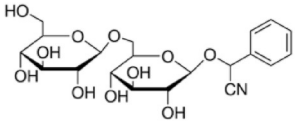
Table 1
Chemical structures and molecular formulas of tested chemicals.

Chemicals	Chemical structure	Molecular formula
Diclofenac InChIKey - DCOPUUMXTXDBNB-UHFFFAOYSA-N		$C_{14}H_{11}Cl_2NO_2$.
Biodegradation metabolite: **4′-hydroxydiclofenac** InChIKey -KGVXVPRLBMWZLG-UHFFFAOYSA-N		$C_{14}H_{11}Cl_2NO_3$
Biodegradation metabolite: **5-hydroxydiclofenac** InChIKey -VNQURRWYKFZKJZ-UHFFFAOYSA-N		$C_{14}H_{11}Cl_2NO_3$
Antibiotic: **Nalidixic acid** InChIKey -MHWLWQUZZRMNGJ-UHFFFAOYSA-N		$C_{12}H_{12}N_2O_3$
Antibiotic: **Ampicillin sodium salt** InChIKey -KLOHDWPABZXLGI-YWUHCJSESA-M		$C_{16}H_{18}N_3NaO_4S$
Antibiotic: **Kanamycin sulfate** InChIKey -OOYGSFOGFJDDHP-KMCOLRRFSA-N		$C_{18}H_{36}N_4O_{11} \cdot H_2O_4S$
Amygdalin InChIKey -XUCIJNAGGSZNQT-UHFFFAOYSA-N		$C_{20}H_{27}NO_{11}$

Explanations: InChIKey - International Chemical Identifier key.

Lonappan et al., 2016; Matejczyk et al., 2020). DCF and its metabolites enter the environment mainly through wastewater treatment systems, where DCF is only partially degraded (Huber et al., 2016; Lonappan et al., 2016; Ivshina et al., 2019).

Antibiotics are a large group of biologically active compounds used extensively in human and veterinary healthcare. Antibiotics are detected in various environmental matrices, such as WWTPs (Wastewater Treatment Plants), surface water, groundwater and drinking water at concentrations ranging from ng/L to μg/L. Due to antimicrobial activity of antibiotics they have ability to inhibit biological activity of activated sludges (Mutiyar and Mittal, 2014; Hanna et al., 2018). Moreover, the presence of antibiotics in the environment contributes to the spread of the phenomenon of antibiotic resistance that is a major threat to public health (Kümmerer, 2009). In addition, antibiotics can affect wildlife, even at trace levels. Therefore, the European Union (EU) recommends the prudent use of antimicrobial agents in medicine (Tahrani et al., 2016).

Ampicillin (AMP) is a broad-spectrum semi-synthetic β-lactam antibiotic that are often used in human and animal treatment and in livestock production (Chen et al., 2019). The residues of β-lactam antibiotics and their metabolites are often detected in wastewaters. Kanamycin belongs to the group of aminoglycoside antibiotics that are widely used in hospitals for treatment of serious human infection by Gram-negative and Gram-positive bacteria and in veterinary medicine. Aminoglicoside residues are present in environmental matrices (Chen et al., 2019).

Amygdalin (D-mandelonitrile-β-D-gluco-sido-6-β-D-glucoside) is naturally occurring cyanogenic glycoside compound present in stone fruits and berries like apricots, plums, peaches, apples, papaya and cherries. Amygdalin is also in plants like rice, unripe sugarcane, sorghum, and certain species of nuts. Amygdalin was also found in the leaves and foliage of plants like Saskatoon berries and mature trees of the Prunus taxa. It was shown that amygdalin has antiinflammatory and antimicrobial activity (Jaswal et al., 2018). Amygdalin is comercially available as dietary supplement and it is popular among consumers (Long et al., 2019).

Table 2
Composition of samples and concentrations of tested chemical compounds.

Lp.	Sample
1.	DCF (3 μg/ml)
2.	4′-OHDCF (0.3 μg/ml)
3.	5-OHDCF (0.3 μg/ml)
4.	Ampicillin (3 μg/ml)
5.	Kanamycin (3 μg/ml)
6.	Amygdalin (3 μg/ml)
7.	Nalidixic acid (3 μg/ml)
8.	DCF (3 μg/ml) + 4′-OHDCF (0.3 μg/ml)
9.	DCF (3 μg/ml) + 5-OHDCF (0.3 μg/ml)
10.	DCF (3 μg/ml) + 4′-OHDCF (0.3 μg/ml) + Ampicillin (3 μg/ml)
11.	DCF (3 μg/ml) + 4′-OHDCF (0.3 μg/ml) + Kanamycin (3 μg/ml)
12.	DCF (3 μg/ml) + 4′-OHDCF (0.3 μg/ml) + Amygdalin (3 μg/ml)
13.	DCF (3 μg/ml) + 4′-OHDCF (0.3 μg/ml) + Nalidixic acid (3 μg/ml)
14.	DCF (3 μg/ml) + 5-OHDCF (0.3 μg/ml) + Ampicillin (3 μg/ml)
15.	DCF (3 μg/ml) + 5-OHDCF (0.3 μg/ml) + Kanamycin (3 μg/ml)
16.	DCF (3 μg/ml) + 5-OHDCF (0.3 μg/ml) + Amygdalin (3 μg/ml)
17.	DCF (3 μg/ml) + 5-OHDCF (0.3 μg/ml) + Nalidixic acid (3 μg/ml)

Recently, many scientific papers have been pointed the subject of the occurrence and degradation of DCF. However, very little is known about the interaction of DCF and its biodegradation metabolites with antibiotics, which next to DCF belong to the most frequently detected pharmaceutical pollution in wastewater (Lonappan et al., 2016; Kümmerer, 2009; Vieno and Sillanpää, 2014; Fatta-Kassinos et al., 2011). DCF and its metabolites have a chance to react with antibiotics and amygdalin in wastewaters and surface waters. Therefore, in this work, it was decided to study the impact of mixtures of DCF and its metabolites with antibiotics and amygdalin on *E. coli* strains. The following parameters were examined in the work: *E. coli* K-12 cells viability assay, *E. coli* K-12 culture growth inhibition test, oxidative stress: ROS generation and superoxide dismutase protein (*sodA*) promoter induction and genotoxicity test. The response pattern of *E. coli* SM *recA:luxCDABE* in artifically contaminated with mixture of DCF and kanamycin raw and treated, filtered and unfiltered wastewaters was also monitored. For genotoxicity and oxidative stress assessment we used two *E. coli* SM biosensor strains with plasmid fusion of *recA* (genotoxicity assay) and *sodA* (oxidative stress assay) promoters to *luxCDABE* promoter gene. In the work of Melamed et al. (2012) and other authors (Melamed et al., 2012; Kessler et al., 2012) it was shown that, *E. coli* SM *luxCDABE* strain with *recA* and *sodA* promoters were successfully used for detection of genotoxic and oxidative stress conditions after bacteria treatment with different class of drugs, including antibiotics.

2. Materials and methods

2.1. DCF transformation products and other tested chemical compounds

In order to select biodegradation metabolites of DCF, a literature review in the PubMed database on this subject was conducted. We selected 4′-OHDCF and 5-OHDCF which were detected and identified by scientists as biodegradation metabolites of DCF in several scientific publications (Moreira et al., 2018; Lonappan et al., 2016; Marco-Urrea et al., 2010; Langenhoff et al., 2013; Vieno and Sillanpää, 2014; Bouju et al., 2016; Haiba et al., 2017; Kawase et al., 2017; Doruk et al., 2018; Schmidt et al., 2018). DCF (Diclofenac sodium salt), 4′-OHDCF, 5-OHDCF, ampicillin (ampicillin sodium salt), kanamycin (kanamycin sulfate), nalidixic acid and amygdalin were commercially obtained from Sigma Aldrich, UK. Antibiotics were selected on the base on their presence in the environment (Chen et al., 2019). Nalidixic acid (NA) was used as a positive control. Chemical structures and molecular formulas of tested chemicals were presented in Table 1.

2.2. Selection of concentrations of DCF and its metabolites

The concentration range of the analyzed chemicals were selected experimentally from the minimum level of promoter-reporter gene *luxCDABE* constructs sensitivity. The most optimal solution would be to use concentrations corresponding to the concentrations of the tested APIs in the environment, on the order of μg/L or ng/L. Unfortunately, at such low concentrations, we did not observe satisfactory results of the reaction of *E. coli* SM biosensor strains with *recA* and *sodA* promoters. Schmidt et al. (2018) reported that in most of the analyzed samples of wastewater or surface water, about 10 times lower concentrations of 4′-OHDCF are detected in comparison to DCF. However, it was also reported that in Spanish wastewaters treatment plants, 4′-OHDCF was present in much higher concentrations than DCF. In relation to the above, excluding DCF biodegradation metabolites (4′-OHDCF and 5-OHDCF), the remaining compounds were used at concentration of 3 μg/ml. DCF biodegradation metabolites 4′-OHDCF and 5-OHDCF were used at concentrations of 0.3 μg/ml.

2.3. Chemical treatment of E. coli cultures

All tested chemicals were dissolved in dimethyl sulfoxide (DMSO) (Chempur). To reduce the impact of DMSO on the bacterial culture, prepared, solutions of tested compounds in DMSO were diluted in the culture medium in a ratio of 1: 9 (1 ml DMSO and 9 ml medium). The compounds were added to the *E. coli* cultures for a final concentrations of 3 μg/ml for DCF, ampicillin, kanamycin, nalidixic acid and amygdalin and 0.3 μg/ml for 4′-OHDCF and 5-OHDCF. A detailed description of the composition of individual samples tested in the work is presented in Table 2. The *E. coli* control cultures were incubated without the tested compounds.

2.4. E. coli cell viability assay

The *E. coli* cell viability assay after treatment with tested chemicals was determined using BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, WI, USA) in *E. coli* K-12 culture. According to this method the determination of the number of viable microbial cells is based on quantitation of the ATP present. The formulation of the reagent supports bacterial cell lysis and generation of a luminescent signal. The stable luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of viable cells in culture. This method allows the detection of growth or toxicity of bacteria culture (Promega instructions). *E. coli* K-12 was grown in Mueller Hinton II (MH II) broth at 37 °C overnight. The overnight culture was diluted 50-fold in fresh MH II Broth and then incubated to reach log phase (OD_{600} = 0.2). Bacterial cell numbers were determined by plate counting of colony forming units on Luria-Bertani agar plates. Tested chemicals were added to 10^7 CFU/ml of *E. coli* culture at appropriate concentrations and incubated for 24 h. Control sample did not contain any chemicals. Nalidixic acid (NA) was used as a positive control. NA is a selective inhibitor of the type II topoisomerase DNA gyrase and topoisomerase IV. These enzymes play a critical role in bacterial cell growth and division. The assay was performed according to the Promega attached protocol. RLU (Relative Luminescence Units) was recorded on a GloMax®-Multi Microplate Multimode Reader, Promega. In all the RLU assays, data were normalized to bacteria concentration measured spectrophotometrically as OD value at 600 nm. The viability of *E. coli* cells was measured after 24 h incubation with tested chemicals and it was calculated as a percentage of control cells, incubated without tested compound. Three independent experiments were done.

2.5. E. coli K-12 culture growth inhibition effect

The growth inhibition effect of DCF, its biodegradation metabolites,

antibiotics and amygdalin was estimated for *E. coli* K-12 culture and with use of spectrophotometric method based on Optical Density (OD) measurement at wavelength of 600 nm. *E. coli* culture was grown in Luria-Bertani (LB) broth (BTL) at 37 °C overnight. The overnight culture was diluted 50-fold in fresh LB broth and then incubated to reach log phase (OD_{600} = 0.2). Tested chemicals and the mixtures were added to 10^7 CFU/ml of *E. coli* culture at appropriate concentrations and incubated for 24 h. Non-treated *E. coli* cultures were used as the control. The values of bacteria growth inhibition (GI) after 24 h treatment with tested compounds and the mixtures were calculated according to the formula: GI (%) = OD_{CS} (%) – D_{ODTS} (%), where: OD_{CS} (%) – Optical Density of control sample = 100%; D_{ODTS} (%) – the percent of decrease in the value of Optical Density of bacteria samples treated with chemicals in relation to OD value of control sample. The results are shown as growth inhibition (GI) values (%) in comparison to the control sample, not treated bacteria. Experiments were conducted in triplicate.

2.6. Genotoxic effect estimation

Genotoxic effects of the DCF, its metabolites, antibiotics, amygdalin and the mixtures were measured with bacterial biosensor *Escherichia coli* SM342/pBRlux-trp:*recA*::*luxCDABE* strain with reporter plasmids, harbouring a *recA* promoter sequence fused to the *luxCDABE* reporter genes in the pBRlux-trp vector. Several previous studies reported that assays based on transcriptional fusions between DNA-damage inducible *recA* promoter and reporter system *luxCDABE* has been used to detect a variety of genotoxic agents. *RecA* promoter belongs to the group of DNA-damage inducible promoters of genes involved in SOS repair system, which is activated in response to DNA damage. These sensors are based on light production resulting from the action of the enzyme, luciferase (Melamed et al., 2012; Kessler et al., 2012; Kostrzyńska et al., 2002; Lee et al., 2013). Nalidixic acid (NA) has been used as a model genotoxin and inducer of DNA-damage SOS response for many bacterial sensors (Melamed et al., 2012; Kessler et al., 2012; Kostrzyńska et al., 2002; Lee et al., 2013; Matejczyk et al., 2017, 2018). *E. coli* strain used in this study was presented in Table 3. *Escherichia coli* SM342/pBRlux-trp:*recA*::*luxCDABE* strain was cultured overnight in M9 medium at 37 °C. Luminescence measurement and data analysis were conducted according to method described by Melamed et al. (2012) and Kessler et al. (2012). Bacteria cultures were diluted 100-fold in fresh LB and regrown at room temperature with shaking (200 r.p.m.) to early log phase (OD_{600} = 0.2). Sensitivity of these strains were characterized by monitoring their bioluminescence as a response to chemical exposure. Culture aliquots (50 μl) were then transferred into the wells of an opaque white 96-well microtiter plate (Greiner Bio-One, Germany) containing 50 μl of either predetermined concentrations of the tested sample or of a sample-free control. Luminescence was measured using a GloMax®-Multi Microplate Multimode Reader (Promega) at time zero and after 5 h of incubation. The luminescence signal is proportional to the level of *recA* promoter induction and genotoxic potency of tested chemical. At each point of measurement, luminescence of each sample was normalized to its bacterial concentration measured spectrophotometrically as OD value at 600 nm. Every experiment included a negative control, chemical-free double deionized water. Nalidixic acid (NA) was used as positive control. Chemicals-free bacterial culture was used as the control. The results are showed as fold induction values in comparison to the control, non treated bacteria culture. Experiment was carried out in three independent series.

2.7. Assessment of luminescence values

Luminescence intensity of each sample was calculated according to the formula:

$$L = IL/OD$$

where: L - Luminescence, IL – The raw luminescence intensity of the sample, OD – Optical Density of the sample measured at 600 nm.

2.8. The fold induction values calculations

The response data was expressed as fold induction (FI) normalized with control and calculated according to the formula:

$$FI = L_{TS}/L_{CS};$$

where: FI – fold induction; L_{TS} – luminescence values of tested sample; L_{CS} - luminescence values of control sample.

2.9. Oxidative stress assay

The imbalance between the generation and the neutralization of reactive oxygen species (ROS) by antioxidant mechanisms within an organism is called oxidative stress (Valavanidis et al., 2006). In environmental toxicology and ecotoxicological studies for the evaluation of the damage induced by pharmaceutical residues, oxidative stress parameters analysis are used. In many cases, the toxic effects of xenobiotics, including pharmaceuticals are mediated by reactive oxygen species (ROS) formation (Islas-Flores et al., 2013, 2017). Organisms are able to adapt to stresses by inducing the synthesis of antioxidant enzymes to regulate oxidative damage (Valavanidis et al., 2006). Next to the ROS generation, the induction of superoxide dismutase (SOD) gene promoter assay is also important biomarker for monitoring environmental pollution (Melamed et al., 2012; Islas-Flores et al., 2013).

2.9.1. ROS generation

The production of ROS by *E. coli* K-12 after treatment with DCF and its metabolites, antibiotics, amygdalin and the mixtures was evaluated using a 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, UK) and according to the method described by Ong et al. (2017) and Díaz-García et al. (2019). DCFH-DA can detect a broad range of ROS including nitric oxide and hydrogen peroxide. *E. coli* K-12 was grown in Luria-Bertani (LB) broth (BTL) at 37 °C overnight. The overnight culture was diluted 50-fold in fresh LB broth and then incubated to reach log phase (OD_{600} = 0.2). 10^7 CFU/ml of *E. coli* culture were treated with tested chemicals used in appropriate concentrations. Subsequently, to bacteria culture DCFH-DA was added at a final concentration of 5 μM and incubated at 37 °C for 35 min. Non-treated bacterial suspensions were used as the control. The DCF fluorescence intensity was measured by GloMax®-Multi Detection System (Promega) at the excitation wavelength of 485 nm and the emission wavelength of 535 nm. The OD values of bacteria cultures was also monitored with use of spectrophotometer at 600 nm. The ROS generation in *E. coli* culture was shown as a percent of ROS increase in comparison to control sample, not treated bacteria. All the experiments were done in triplicates.

2.9.2. SodA promoter induction

Oxidative stress after bacteria treatment with tested chemicals and the mixture was investigated by analysis of the induction of *sodA*-

Table 3
E. coli SM strains used in this study.

Strain	Plasmid	Phenotype/genotype	Sensing element information	Stress detected	Reference
SM342	pBRlux-trp:*recA*::*luxCDABE*	*ΔtrpE/ptrpED*	DNA recombination protein, induce the SOS response to DNA damage	DNA damage	Melamed et al. (2012)
SM345	pBRlux-trp:*sodA*::*luxCDABE*	*ΔtrpE/ptrpED*	Superoxide dismutase protein	Superoxide stress	

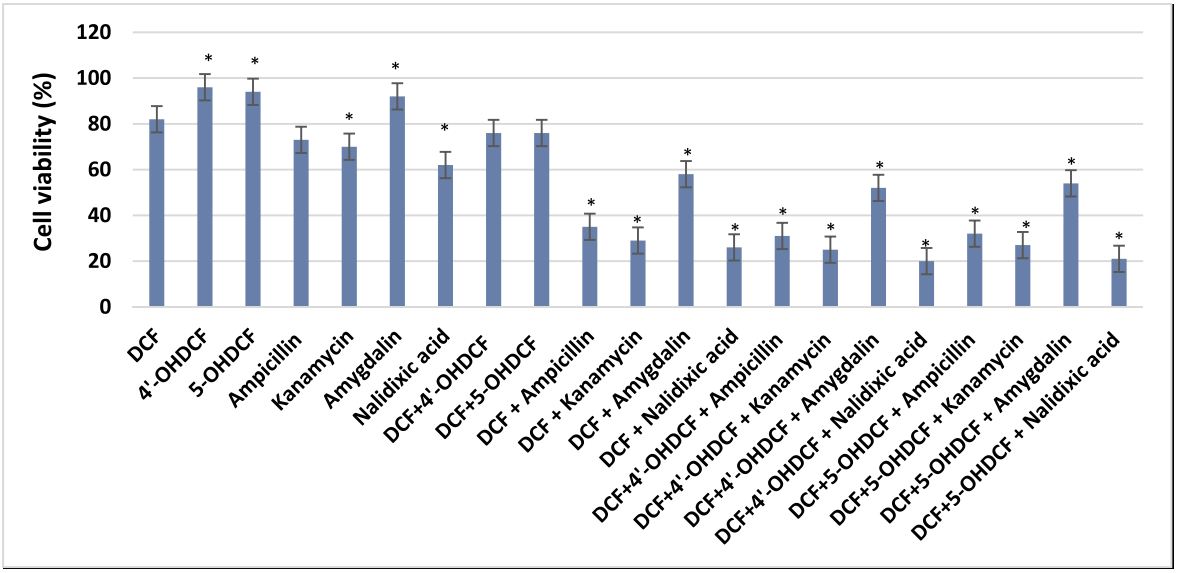


Fig. 1. The effect of DCF, 4′-OHDCF, 5-OHDCF, ampicillin, kanamycin, nalidixic acid amygdalin and their mixtures on *E. coli* K-12 cells viability after 24 h of incubation. DCF, ampicillin, kanamycin, nalidixic acid and amygdalin were applied at concentrations of 3 μg/ml; 4′-OHDCF, 5-OHDCF were used at concentrations of 0.3 μg/ml. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

luxCDABE fusion-gene products and the luminescence assay. *SodA* promoter is a promoter of superoxide dismutase gene. Superoxide dismutase (SOD) is one of the key enzyme in oxygen defense systems. SOD catalyzes the dismutation of superoxide anion into oxygen and H_2O_2, the latter being broken in turn to water by catalase or peroxidase (Merkamm and Guyonvarch, 2001). Exposure to superoxide stress activates the *sodA* promoter resulting in luminescence emission. The level of *sodA* promoter induction in *sodA-luxCDABE* genetic construct in *E. coli* SM is proportional to the level of superoxide stress and luminescence signal. Bacterial biosensor *Escherichia coli* SM342/pBRlux-trp:*sodA*::*luxCDABE* strain with reporter plasmids, harbouring a *sodA* promoter sequence fused to the *luxCDABE* reporter genes in the pBRlux-trp vector was used. *E. coli* strain used in this study was presented in Table 3. *Escherichia coli* SM342/pBRlux-trp:*sodA*::*luxCDABE* strain was cultured overnight in M9 medium at 37 °C. Luminescence measurement and data analysis were conducted according to method described by Melamed et al. (2012) and Kessler et al. (2012). Bacteria cultures were diluted 100-fold in fresh LB and regrown at room temperature with shaking (200 r.p.m.) to early log phase (OD_{600} = 0.2). Sensitivity of the strain was characterized by monitoring their bioluminescence as a response to chemical exposure. Culture aliquots (50 μl) were then transferred into the wells of an opaque white 96-well microtiter plate (Greiner Bio-One, Germany) containing 50 μl of either predetermined concentrations of the tested sample or of a sample-free control. Luminescence was measured using a GloMax®-Multi Microplate Multimode Reader (Promega) at time zero and after 5 h of incubation. At each point of measurement, luminescence of each sample was normalized to its bacterial concentration measured spectrophotometrically as OD value at 600 nm. Every experiment included a negative control, chemical-free double deionized water. Nalidixic acid (NA) was used as positive control. Chemicals-free bacterial culture was used as the control. The results are showed as fold induction values in comparison to the control, non treated bacteria culture. Experiment was carried out in three independent series.

2.10. Estimation of recA promoter induction in the matrice of raw and treated wastewaters

E. coli SM *recA:luxCDABE* biosensor strain reactivity in raw and treated municipal wastewaters from the Stawisk Wastewaters Treatment Plant (WWTP) was observed. Wastewaters samples were filtered using a bacteriological filter with a diameter of 0.45 μm (Sartorius). Next, the raw and treated, filtered and no filtered wastewaters in the sterile flasks were inoculated with *E. coli* biosensor strain with the *recA* promoter. *E. coli* SM *recA:luxCDABE* culture were then artificially contaminated with DCF, kanamycin and the mixture of the both chemicals used at concentrations of 3 μg/ml. Samples were incubated at 37 °C. Measurements of luminescence and OD values of *E. coli recA* cultures in raw and filtered wastewaters were made at time zero and after 2, 4, 24 and 48 h of incubation. The results are showed as luminescence intencity values (%) in comparison to the control, non treated with tested compounds *E. coli* SM *recA:luxCDABE* culture inoculated in raw and treated filtered and no filtered wastewaters. Experiment was conducted in triplicate.

2.11. Statistical analysis

The results of laboratory studies were subjected to statistical analysis in order to indicate statistically significant differences in selected groups of variables. Statistically significant differences between individual values were indicated using Tukey's test for the same group size. In the analysis of cell viability, growth inhibition, ROS, genotoxcity and oxidative stress values, the values of these parameters obtained for particular chemical compounds were compared separately. In the case of toxcity assay, the percentage of mortality of organisms in relation to DCF concentration and incubation time was compared. In the case of biosensor incubation *recA:luxCDABE* in wastewater, the type of wastewater and incubation time were taken into account. All variables included in the analysis were characterized by a normal distribution according to the Shapiro-Wilk test and homogeneity of variance according to the Bartlett test. The calculations were performed with the use of Statistica 13.1 software running on Windows 10 platform.

3. Results

3.1. E. coli cells viability

The assay of *E. coli* K-12 cells viability showed that all tested compounds reduced cells viability as compared to the control, non-treated cells after 24 h treatment (Fig. 1). Obtained results presented the strongest decrease in live cell number of 38% for nalidixic acid (NA), compared to the untreated control cells. Considering the DCF mixture

with metabolites 4′-OHDCF and 5-OHDCF and NA the inhibition of *E. coli* cells viability was observed on the level of 74 and 80%, respectively. For the remaining DCF mixtures with metabolites and antibiotics, the strongest reduction in *E. coli* cells viability was observed for ampicillin and kanamycin, which was 69 and 75% for 4′-OHDCF and 68 and 73% for 5-OHDCF. Compared to tested chemicals used separately, mixtures of DCF with metabolites and antibiotics had the strongest inhibition of *E. coli* cells viability. The mixture of DCF with metabolites and amygdalin had less effect on *E. coli* cells viability compared to antibiotics. The obtained results indicate that ampicillin, kanamycin, NA and amygdalin enhance the inhibitory activity of DCF and its metabolites in relation to *E. coli* K-12 cells viability. Similar results were achieved with amygdalin. The results are presented as a percentage of cell viability of treated *E. coli* culture in comparison to the control sample, not treated bacteria. In the conducted studies lower cell viability values were observed for mixtures of compounds in comparison to pure compounds. There were statistically significant ($p \leq 0.05$) differences between pure compounds between kanamycin and 4′-OHDCF and 5-OHDCF and between amygdalin and nalidixic acid. On the other hand, cell viability value for particular mixtures differed statistically significant ($p \leq 0.05$) in comparison with basic compounds. Taking into account the fact that the cell viability values obtained were significantly ($p \leq 0.05$) lower than those observed for pure compounds, the Tukey test confirmed the more complex and toxic character of the compounds' activity in relation to *E. coli* cell viability.

3.2. E. coli growth inhibition

The growth inhibition effect of tested chemicals and the mixtures was observed with use of *E. coli* K-12 culture. The increase in growth inhibition (GI) values of *E. coli* culture was discovered in the case of all tested chemicals (Fig. 2). The strongest inhibition of *E. coli* K-12 culture growth about 82% was obtained after 24 h treatment with mixtures of DCF with 4′-OHDCF and NA and about 81% for the mixtures of DCF with 5-OHDCF and NA. Ampicillin, kanamycin and NA in the mixture with DCF and its metabolites significantly enhanced the inhibitory effect of DCF on *E. coli* culture development. A clear decrease in *E. coli* culture development was also noted after bacteria incubation with the mixture of DCF and its biodegradation metabolites 4′-OHDCF and 5-OHDCF and amygdalin, even to 50 and 48%, comparable to the control sample. Our results showed that ampicillin, kanamycin, NA and amygdalin in the mixture with DCF support the inhibitory effect of DCF against *E. coli* K-12 culture development. The results are showed as a percent of growth inhibition of treated *E. coli* culture in comparison to the control sample, not treated bacteria culture. The value of growth inhibition in the conducted studies was higher for mixtures of compounds in comparison with single compounds. Statistically significant differences ($p \leq 0.05$) in the group of pure compounds were found between DCF and nalidixic acid, between 4′-OHDCF and ampicillin and kanamycin and between 5-OHDCF and ampicillin and kanamycin. In each case the statistically significant difference ($p \leq 0.05$) was related to the growth inhibition. However, the values of growth inhibition observed between single compounds and their mixtures differed significantly statistically ($p \leq 0.05$) and indicated a higher value of growth inhibition. On this basis it can be concluded that mixtures of compounds show more toxic character in relation to single compounds.

3.3. Genotoxic effect estimation

The genotoxic effects of the tested chemicals and the mixtures were estimated with use of *Escherichia coli* SM342/pBRlux-trp:*recA:luxCDABE* biosensor strain by analysis of the induction of *recA* promoter in fusion with *luxCDABE* reporter gene and the luminescence assay. Obtained results showed that all tested chemicals and the mixtures induced *recA* promoter with different efficiency. We noticed the strongest induction of reporter strain *recA:luxCDABE* in response to the mixtures of DCF with its metabolites and NA with Fold Induction (FI) values 7.01 for DCF + NA, 7.78 for DCF + 4′-OHDCF + NA and 7.79 for DCF + 5-OHDCF + NA (Fig. 3). The strong induction of *recA:luxCDABE* by the mixture of DCF with its metabolites and ampicillin and kanamycin was also observed with the highest values of FI = 4.85 for 4′-OHDCF and FI = 5.01 for 5-OHDCF. Our studies also showed the sensitivity of *recA:luxCDABE* reporter strain to the mixture of DCF with its metabolites and amygdalin. The genotoxic potency of tested compounds is directly proportional to the induction of *recA* promoter and luminescence signal emission. The obtained results indicate that DCF mixtures with its biodegradation metabolites and NA, ampicillin and kanamycin had the strongest genotoxic effect on *E. coli* cultures. The results are presented at Fig. 3 as Fold Induction (FI) value of treated bacteria culture in comparison to the contol sample, not treated. In the case of genotoxcity, a relationship similar to that described for growth inhibition was observed, with the difference that no statistically

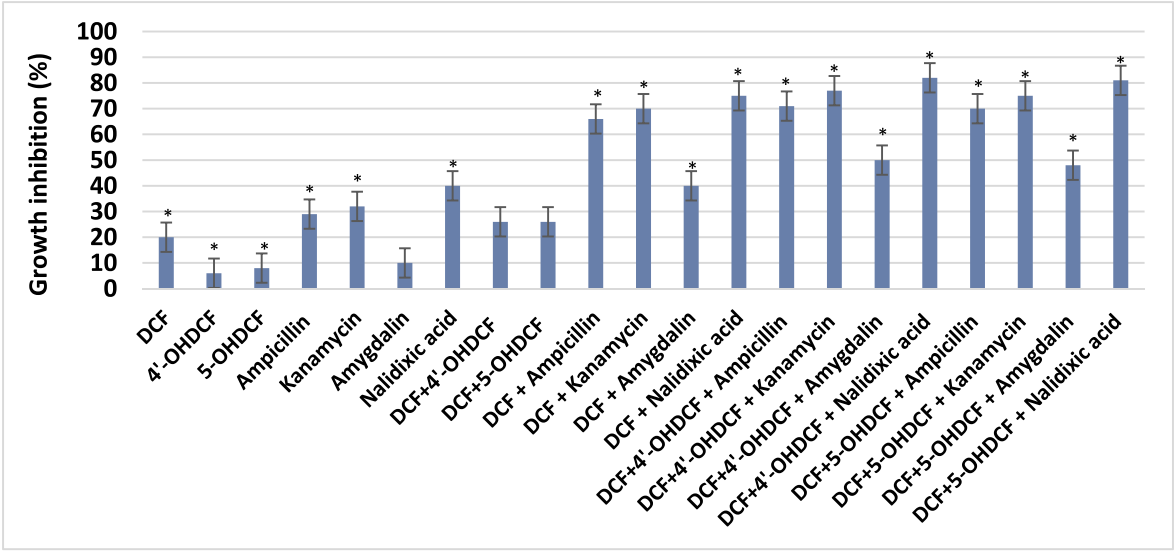


Fig. 2. The growth inhibition of *E. coli* K-12 cultures after 24 h incubation with DCF, 4′-OHDCF, 5-OHDCF, ampicillin, kanamycin, nalidixic acid, amygdalin and their mixtures. DCF, ampicillin, kanamycin, nalidixic acid and amygdalin were applied at concentrations of 3 μg/ml; 4′-OHDCF, 5-OHDCF were used at concentrations of 0.3 μg/ml. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

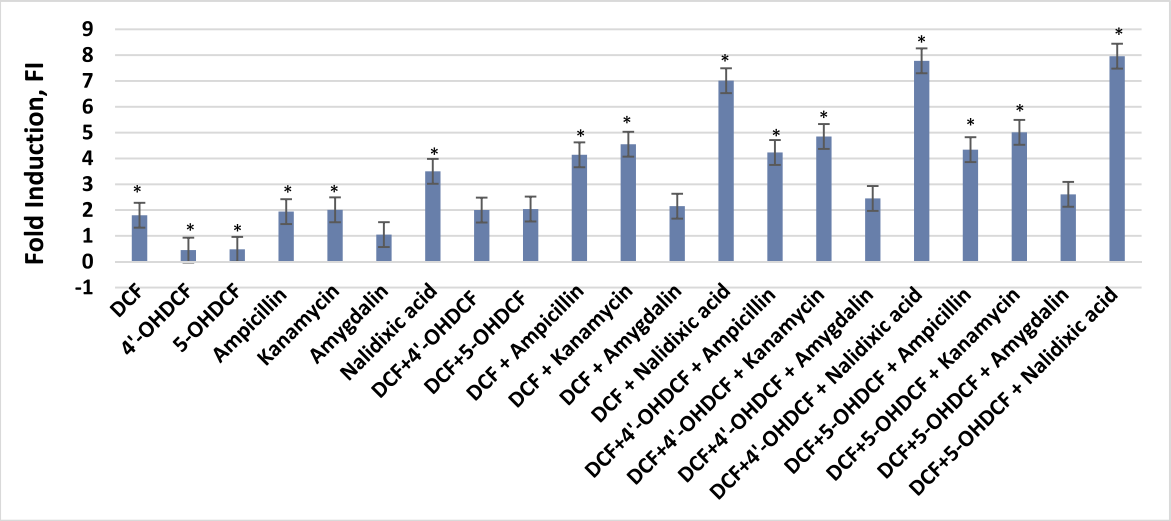


Fig. 3. The genotoxic response pattern of *E. coli* SM *recA:luxCDABE* after 5 h incubation with DCF, 4′-OHDCF, 5-OHDCF, ampicillin, kanamycin, nalidixic acid amygdalin and their mixtures. DCF, ampicillin, kanamycin, nalidixic acid and amygdalin were applied at concentrations of 3 μg/ml; 4′-OHDCF, 5-OHDCF were used at concentrations of 0.3 μg/ml. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

significant differences ($p \leq 0.05$) were observed between the mixtures of metabolites with amygdalin and DCF.

3.4. Oxidative stress assay

3.4.1. ROS generation

Reactive oxygene species (ROS) generation is a basic parameter of oxidative stress. Obtained results after the incubation of *E. coli* K-12 culture with the mixtures of DCF with its biodegradation metabolites and NA, ampicillin and kanamycin ROS generation reaches the highest values, even to 194% for DCF + 5-OHDCF + NA. Considering the DCF mixtures with amygdalin, our research showed that DCF mixture with 4′-OHDCF and 5-OHDCF and amygdali had a similar effect on ROS generation in bacteria culture. These results are in agreement with a cell viability assay and growth inhibition effect, where the strongest decrease in *E. coli* cell viability and the strongest growth inhibition effect, after bacteria treatment with the mixtures of DCF with its biodegradation metabolites 4′-OHDCF, 5-OHDCF and NA, ampicillin and kanamycin was noted. It indicated that oxidative stress is a possible underlying mechanism of these chemicals action. The results are presented at Fig. 4 as percent of ROS increase of treated sample in comparison to control, not treated. In case of ROS, statistically significant differences ($p \leq 0.05$) were observed between DCF and nalidixic acid, between 4′-OHDCF and ampicillin and kanamycin, and between 5-OHDCF and ampicillin and kanamycin. In each case, the statistically significant difference was related to the increase in ROS value.

3.4.2. SodA promoter induction

To protect cells from oxidative stress caused by ROS, organisms have evolved a variety of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase is a key enzyme involved in oxygen radical scavenging (Chang and Zhou, 2011).

We examined the induction of *sodA* promoter of superoxide dismutase gene in *sodA-luxCDABE* genetic construct in *E. coli* SM biosensor strain, after incubation with tested compounds and the mixtures. *SodA* promoter is activated in response to exposure to superoxide stress, resulting in luminescence emission. The level of *sodA* promoter induction in *sodA-luxCDABE* genetic construct in *E. coli* SM is proportional to the level of superoxide stress and luminescence signal. Obtained results presented that the mixtures of DCF with biodegradation metabolites and NA, ampicillin and kanamycin induced *sodA* promoter. The strongest induction of *sod*A promoter was obtained for the mixtures DCF + NA, DCF +4′-OHDCF + NA and DCF + 5-OHDCF + NA with FI values 6.23, 6.87 and 7.12, respectively. Also ampicillin and kanamycin efficiently induced *sodA* promoter. Amygdalin in the mixtures with DCF significantly less affected *sodA* promoter in *E. coli* cultures. The results are presented at Fig. 5 as Fold Induction (FI) value of treated bacteria culture in comparison to the contol sample, not treated.

3.5. RecA promoter induction in the matrice of wastewaters

Obtained results indicated a different pattern of response of *recA* promoter in matrices of raw and treated, filtered and unfiltered wastewater contaminated with DCF, kanamycin and the mixture of DCF with kanamycin (Fig. 6). DCF and kanamycin at concentration of 3 μg/ml were applied. For each sample luminescence and OD values at time 0 and after 2, 4, 24 and 48 h of incubation was measured. The strongest *recA* promoter induction of 168, 223 and 240% in the case of treated, filtered wastewaters contaminated with the mixture of DCF with kanamycin, in comparison to the control sample was obtained. Clear response from the *recA* promoter about 187 (after 24 h of incubation) and 210% (after 48 h of incubation) after *E. coli* SM *recA:luxCDABE* treatment with the mixture of DCF with kanamycin, comparable to the control sample was also noted. A much lower level of luminescent response of the *E. coli* SM *recA:luxCDABE* microbial biosensor was obtained in unfiltered raw and treated wastewaters contaminated with DCF and the mixture of DCF with kanamycin. Generally, the strongest luminescence response of *E. coli recA* cultures in filtered raw and treated wastewaters than in unfiltered samples was noted. The obtained results indicate a clear influence of the composition of the wastewaters matrices on the level of induction of the *recA* promoter and expression of the *luxCDABE* gene. The results are presented at Fig. 6 as the percentage of luminescence signal of treated sample in comparison to the control, not treated. In all types of wastewater used in the studies, an increase in luminescence of the biosensor with exposure time was observed. The most visible changes in luminosity were obtained after 24 and 48 h. In all cases the differences in luminescence values after 24 and 48 h were statistically significant ($p \leq 0.05$) in comparison to other exposure times. The observed regularity indicates higher toxicity of chemical compound mixtures after longer exposure time. Luminescence values in raw wastewater contaminated only with DCF were a deviation from this regularity, where no statistically significant differences ($p \leq 0.05$) between individual exposure times were observed. It should also be

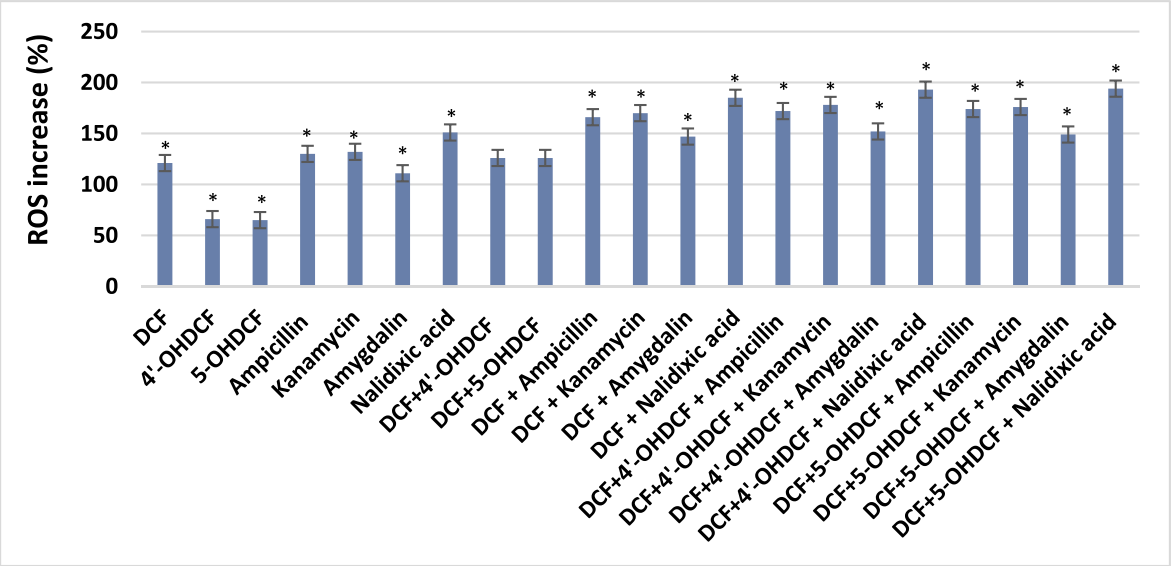


Fig. 4. Reactive oxygen species (ROS) increase induced in *E. coli* K-12 cultures incubated with DCF, 4′-OHDCF, 5-OHDCF, ampicillin, kanamycin, nalidixic acid, amygdalin and their mixtures. DCF, ampicillin, kanamycin, nalidixic acid and amygdalin were applied at concentrations of 3 μg/ml; 4′-OHDCF, 5-OHDCF were used at concentrations of 0.3 μg/ml. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

noted here that the luminescence values obtained in the filtered raw wastewater and filtered treated wastewater containing a mixture of DCF and kanamycin after 48 h of exposure were statistically significant ($p \leq 0.05$) in comparison to all other observed values.

4. Discussion

DCF and antibiotics such as ampicillin and kanamycin belong to one of the most commonly detected pharmaceutical residues in wastewater and surface waters around the world. The current scientific literature mainly concerns the environmental impact of DCF and antibiotics. However, we do not know much about biological activity and interactions between drug metabolites and other chemical compounds present in wastewater. In environmental matrices, diclofenac may interact with other chemical compounds and form more toxic complexes compared to parent chemicals (Lonappan et al., 2016; Vieno and Sillanpää, 2014).

The results of our studies (*E. coli* K-12 viability assay, *E. coli* K-12 growth inhibition), genotoxicity (*recA* promoter induction) and the oxidative stress parameters (ROS generation, *sodA* promoter induction) indicate that the mixtures of DCF, its metabolites with antibiotics had the strongest effect on tested parameters compared to parent compounds. Obtained results indicated that DCF and its biodegradation metabolites have ability to interact with ampicillin, kanamycin and amygdalin and their mixtures have stronger toxicity to *E. coli* than chemicals used individually. Our results indicate that there is a high probability that DCF and its metabolites combine with antibiotics in wastewater and surface waters to form complexes that have a much stronger impact on living organisms than parent chemicals. The results obtained during our experiment support the observations of earlier authors (Lonappan et al., 2016; Kümmerer, 2009) about the possibility for pharmaceuticals and their metabolites to form more toxic complexes with other pollutants found in wastewaters.

Diclofenac (DCF) is one of the best studied pharmaceutical residues present in the environment. It is estimated that about 65% of DCF is in the form of metabolites, containing –OH groups in their structure in

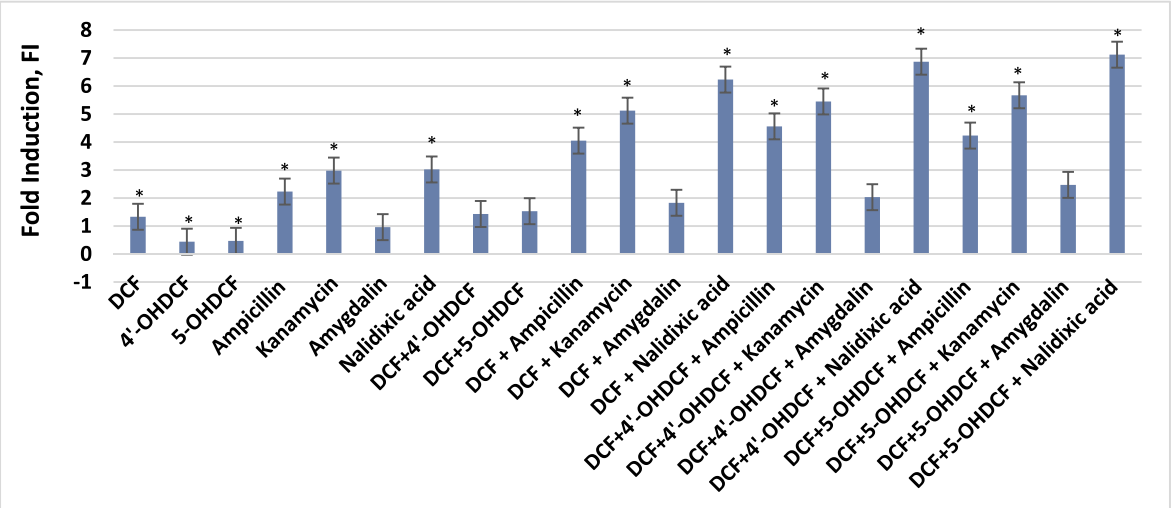


Fig. 5. The response pattern of *E. coli* SM *sodA:luxCDABE* after 5 h incubation with DCF, 4′-OHDCF, 5-OHDCF, ampicillin, kanamycin, nalidixic acid, amygdalin and their mixtures. DCF, antibiotics and amygdalin were applied at concentrations of 3 μg/ml; 4′-OHDCF, 5-OHDCF were used at concentrations of 0.3 μg/ml. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

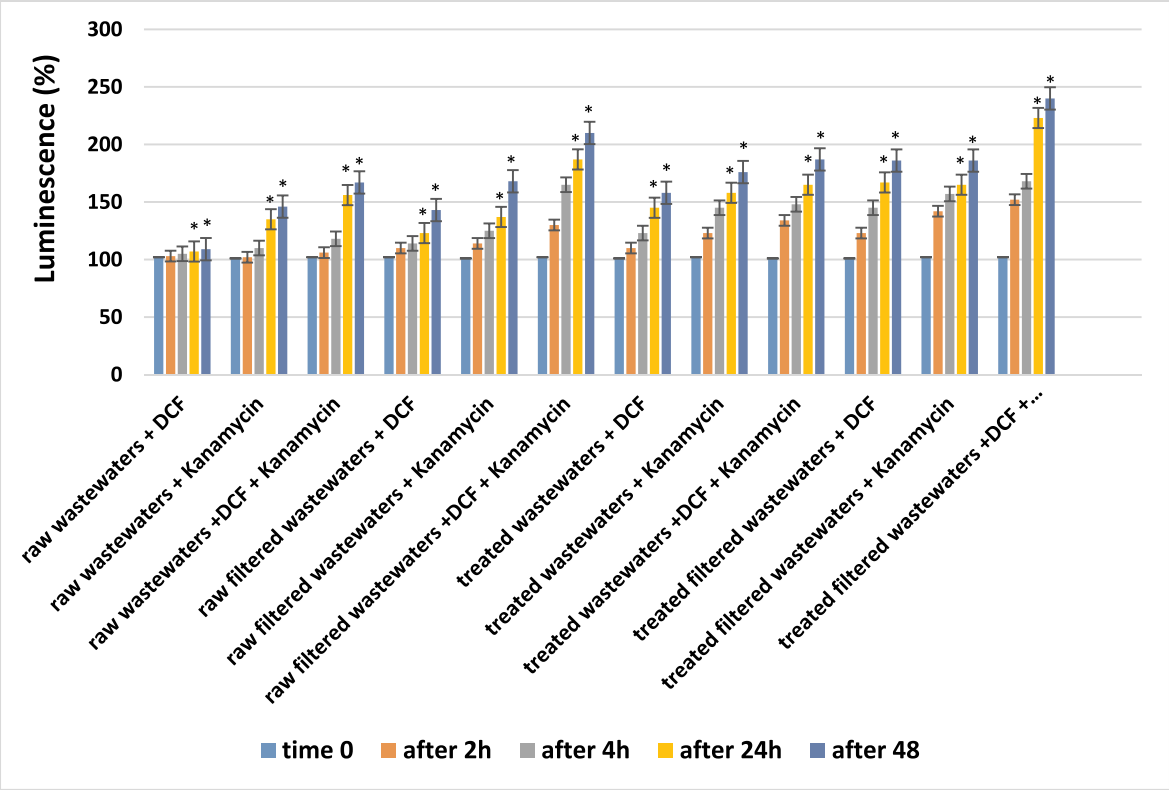


Fig. 6. The response pattern of *E. coli* SM *recA:luxCDABE* biosensor strain to raw and treated, filtered and no filtered wastewaters contaminated with 3 μg/ml of DCF, kanamycin and the mixture of DCF with kanamycin. The results are presented as the luminescence values (%) of treated sample in comparison to control, not treated bacteria cultures. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

various positions. The presence of hydroxyl groups strengthens the potential for interactivity of metabolites with metals and the formation of complexes that are new, emerging contaminants (ECs) whose toxicity may be higher than that of parent compounds. In addition, DCF and its metabolites may interact with other ECs found in wastewater and surface waters, such as pesticides or surfactants. ECs are often compounds having several active groups in their structure. Thus, multiple interactions of DCF and its metabolites with one or more other ECs are possible (Eriksson et al., 2010; Schulze et al., 2010; Lonappan et al., 2016). Perhaps this mechanism is also responsible for the synergistic interaction of DCF and its metabolites with the antibiotics and amygdalin tested in this study.

In our studies the strongest inhibition of *E. coli* cells viability and bacteria culture growth after their treatment with mixtures of DCF with antibiotics and mixtures of DCF, its biodegradation metabolites 4′-OHDCF and 5-OHDCF with antibiotics was noticed. Antimicrobial activity of DCF against both Gram-positive and Gram-negative bacteria has been reported in various earlier studies. Probably, the antimicrobial potential of DCF is related to its ability to inhibit DNA replication and membrane activity disruption (Dutta et al., 2007; Padma and Yalavarthy, 2015). Moreover, collected reports presented that some of the metabolites of DCF are more toxic compared to the parent drugs. In the algae test 6-fold more toxic activity of DCF phototransformation products than DCF was documented (Aristi et al., 2016; Lonappan et al., 2016). In addition, it was showed that DCF metabolites are biologically active to aquatic organisms. In connection to DCF interactions with different drugs, in the toxicity assays on *D. magna* the synergistic effect of the mixtures of DCF, ibuprofen, naproxen and acethylsalicylic acid in comparison to the drugs used individually was noticed. In experimental studies the changes in the biological activity of pharmaceutical residues have also been noticed when used simultaneously with other stressors, i.e. pH and temperature. In *R. philippinarum* mussels exposed to DCF, increasing the pH value to 7.8 caused a higher mortality rate of the tested organisms. It is likely that changes in pH were a factor that influenced the bioavailability of drugs and the response of aquatic organisms to them (Backhaus et al., 2014; Lonappan et al., 2016).

Due to the very dynamic development of the pharmaceutical industry, it is estimated that in the past 50 years, more than one million tons of antibiotics have been introduced into the environment. The high usage and continuous release of antibiotics into the environment can generate a number of ecotoxicological effects, including frequently observed drug-resistant bacteria (Łukaszewicz et al., 2017). Antibiotic resistance genes (ARGs) are spread by plasmid horizontal transfer mechanism. The migration and transformation of ARGs in the environmental matrices is harmful (Chen et al., 2019). Moreover, there is no regulations regarding acceptable levels of drug concentrations in the environment. In addition to human and animal healthcare, another source of APIs release into environment is the increasing development of agriculture and the intensification of livestock farming. APIs are not only used for therapeutic purposes but are also administered prophylactically or used as animal weight growth promoters (Łukaszewicz et al., 2017).

In earlier studies the antibacterial effect of DCF in synergism with antibiotics both *in vitro* and *in vivo* studies was shown (Padma and Yalavarthy, 2015; Annadurai et al., 2002). In presented studies it was also noticed that DCF and DCF with the mixture with its biodegradation metabolites enhanced the antibacterial activity of ampicillin, nalidixic acid and kanamycin. Ampicillin is one of representative of the group of β-lactam antibiotics that are detected in environmental matrices (Mutiyar and Mittal, 2014; Faleye et al., 2017). The inhibition of bacterial cell wall synthesis that ultimately leads to cell lysis is the main mechanism of ampicillin action. Kanamycin inhibits protein synthesis. NA is a specific and effective inhibitor of cellular deoxyribonucleic acid synthesis. The strengthening, antibacterial effect of DCF with selected antibiotics has been described in the scientific literature (Padma and Yalavarthy, 2015; Annadurai et al., 2002). However, effects of DCF in

combination with its biodegradation metabolites and the antibiotics tested is not fully understood. In previous laboratory studies it was detected that diclofenac sodium had antibacterial activity towards not only Gram-positive and Gram-negative bacteria but also *Mycobacteria*. DCF was synergistic with Streptomycin (STM) against *Mycobacterium smegmatis*, *E. coli* and *Staphylococcus aureus*. Similarly, DCF exhibited synergistic effect with trifluoperazine against some Gram-positive and Gram-negative bacteria (Dutta et al., 2004, 2007).

In the present experiment also it was detected that amygdalin, a toxic cyanogenic glycoside enhanced antibacterial activity of DCF and its metabolites against *E. coli*. Earlier experimental studies revealed antimicrobial potency of amygdalin against *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, and *Streptococcus pyogenes* at concentrations ranging from 4 to 10% (Abtahi et al., 2008; Al-Bakri et al., 2010). Amygdalin is hydrolyzed by β-glucosidase into d-glucose, benzaldehyde, and prussic acid (hydrogen cyanide). It has been established that antibacterial activity of amygdalin is due to its decomposition products hydrogen cyanide and benzaldeyde, the toxic compounds which killed the bacteria (Al-Bakri et al., 2010).

Oxidative stress occurs when there is an imbalance between ROS production and the antioxidant defense mechanisms of a cell or tissue. These conditions lead to oxidation of proteins, lipids, and DNA, what causes the inactivation of many enzymes and cell death (Islas-Flores et al., 2017). Recently, oxidative toxicity studies of various environmental pollutants are very important biomarkers for assessing the impact of pollution on the environment. Reactive oxygen species (ROS) generation is a important parameter for oxidative stress assessment. In previous works it was shown that chemical toxic pollutants are important sources of ROS in biological systems. Superoxide anion and hydroxyl radicals, which are known to cause oxidative damage to important cellular biomolecules, are the two most studied ROS (Vlahogianni et al., 2007). Organisms exhibit a variety of changes in enzymatic antioxidant defences after exposure to pollutants with oxidative potential. Superoxide dismutase (SOD) is a key antioxidant defence enzyme that is used as biomarker of oxidative damage. SOD converts the superoxide anion into hydrogen peroxide. Earlier studies showed that SOD is induced by various environmental pollutants (Valavanidis et al., 2006; Vlahogianni et al., 2007). In our studies we showed that DCF, its biodegradation metabolites and the mixtures of DCF with metabolites, antibiotics and amygdalin expressed oxidative potency in *E. coli* strains. Two oxidative stress biomarkers ROS generation in *E. coli* K-12 strain and *sodA* promoter induction in biosensor strain *E. coli* SM *sodA:luxCDABE* were measured. The mixtures of DCF and its metabolites with tested antibiotics strongly induced oxidative stress, comparable to compounds used individually. Presented results are in agreement with the results of previous works, where it was shown that DCF induce oxidative stress on microcrustaceans such as *Daphnia magna*, amphipods such as *Hyalella azteca*, and commercially valuable freshwater fish such as *Cyprinus carpio*, negatively affecting biomolecules such as proteins, membrane lipids, and DNA (Gomez-Olivan et al., 2014). In the work of Islas-Flores et al. (2017), it was presented that DCF hydroxylation metabolism ROS generation occured in different organs of common carp *Cyprinus carpio*. Considering to DCF metabolites 4′-OHDCF and 5-OHDCF it was established that they can be oxidized to intermediates of a reactive quinone imine that reacts with protein nucleophilic groups to form adducts (Islas-Flores et al., 2017). Considering the activity of ampicillin, kanamycin and NA, our results support the hypothesis advanced in the works of earlier authors where different classes of bactericidal antibiotics, generate ROS, that contribute to cell killing (Dwyer et al., 2014). Similarly, obtained results showed the potency of induction of genotoxic response of biosensor strain *E. coli* SM *recA:luxCDABE*, after bacteria treatment with tested chemicals and their mixtures. Genotoxic activity of DCF was also detected in *Cyprinus carpio* and *Daphnia magna*. Oxidative stress affects the level of gene expression and cellular signal transduction pathways (Islas-Flores et al., 2017; Gomez-Olivan et al., 2014). Conducted genotoxic studies are in agreement with previous studies with use of *E. coli recA:luxCDABE*, where authors confirmed the positive reaction of this biosensor in monitoring chemical, physical, and genotoxic agents. It was revealed that oxidative stress triggers the *E. coli* SOS response system and *recA* promoter induction (Melamed et al., 2012; Kostrzyńska et al., 2002; Rosen et al., 2000).

In the present studies we used *E. coli* SM *recA:luxCDABE* strain as microbial biosensor for bacteria living cells monitoring in the environment of raw and treated wastewaters contaminated with DCF and kanamycin. It was noticed that the different efficiency of induction of *recA* promoter in the all types of wastewaters raw, treated, filtered and unfiltered. In previous works it was shown that *recA* promoter is sensitive to stress conditions and genotoxins (Long et al., 2019; Kostrzyńska et al., 2002; Rosen et al., 2000). The strongest luminescence signals as *E. coli recA:luxCDABE* biosensor strain response were detected in the environment of raw and treated filtered wastewaters, comparable to unfiltered wastewaters. Many studies have shown that the composition of wastewaters, especially the content of organic matter as well as the quantitative and qualitative composition of microflora affects the efficiency of biodegradation of pharmaceuticals and other chemical compounds contained in wastewaters (Lonappan et al., 2016; Vieno and Sillanpää, 2014). Wastewater filtration through a bacteriological filter leads to removal of the competitive microbial consortia in the wastewater, which facilitates the free growth and reaction of *E. coli* SM *recA: luxCDABE*. In obtained results it was observed that the wastewaters matrices infuenced the level of *recA* promoter induction and *luxCDABE* gene expression. Stronger luminescence response in the case of pure mixtures of DCF with kanamycin than in the wastewaters matrices was obtained. It suggests that the diverse nature of the wastewater matrices and the presence of potential inhibitors affect the luminescence response of *recA:luxCDABE* system in *E. coli*. Presented research has shown that *E. coli* SM *recA:luxCDABE* microbial biosensor is a useful tool in monitoring live bacteria in the wastewater environments.

A very important problem that undoubtedly requires further research is the ability of drugs and their metabolites to interact with other chemical compounds found in wastewater. Wastewater treatment plants are comprehensive systems to which pollution flows from various sources. Drug metabolites can become a new category of potential emerging micro-pollutants, often with biological activity similar to parent drugs. Pharmaceutical residues, although present in environmental matrices at low concentrations, are molecules showing a high degree of reactivity and even at such low microconcentration may adversely affect the environment. In addition, pharmaceutical residues may interact, combine and aggregate with other pollutants of the same or different class present in wastewater. Future research on the estimation of the risk of environmental effects of pharmaceutical residues should be more focused on determining the toxicity of drug mixtures compared to parent drugs. Considering the fact that drugs and their metabolites get into wastewater with human excreta, the biological activity of metabolites and their environmental impact should also be studied (Schulze et al., 2010; Lonappan et al., 2016; Mezzelani et al., 2018).

Our research results indicate that DCF and its metabolites can interact with ampicillin, kanamycin and amygdalin and these mixtures have more toxic potency to *E. coli* strains than parent compounds. The results of our research are very important, especially in relation to possible technological solutions. Considering the possibility of transferring the results obtained in our work from the laboratory scale to specific technological solutions aimed at removing pharmaceutical residues, their metabolites as well as toxic complexes, especially in the case of wastewaters with a high pharmaceutical load (hospital wastewaters, wastewaters from the pharmaceutical industry), it is recommended to use the biofiltration process or reverse osmosis after biological wastewaters treatment. Research from recent years also indicates high elimination of pharmaceutical residues using technology

based on advancess oxidation processess (AOPs) (Vogna et al., 2004; Kanakaraju et al., 2018). However, it should be emphasized that during these processes toxic by-products are formed, which are recommended to be removed by e.g. reverse osmosis or biofiltration. As for surface waters, pharmaceutical residues in these waters can be a potential threat to drinking water intakes. It seems that a good solution in water treatment may be the use of biofiltration as the first stage of water treatment, activated carbon as the second to last stage of purification, and reverse osmosis as last stage.

5. Conclusion

In the present experiment, it has been shown that:

1. DCF and its biodegradation metabolites 4′-OHDCF and 5-OHDCF can interact with tested antibiotics and compounds of natural origin, i.e. amygdalin to form mixtures showing stronger biological activity against *E. coli* than parent chemicals.
2. The mixtures of DCF and its metabolites with tested antibiotics have more toxic potency to *E. coli* strains, comparable to chemicals used individually.
3. Additionally, the mixtures of DCF and its metabolites with tested antibiotics have more genotoxic and oxidative stress generation potency to *E. coli* strains, than compounds used separetely.
4. The obtained results indicate the need to study biological activity and the impact on living organisms not only of the parent drugs but also of their metabolites and their mixtures. In addition, drug and metabolite interactions with other compounds in the environment, e.g. wastewater, surface waters, should also be investigated.
5. Another important step in the elimination of pharmaceutical residues from wastewater is to increase the efficiency of wastewater treatment and introduce additional technological solutions.

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Declaration of competing interest

No conflict of interest have been declared.

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