Archives of Environmental Protection
Vol. 46 no. 4 pp. 10–22

PL ISSN 2083-4772
DOI 10.24425/aep.2020.135760

Evaluation of the biological impact of the mixtures of diclofenac with its biodegradation metabolites 4'-hydroxydiclofenac and 5-hydroxydiclofenac on *Escherichia coli*. DCF synergistic effect with caffeic acid

Marzena Matejczyk[1]*, Piotr Ofman[2], Katarzyna Dąbrowska[3], Renata Świsłocka[1], Włodzimierz Lewandowski[1]

[1]Bialystok University of Technology, Faculty of Civil Engineering and Environmental Sciences, Division of Chemistry, Biology and Biotechnology, Bialystok, Poland,
[2]Bialystok University of Technology, Faculty of Environmental Engineering Technology and Systems, Bialystok University of Technology, Bialystok, Poland.
[3]Department of Microbiology, Institute of Agricultural and Food Biotechnology, Warsaw, Poland.

*Corresponding author's e-mail: m.matejczyk@pb.edu.pl

Keywords: diclofenac, biodegradation, metabolites, wastewaters, microbial biosensors.

Abstract: In environmental matrices there are mixtures of parent drug and its metabolites. The majority of research is focused on the biological activity and toxic effect of diclofenac (DCF), there is little research on the biological activity of DCF metabolites and their mixtures. The study focused on the assessment of the biological impact of DCF, its metabolites 4'-hydroxydiclofenac (4'-OHDCF) and 5-hydroxydiclofenac (5-OHDCF) and their mixtures on *E. coli* strains. The biological effects of tested chemicals were evaluated using the following: *E. coli* K-12 cells viability assay, the inhibition of bacteria culture growth, ROS (reactive oxygene species) generation and glutathione (GSH) content estimation. Moreover, we examined the influence of the mixture of DCF with caffeic acid (CA) on *E. coli* cells viability. Our results showed the strongest impact of the mixtures of DCF with 4'-OHDCF and 5-OHDCF on *E. coli* SM biosensor strains in comparison to parent chemicals. Similar results were obtained in viability test, where we noticed the highest reduction in *E. coli* cell viability after bacteria incubation with the mixtures of DCF with 4'-OHDCF and 5-OHDCF. Similarly, these mixtures strongly inhibited the growth of *E. coli* culture. We also found synergistic effect of caffeic acid in combination with DCF on *E. coli* cells viability. After bacteria treatment with the mixture of DCF and its metabolites we also noted the strongest amount of ROS generation and GSH depletion in *E. coli* culture. It suggests that oxidative stress is the most important mechanism underlying the activity of DCF and its metabolites.

Introduction

Diclofenac (DCF) is a non-steroidal anti-inflammatory (NSAID) and pain relieving drug widely used throughout the world. The chemical name of DCF is 2-(2,6-dichloroanilino) phenylacetic acid and it was discovered in 1973 by Ciba--Geigy pharmaceutical company. It is estimated that the global consumption of DCF is about 940 t per year. In 2012 DCF was listed as the 12th bestselling generic molecule globally. DCF is one of the most popular anthropogenic micropollutant in wastewaters and aquatic environment. Additionally, this drug was found in drinking water across Japan (Simazaki et al., 2015). DCF is detected in different environmental compartments at concentration ranging from ng L^{-1} to µg L^{-1}. Due to poor degradation and higher consumption rates, DCF is one of the most frequently detected drugs in wastewater treatment plants (WWTPs) where it has low removal efficiency and it is often found in effluent water. Moreover, DFC residues have been detected in sewage sludge with concentration even to 140 ng g^{-1}, sediments and in aqueous environment. In surface water DCF has been detected in ng L^{-1}, whereas in wastewater, the concentration has been as high as µg L^{-1}. The highest concentration of DCF was detected in rivers in Pakistan and Germany at the concentrations of 4900 ng L^{-1} and 1030 ng L^{-1}, respectively (Lonappan et al., 2016).

After consumption of DCF for both human and veterinary purposes unmetabolized pharmaceutical and its metabolites end up in wastewater treatment plants or in landfills as biologically active substances, which may affect homeostatic mechanisms in the human body at very low concentrations (Beltran et al., 2009; Bouju et al., 2016). The metabolism of DCF has been studied and described in mammals, fungi and microorganisms. Some

studies reported about negative effect of DCF, its metabolites and DCF-containing mixtures on the microrganisms' biodiversity in activated sludge. Proper maintenance of the biomass diversity of the activated sludge is the basis for the good functioning of activated sludge and the biodegradation of pharmaceutical residues and other micropollutants. Studies have shown that pharmaceutical residues, including DCF, can disturb quantitative and qualitative microbial composition in activated sludge and lead to disturbances in its functioning. Moreover, the majority of laboratory experiments are focused on the parent compound DCF only. The major metabolites of DCF are hydroxy-products, such as 4'-OHDCF and 5-OHDCF (Marco-Urrea et al., 2010; Memmert et al., 2013; Lonappan et al., 2016; Haibal et al. 2017).

The concentration of DCF in environmental compartments decreased by natural processes, such as soil retention, biodegradation and phototransformation, and also by physico--chemical processes in wastewater treatment plants. The removal efficiencies of DCF by WWTPs lies mainly within the 21–40% range. The efficiency of DCF degradation depends on the method used. It was shown that the maximum removal of about 93% of DCF was obtained by adsorption on activated carbon followed by ozonation (Marco-Urrea et al., 2010; Memmert et al., 2013). The use of advanced oxidation processes (AOPs) in wastewater treatment leads to formation of toxic transformation products (by-products) (Felis et al., 2009). A very promising method of DCF degradation is the use of enzymes that do not create harmful by-products (Lonappan et al., 2016).

At typical detected environmental concentrations, DCF does not exhibit toxic effects towards living organisms, albeit chronic exposure may lead to severe effects (Felis et al., 2005; Marciocha et al., 2013; Acuña et al., 2015; Lonappan et al., 2016; Desbiolles et al., 2018; Klopčič et al., 2018). Diclofenac due to frequent detection in the environment enters the food chain and accumulates in aquatic organisms, mainly fish. In addition, residues of diclofenac, pyrazoline derivatives, were detected in edible tissues of farm animals, mainly in the kidneys at the concentration of 28.4 mg/kg. The presence of diclofenac and its derivatives in fish meat and animal tissues poses a threat to food quality and safety (Lonappan et al., 2016; Desbiolles et al., 2018; Klopčič et al., 2018). The toxic effect of DCF is connected with its chemical structure with a chlorine atom and, therefore, its residues are not readily biodegradable in the environment. In 2018, Klopčič et al. discovered that DCF and its metabolite 4'-OHDCF have endocrine disrupting activities and immunomodulatory effects in lymphoblastoid cell lines (Behera et al., 2011; Klopčič et al., 2018). It was reported that some of the metabolites and their mixtures could be more toxic than DCF and they can affect living organisms (Lonappan et al., 2016; Klopčič et al., 2018). In connection to the above, there is need to investigate the potential impact and toxicity of DCF metabolites and their mixtures during toxicological and environmental monitoring experiments. Similarly, by--products that are created during advanced oxidation processes (O_3, O_3/H_2O_2 and photo-Fenton, UV/H_2O_2) of DCF should be subjected to toxicological tests. The toxicity concerns over DCF metabolites are still valid. Moreover, in wastewaters or aquatic waters there is a mixture of DCF and its metabolites. The mixture toxicity effects of these chemicals are still unknown (Felis et al., 2009; Kümmerer 2009; Fatta-Kassinos and Nikolaou 2011; Acuña et al., 2015; Lonappan et al., 2016). Due to DCF direct and indirect toxicity to vertebrates, it has been suggested to be added into the list of priority substances in EU's Water Framework Directive (Directive 2013/39/EU) (Cunha et al., 2017).

The next very important problem are the possible interactions of pharmaceuticals with different comopunds that are present in environmental matrices. DCF and its metabolites can interact with other inorganic and organic contaminants in wastewaters or surface waters leading to the creation of another possible emerging contaminant, that could be more resistant to biodegradation. We know very little about DCF interactions with other compounds found in wastewaters or surface waters (Lonappan et al., 2016; Osorio et al., 2016; Sochacki et al., 2018)

In our resea.rch, we chose caffeic acid (CA) as a substance potentially interacting with DCF. CA is one of the main representatives of phenolic acids, the compounds widespread in the plant world, with diverse structure and chemical and biological properties. Phenolic acids belong to plant secondary metabolites and are popular in human diet. CA is present in many food sources, including coffee drinks, blueberries, apples and cider. Moreover, CA in combination with other compounds of natural origin, e.g. propolis, is used in several medications of popular use (Magnani et al., 2014). CA is discharged with wastewaters into the environment, where it can interact with DCF and its metabolites (Gernjak et al., 2003; Mantazavinos et al., 2005). It has been shown in many earlier works that CA showed antimicrobial potential and/or synergistic effects with antibiotics against Gram-positive and Gram-negative bacteria and *C. albicans* strains. The antimicrobial activity of CA is probably associated with one more hydroxyl group substituted at the CA phenol ring (Magnani et al., 2014). Similarly, antimicrobial properties were observed in DCF. Both CA and DCF due to their antimicrobial activity may affect the activated sludge microflora in wastewater treatment plant (Lonappan et al., 2016). Therefore, in our experiment, we decided to explore potential CA interactions with DCF.

The aim of our experiment was the assessment of the biological actvity of DCF and its main metabolites 4'-OHDCF and 5-OHDCF and their mixture toward *E. coli* strains. We investgated the influence of the tested chemicals on the level of stress-responsive promoters *recA*, *micF*, *katG*, *sodA* and *inaA* induction in *E. coli* SM strains. We also studied *E. coli* K-12 cells viability, growth development, ROS generation potency and glutathione (GSH) level. Moreover, we examined the influence of the DCF with CA mixture on *E. coli* cells viability. Each of *E. coli* SM strains harbors a plasmid that carries a fusion of selected stress-responsive promoters (*recA*, *micF*, *katG*, *sodA* and *inaA*) to *Photorhabdus luminescens luxCDABE* reporter genes. Genetically engineered microbial reporter strains are based upon the fusion of an inducible sensing element upstream of a reporting element, so that the construct emits a luminescence signal when exposed to the inducing compounds. Microbial cell-based luminescence reporter gene assays is rapid, effective and not-expensive method for biological activity study of different chemicals (Yagur-Kroll et al., 2010; Yagur-Kroll and Belkin 2011; Janion 2008; Hassan et al., 2016). As it was previously shown, the *E. coli luxCDABE* strains with five promoters (*recA*, *micF*, *katG*, *sodA* and *inaA*) used in this study, are useful to detect antibiotics, genotoxins, environmental chemicals, anticancer drugs and some new

candidate drugs. Genetically engineered bacteria successfully work as biosensors and chemical pollution bioindicators for the detection and monitoring of toxic compounds in wastewaters, surface waters, drinking water, soil and sediments (Melamed et al., 2012; Kessler et al., 2012; Lee and Mitchell et al., 2012).

This work is in the range of broader theme, which focuses on investigations of biological activity of natural origin compounds (e. g. caffeic acid) as well as biosensors (e. g. *E. coli*) in aspect of conservants, antioxidants and biodegradation properties.

Materials and methods

DCF biodegradation metabolites

In order to select biodegradation metabolites of DCF, a literature review in the PubMed database on this subject was conducted. We selected these chemical compounds which were detected and identified by scientists as biodegradation metabolites of DCF in at least 10 scientific publications (Marco-Urrea et al., 2010; Langenhoff et al., 2013; Vieno and Sillanpää 2014; Bouju et al., 2016; Lonappan et al., 2016; Haibal et al., 2017; Kawase et al., 2017; Doruk et al., 2018; Moreira et al., 2018; Schmidt et al., 2018). DCF (Diclofenac sodium salt), 4'-OHDCF, 5-OHDCF were commercially obtained (Sigma Aldrich, UK). Chemical structures and molecular formulas of the tested chemicals are presented in Table 1 and also included in the supplementary material.

Selection of concentrations of DCF and its metabolites

In relation to the literature (Schmidt et al., 2018), we decided to treat of *E. coli* strains with DCF and its metabolites in equal concentrations of 5 µg/ml. The study analyzed DCF at the concentration of 5 µg/ml in order to obtain expressive response to the tested biosensors with the *luxCDABE* gene. At lower DCF concentrations, corresponding to the environmental concentrations of DCF, the response of *E. coli* strains to the analyzed parameters was quite weak. The most reactive *E. coli* SM strain with the *recA* and *micF* promoters was affected by the tested chemicals at a ratio of 10-times lower metabolites' concentrations in relation to DCF (0.5 µg/ml of metabolite and 5 µg/ml of DCF).

Chemical treatment of E. coli cultures

DCF, its metabolites and their mixtures were dissolved in dimethyl sulfoxide (DMSO) (Chempur). To reduce the impact of DMSO on the bacterial culture, prepared solutions of the tested compounds in DMSO were diluted in the culture medium (Luria Bertani broth or Müeller Hinton II broth) 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 5 µg/ml for DCF and 5 µg/ml and 0.5 µg/ml for 4'-OHDCF and 5-OHDCF. Caffeic acid (CA) (Sigma Aldrich) was dissolved in water and then it was added to the *E. coli* cultures for a final concentration of 10 µg/ml. In the mixtures we added DCF at the concentration of 5 µg/ml and CA at the concentration of 10 µg/ml. The scheme of the experiment containing detailed composition of samples and concentrations of the tested chemical compoundsis is presented in Table 1 in supplementary material.

E. coli cell viability assay

The *E. coli* cell viability assay after treatment with the tested chemicals and their mixtures 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 for the detection of growth or toxicity of bacteria culture (Promega instructions). *E. coli* K-12 was grown in Müeller 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. The tested chemicals and their mixtures were added to 10^7 CFU/ml of *E. coli* culture at appropriate concentrations and incubated for 24 and 48 h. The assay was performed according to the Promega attached protocol. RLU (Relative Luminescence Unit) 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 and 48 h incubation with tested chemicals and it was calculated as a percentage of control cells, incubated without the tested compound. Three independent experiments were done.

E. coli K-12 culture growth inhibition effect

The growth inhibition effect of DCF, its metabolites and their mixtures 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). The tested chemicals and their mixtures were added to 10^7 CFU/ml of *E. coli* culture at appropriate concentrations and incubated for 24 and 48 h. Non-treated *E. coli* cultures were used as the control. The values of bacteria growth inhibition (GI) after 24 and 48 h treatment with the tested compounds and their 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.

E. coli stress-response promoters induction

Stress-response bioreporter panel of five *Escherichia coli* strains: *Escherichia coli* SM338/pBRlux-trp:*inaA::luxCDABE*, *Escherichia coli* SM342/pBRlux-trp:*recA::luxCDABE*, *Escherichia coli* SM343/pBRlux-trp:*micF::luxCDABE*, *Escherichia coli* SM344/pBRlux-trp:*katG::luxCDABE*, *Escherichia coli* SM345/pBRlux-trp:*sodA::luxCDABE* with

reporter plasmids, harboring a different promoter sequence fused to the *luxCDABE* reporter genes in the pBRlux-trp vector were used. Previously laboratory experiments showed that *inaA*, *recA*, *micF*, *katG* and *sodA* promoters were involved in drug response circuits or in response to global stress factors (Melamed et al., 2012; Kessler et al., 2012). All strains were cultured overnight in M9 medium at 37°C. Luminescence measurement and data analysis were conducted according to the method described by Melamed et al., 2012 and Kessler et al., 2012. Bacteria cultures were diluted 100-fold in fresh LB and re-grown at room temperature with shaking (200 r.p.m.) to early log phase (OD_{600}=0.2). Sensitivity of these strains 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 1, 2, 3 and 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. For each reporter strain nalidixic acid (NA) was used as positive control. The results are showed as fold induction values in comparison to the control (untreated bacteria culture) and to the time zero. Experiment was carried out in three independent series.

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.

The fold induction values calculations

The response data were expressed as fold induction (FI) normalized with control or tested sample at time 0 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 or tested sample at time 0.

Oxidative stress studies

ROS generation

The production of ROS by *E. coli* K-12 after treatment with DCF, its metabolites and their mixtures was evaluated using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, UK) and according to the method described by Ong et al., 2017 and Diaz-Garcia 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 a log phase (OD_{600}=0.2). 10^7 CFU/ml of *E. coli* culture was treated with the tested chemicals and their mixtures used in appropriate concentrations. Subsequently, DCFH-DA was added to bacteria culture at a final concentration of 5 µM and incubated at 37°C for 35 minutes. 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 percentage of ROS increase in comparison to the control sample (untreated bacteria). All the experiments were done in triplicates.

Total GSH assay

Total glutathione in *E. coli* K-12 suspensions was determined using GSH/GSSG-Glo™kit (Promega, Madison, WI, USA). *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 a log phase (OD_{600}=0.2). The tested chemicals and their mixtures were added to 10^7 CFU/ml of *E. coli* culture at appropriate concentrations and incubated at 37°C for 3 h. The optical density (OD) of bacteria culture was monitored with use of spectrophotometer at the wavelength of 600 nm. After incubation, *E. coli* K-12 culture was pelleted, the bacterial cell pellet was lysed in 1X FastBreak™ Cell Lysis Reagent (Promega), according to the instructions provided by the manufacturer. The GSH level in *E. coli* K-12 lysate was determined according to the instructions provided by the manufacturer. Assay is a luminescence-based system to detect and quantify total glutathione. Stable luminescent signals are correlated with the GSH concentration of a sample. According to this method GSH-dependent conversion of a GSH probe, Luciferin-NT, to luciferin by a glutathione-S-transferase enzyme is coupled to a firefly luciferase reaction. Light from luciferase is dependent on the amount of luciferin formed, which is in turn dependent on the amount of GSH present. The obtained luminescent signal is proportional to the amount of GSH. The GSH level in *E. coli* K-12 suspensions treated with the tested chemicals was shown as a percentage of GSH level of control cells, incubated without the tested compounds. The experiment was done in triplicate.

Statistical analysis

Laboratory analysis results were statistically processed in order to determine the statistical significance between the absorbance and luminescence values. Variance analysis is one of the methods allowing for this type of comparison. Within the statistical analysis, the obtained database was divided into two groups of variables. First of them were qualitative factors, in which the criteria variables of the experiment were taken into account, including the type of genome structure, reaction time and biosensor used. The second group of variables, the subsidiary variables, were the values of absorbance or luminescence obtained for particular qualitative variables. In order to analyze the differences between the obtained results,

Tukey's HSD test for the same population in groups was used. Due to high precision of measurement results, the level of statistical significance (α) was assumed to be 0.05. In total, 2700 measurement results were used for statistical analysis. 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 use of Statistica 13.1 software running on Windows 10 platform.

Results

DCF, its metabolites and their mixtures influence on E. coli cells viability

In the case of estimation of *E. coli* cells viability for the all tested compounds the reduction in cells viability as compared to the control, non-treated cells after 24 and 48 h treatment with chemicals was noticed (Fig. 1). Our results showed the strongest decrease in live cell number of above 74%, after 48 h treatment, and above 64% after 24 h treatment with the mixture of DCF (5 μg/ml) with 4'-OHDCF (5 μg/ml) and 5-OHDCF (5 μg/ml), compared to the untreated control cells. Significant decreases in cells viability in the case of DCF and its mixtures with 4'-OHDCF or 5-OHDCF in lower concentrations of metabolites (0.5 μg/ml) were also noted. In general, it was detected that the mixtures of DCF with metabolites and the mixtures of metabolites significantly reduced the viability of *E. coli* cells compared to parent chemicals. We also found the synergistic effect of caffeic acid, used at the concentration of 10 μg/ml in combination with DCF at the concentration of 5 μg/ml. The mix of both compounds resulted in a decrease in *E. coli* cells viability of 68% (after 24 h) and 64% (after 48 h) compared to DCF alone.

E. coli growth inhibition potency estimation

The growth inhibition effect of tested compounds was observed with use of *E. coli* K-12 culture. The increase in growth inhibition (GI) values of *E. coli* culture was observed in the case of all tested chemicals and in two different concentrations of metabolites and their mixtures with DCF (Fig. 2). The strongest inhibition of *E. coli* K12 culture growth of about 79% was obtained after 48h treatment with the mixture of DCF (5 μg/ml) with 4'-OHDCF (5 μg/ml) and 5-OHDCF (5 μg/ml), compared to the control (untreated bacteria). A clear decrease in *E. coli* culture development was also noted after bacteria incubation with DCF and its mixtures with 4'-OHDCF or 5-OHDCF in lower concentrations of metabolites (0.5 μg/ml), even to 67% of inhibition, comparable to control sample. Our results showed that the mixtures of DCF with metabolites and the mixtures of metabolites significantly reduced the rate of *E. coli* culture growth compared to parent chemicals. Results of the influence of the tested compounds and their mixtures on bacteria culture development are in agreement with *E. coli* cells viability assay, where we noticed strongest decrease in live cell number after *E. coli* culture treatment with mixtures of DCF with metabolites, applied in two different concentrations.

The assessment of the effect of DCF, its metabolites and their mixtures on E. coli stress-responsive promoters induction

The obtained in our experiments results showed that all promoters were induced by DCF, its metabolites and their mixtures, exhibiting several response patterns. We applied DCF at the concentration of 5 μg/ml and two different concentrations of metabolites 5 and 0.5 μg/ml. For each sample we measured luminescence and OD values at time 0 and after 1, 2, 3 and 5 h of incubation with tested chemicals. Table 3 and Figures 3

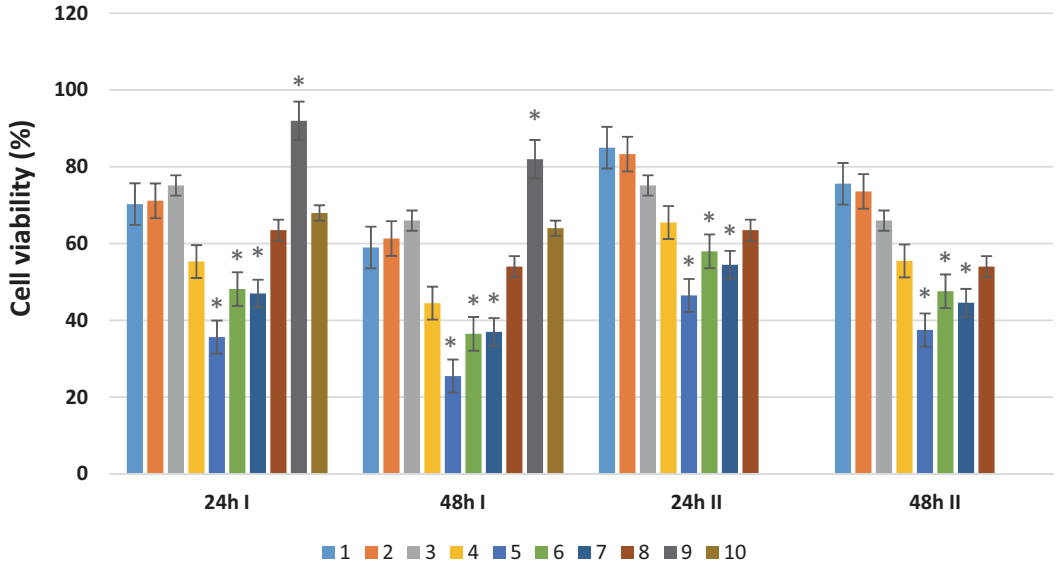

Fig. 1. The effect of DCF, 4'-OHDCF, 5-OHDCF, their mixtures, CA and CA mixture with DCF on *E. coli* K-12 cells viability. Bacteria cultures were incubated 24 and 48 h with 5 μg/ml of DCF and two variants of concentrations of metabolites 5 μg/ml and 0.5 μg/ml. Different concentrations of metabolites are marked with **I** (5 μg/ml of metabolite+5 μg/ml DCF) and **II** (0.5 μg/ml of metabolite+5 μg/ml DCF). 1) 4'-hydroxy-DCF, 2) 5-hydroxy-DCF, 3) DCF, 4) 4'-hydroxy-DCF+5-hydroxy-DCF, 5) 4'-hydroxy-DCF+5-hydroxy-DCF+DCF, 6) 4'-hydroxy-DCF+DCF, 7) 5-hydroxy-DCF+DCF, 8) nalidixic acid, 9) caffeic acid (10 μg/ml), 10) caffeic acid (10 μg/ml)+DCF (5 μg/ml). Mean values from three independent experiments ± SD are shown, * statistically significant result p≤0.05.

and 4 present several examples of these responses. We noticed the strongest induction of reporter strains *recA:luxCDABE* and *micF:luxCDABE* in response to the tested chemicals (Table 1). The fast and strong induction of *recA:luxCDABE* by nalidixic acid, 4-hydroxydiklofenac, 5-hydroxydiclofenac and the mixture of DCF and its metabolites was observed with the highest values of FI for NA (8.51) and the mixture of metabolites with DCF (FI=6.67) after 5 h of incubation. We also detected a more rapid (after 60 min) and stronger response of the *recA* promoter to the metabolite mixtures with DCF with FI= 3.96 compared to the *micF* promoter with FI=3.54. Our studies also showed a strong sensitivity of *micF:luxCDABE* reporter strain to NA, metabolites and the mixture of metabolites with DCF with maximum induction FI=7.64 after 3 h incubation with the mixture of metabolites with DCF. In the case of the both reporter strains *recA:luxCDABE* and *micF:luxCDABE* we gained the strongest response to the mixture of 4'- and 5-OHDCF with DCF in comparison to the mixtures of DCF with only one metabolite. In the conducted experiment we also noticed an increase sensitivity of both *recA* and *micF* promoters after 3 and 5 hours of incubation with the tested chemicals compared to 1 and 2 hours. We also compared the response patterns of the applied reporter strains in relation to control samples (Table 1) and to the samples in time zero (Fig. 1). The quite similar response patterns were observed with maximal induction for *recA* and *micF* promoters. With the application of *katG, sodA* and *inaA* promoters we also obtained less strong response to the tested chemicals. The three promoters reacted with a similar efficiency. Given the above results, the *recA* and *micF* bacteria strains were selected for comparison of the response patterns to different concentrations (5 and 0.5 µg/ml) of 4'- and 5-OHDCF and their mixtures with DCF (Fig. 4). Our results showed that the changes in metabolite concentrations differentiated the reactivity of reporter strains to metabolites and their mixtures with DCF. The analysis of obtained laboratory results with Tukey's test, considering the biosensor and reaction time as qualitative factors, showed that there were no statistically significant differences in the value of absorbance of individual genetic constructs. However, the value of luminescence of individual genetic constructs using the above mentioned quality factors differed significantly among each studied construct.

Oxidative stress studies

ROS generation

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and antioxidant defenses. Previous experimental studies have shown that diverse toxicants, such as heavy metals, hydrocarbons and pharmaceuticals, including DCF, induce ROS generation, making oxidative stress one of the major mechanisms of action (Nava-Álvarez et al., 2014). Thus, we studied the production of reactive oxygen species (ROS) in *E. coli* K-12 suspensions incubated with DCF, its metabolites and their mixtures. Figure 5 shows that the mixture of DCF with two different concentrations of metabolites 4'- and 5-OHDCF generate in *E. coli* K-12 112% (at metabolites concentration 5 µg/ml) and 57% (at metabolites concentration 0.5 µg/ml) more ROS, respectively, comparable to control sample (untreated bacterial culture). Other variants of DCF mixtures with 4'- or 5-OHDCF also strongly stimulated ROS synthesis in *E. coli* K-12. The ROS generation results obtained in our experiment are in agreement with cell viability assay and growth inhibition effect, as we noted the strongest decrease in *E. coli* cell viability and the strongest growth inhibition effect, after bacteria treatment with mixtures of the tested compounds than parent chemicals. It may indicate that the mechanism

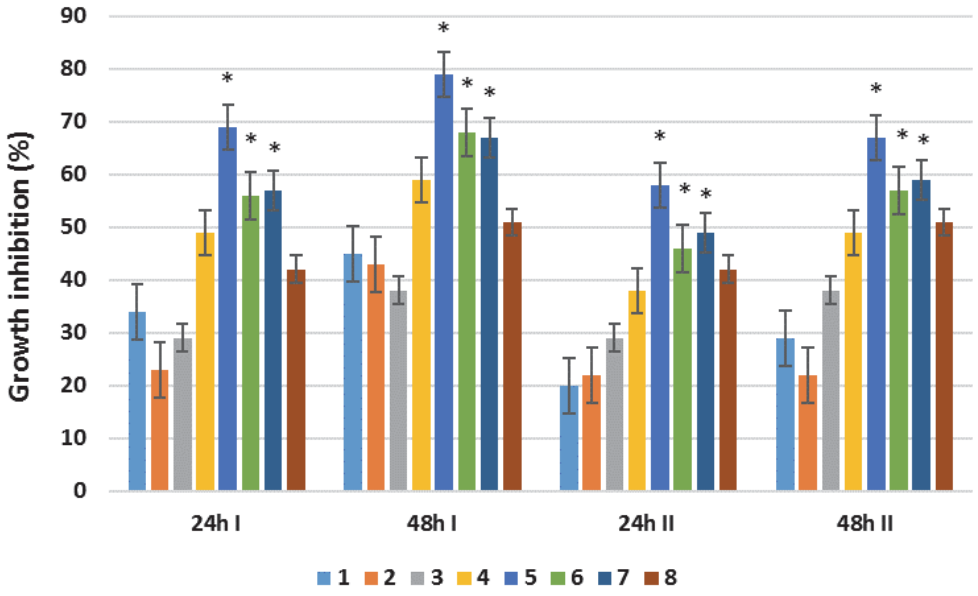

Fig. 2. The growth inhibition of *E. coli* K-12 cultures after 24 and 48 h incubation with DCF, 4'-OHDCF, 5-OHDCF and their mixtures. Bacteria cultures were incubated with 5 µg/ml of DCF and two variants of concentrations of metabolites 5 µg/ml and 0.5 µg/ml. Different concentrations of metabolites are marked with **I** (5 µg/ml of metabolite+5 µg/ml DCF) and **II** (0.5 µg/ml of metabolite+5 µg/ml DCF). 1) 4'-hydroxy-DCF, 2) 5-hydroxy-DCF, 3) DCF, 4) 4'-hydroxy-DCF+5-hydroxy-DCF, 5) 4'-hydroxy-DCF+5-hydroxy-DCF+DCF, 6) 4'-hydroxy-DCF+DCF, 7) 5-hydroxy-DCF+DCF, 8) nalidixic acid. Mean values from three independent experiments ± SD are shown, * statistically significant result p≤0.05.

Table 1. Maximal induction of each *E. coli* SM reporter strain with five promoters by DCF, its metabolites and the mixtures at the same concentrations of 5 µg/ml for DCF and metabolites. The results are presented as the FI (Fold Induction) of the induced sample to that of the non-induced control

Promoter and time of exposure	*4'-hydroxy-DCF*	*5-hydroxy-DCF*	*DCF*	*4'-hydroxy-DCF + 5-hydroxy-DCF*	*4'-hydroxy-DCF + 5-hydroxy-DCF + DCF*	*4'-hydroxy-DCF + DCF*	*5-hydroxy-DCF + DCF*	*Nalidixic acid*
inaA 1 h	1.07	1.0	1.56	1.52	2.51	1.84	1.61	1.66
inaA 2 h	1.75	2.05	2.11	1.81	2.65	1.96	1.99	3.38
inaA 3 h	2.45	1.81	2.31	2.34	3.04	2.33	2.14	4.81
inaA 5 h	2.50	2.66	2.55	2.56	3.24	3.05	2.33	4.06
recA 1 h	**1.92**	**2.25**	**2.13**	**3.05**	**3.96**	**2.67**	**2.54**	**3.90**
recA 2 h	**3.04**	**3.46**	**3.00**	**2.90**	**4.96**	**5.36**	**4.73**	**6.88**
recA 3 h	**3.41**	**2.74**	**3.00**	**3.19**	**4.60**	**3.40**	**3.63**	**6.43**
recA 5 h	**3.67**	**4.35**	**4.26**	**4.95**	**6.67**	**4.94**	**5.95**	**8.51**
micF 1 h	**1.70**	**1.85**	**1.87**	**2.80**	**3.54**	**2.86**	**2.30**	**2.85**
micF 2 h	**3.22**	**2.28**	**2.86**	**3.62**	**4.93**	**4.66**	**3.85**	**5.80**
micF 3 h	**3.14**	**3.42**	**3.62**	**3.90**	**7.64**	**6.73**	**5.27**	**6.50**
micF 5 h	**2.76**	**4.06**	**3.76**	**2.97**	**4.14**	**4.97**	**4.61**	**4.61**
katG 1 h	1.63	1.07	1.35	2.78	3.51	2.20	1.90	1.86
katG 2 h	2.22	1.83	2.44	3.07	2.94	2.83	2.40	3.40
katG 3 h	2.60	3.44	2.30	2.91	3.43	3.23	2.70	6.45
katG 5 h	3.42	3.66	3.94	3.51	3.54	3.16	3.41	5.19
sodA 1 h	1.25	1.00	1.10	1.67	1.98	1.42	1.50	1.60
sodA 2 h	1.91	1.30	1.92	1.70	2.33	1.85	1.78	2.76
sodA 3 h	2.95	3.54	2.68	2.73	3.96	2.87	3.08	4.03
sodA 5 h	2.81	3.03	2.57	2.83	3.31	2.43	2.32	3.78

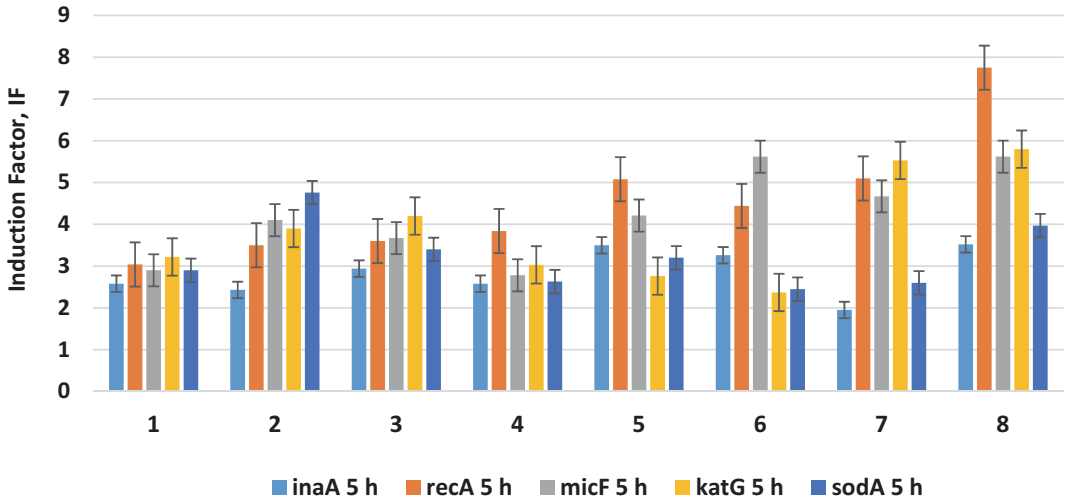

Fig. 3. Response patterns of 5 *E. coli* SM reporter strains with *recA*, *micF*, *katG*, *sodA* and *inaA* promoters to DCF, 4'-hydroxy-DCF, 5-hydroxy-DCF and their mixtures, following 5 h exposure. 1) 4'-hydroxy-DCF, 2) 5-hydroxy-DCF, 3) DCF, 4) 4'-hydroxy-DCF+5-hydroxy-DCF, 5) 4'-hydroxy-DCF+5-hydroxy-DCF+DCF, 6) 4'-hydroxy-DCF+DCF, 7) 5-hydroxy-DCF+DCF, 8) nalidixic acid. The results are presented as the FI (Fold Induction) of the induced sample to the induced sample at time zero. Mean values from three independent experiments ± SD are shown. A description of the statistics is provided in the sections statistical analysis and results.

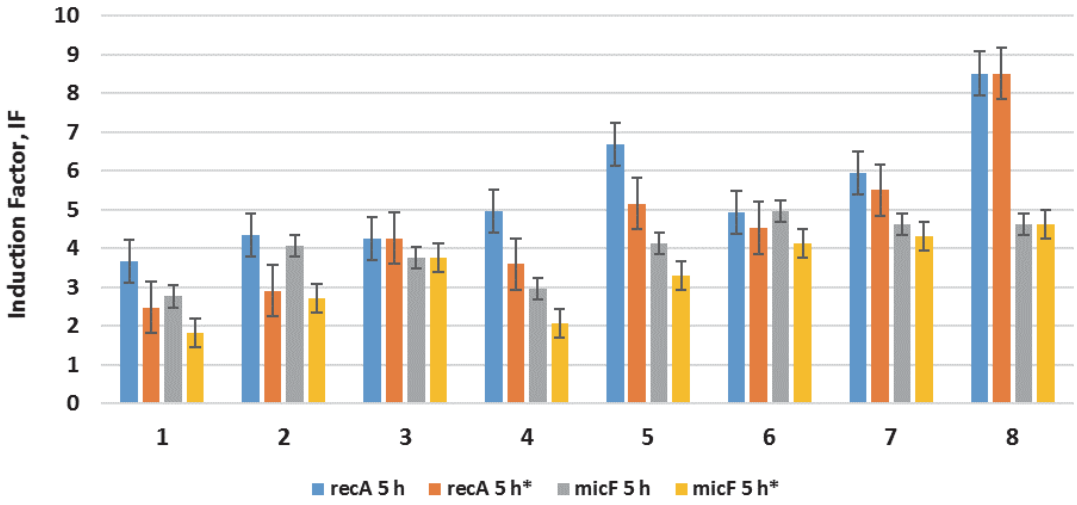

Fig. 4. The comparison of response patterns of *E. coli* SM *recA* and *micF* reporter strains to DIF, 4-hydroxy-DIF, 5-hydroxy-DIF and their mixtures, following 5 h exposure with application of two different concentrations of metabolites 5 µg/ml and 0.5 µg/ml (marked with an asteriks *). 1) 4'-hydroxy-DCF, 2) 5-hydroxy-DCF, 3) DCF, 4) 4'-hydroxy-DCF+5-hydroxy-DCF, 5) 4'-hydroxy-DCF+5-hydroxy-DCF+DCF, 6) 4'-hydroxy-DCF+DCF, 7) 5-hydroxy-DCF+DCF, 8) nalidixic acid. The results are presented as the FI (Fold Induction) of the induced sample to that of the non-induced control. Mean values from three independent experiments ± SD are shown. A description of the statistics is provided in the sections statistical analysis and results.

of bacterial death promoted by DCF, its metabolites and their mixtures is oxidative stress generation mediated by an increment of ROS.

Glutathione assay

Glutathione (GSH) is a low molecular weight thiol antioxidant that plays critical roles in protecting cells from oxidative stress and damage. Therefore, the estimation of GSH level is essential in oxidative stress parameters research. The mixtures of DCF with both metabolites 4'- and 5-OHDCF at the concentration of 5 µg/ml caused the highest 19% decrease in GSH level after 3 h of *E. coli* K-12 culture incubation as compared to the control. *E. coli* K-12 treatment with the mixtures of DCH with one metabolite 4'- or 5-OHDCF resulted in 15 and 16% decrease of GSH level in comparison to the control sample (untreated bacteria cells). In general, the mixtures of DCF and its metabolites at two different concentrations (5 and 0.5 µg/ml) inhibited the amount of GSH in *E. coli* stronger than chemicals used in isolation. The effect of DCF, 4'- and 5-OHDCF and their mixtures on GSH level in *E. coli* K-12 culture is shown in Figure 6. The obtained results revealed an inhibitory influence of DCF, 4'- and 5-OHDCF and their mixtures on GSH amount in *E. coli* culture.

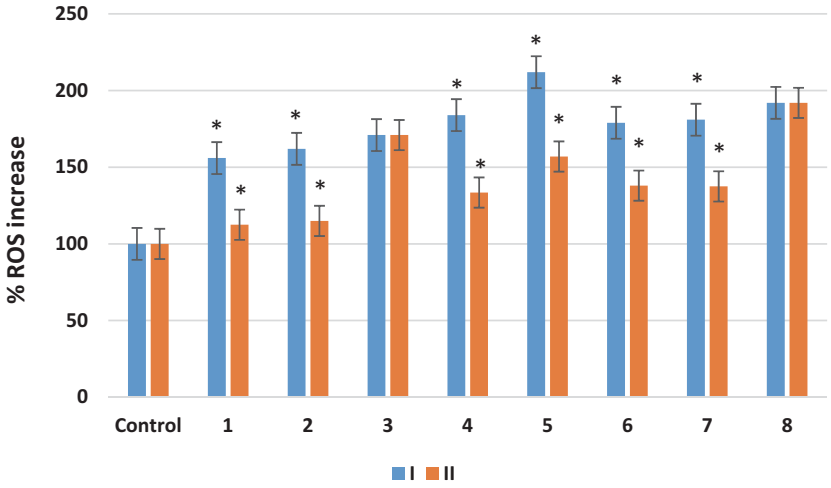

Fig. 5. Reactive oxygen species (ROS) increase induced in *E. coli* K-12 cultures incubated with 5 µg/ml of DCF and two variants of concentrations of metabolites 5 µg/ml and 0.5 µg/ml. Different concentrations of metabolites are marked with I (5 µg/ml) and II (0.5 µg/ml). 1) 4'-hydroxy-DCF, 2) 5-hydroxy-DCF, 3) DCF, 4) 4'-hydroxy-DCF+5-hydroxy-DCF, 5) 4'-hydroxy-DCF+5-hydroxy-DCF+DCF, 6) 4'-hydroxy-DCF+DCF, 7) 5-hydroxy-DCF+DCF, 8) nalidixic acid. Mean values from three independent experiments ± SD are shown, * statistically significant result p≤0.05.

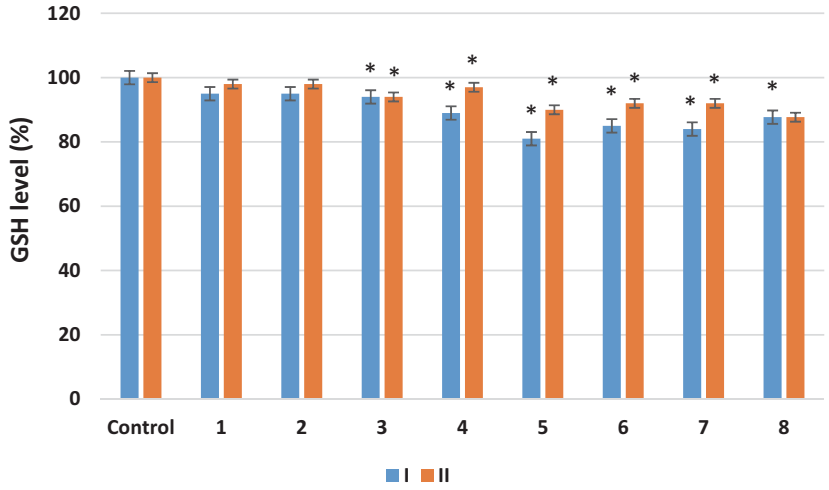

Fig. 6. GSH level (%) in *E. coli* K-12 suspensions incubated with 5 µg/ml of DCF and two variants of concentrations of metabolites 5 µg/ml and 0.5 µg/ml. Different concentrations of metabolites are marked with I (5 µg/ml) and II (0.5 µg/ml). 1) 4'-hydroxy-DCF, 2) 5-hydroxy-DCF, 3) DCF, 4) 4'-hydroxy-DCF+5-hydroxy-DCF, 5) 4'-hydroxy-DCF+5-hydroxy-DCF+DCF, 6) 4'-hydroxy-DCF+DCF, 7) 5-hydroxy-DCF+DCF, 8) nalidixic acid. Mean values from three independent experiments ± SD are shown, * statistically significant result p≤0.05.

Discussion

The results obtained in our experimental studies showed the strongest inhibition of cells viability and cytotoxic effect of the mixtures of DCF with its metabolites on *E. coli* K-12 culture comparable to parent chemicals. Similarly, we noticed the most intense interaction of the mixtures of DCF and its metabolites with *recA*, *micF*, *katG*, *sodA* and *inaA* promoters in *E. coli* SM biosensor strains. Previous laboratory studies showed DCF antibacterial effect against both gram-positive and gram--negative bacteria. Moreover, synergistic effect of DCF with other antibiotics was detected. The mechanism of molecular activity of DCF is still unclear. Some authors described DCF ability to inhibit DNA replication and membrane activity disruption as possible cytotoxic mechanisms (Kristiansen et al., 2007; Dutta et al., 2007). In our work we also found a synergistic effect of CA in combination with DCF. In environmental matrices, pharmaceuticals can interact with many chemicals to form more reactive and toxic complexes (Lonappan et al., 2016).

We used *E. coli* SM biosensor strains with five stress-responsive promoters (*recA*, *micF*, *katG*, *sodA* and *inaA*) to monitor a biological impact of DCF, its metabolites and their mixtures on living bacteria cells. We noticed the positive reaction of the used promoters to the analysed compounds with the strongest induction of reporter strains *recA:luxCDABE* and *micF:luxCDABE* by nalidixic acid, DCF metabolites and the mixture of DCF with its metabolites 4'-OHDCF, 5-OHDCF. RecA is a known regulatory protein that is involved in DNA damage response. RecA induces the SOS response to DNA damage by promoting the autocatalytic cleavage of the repressor

protein LexA. Additionally, in many scientific experiments, it has been shown that the activity of the *recA* promoter is proportional to the genotoxic potency of the tested chemical. Therefore, bacteria biosensor strains with *recA* promoter are commonly used to study the genotoxic effects of different chemical compounds in the environment (Lusetti and Cox 2002; Maul and Sutton 2005). Our results are in agreement with previous laboratory studies, where authors obtained a high level of *recA* and *micF* promoters induction in response to drugs and other toxic compounds. Oxidative damage in response to the analyzed chemicals and abnormal protein accumulation could be a possible mechanism of *recA* and *micF* induction. The *micF* gene belongs to the group of stress response genes and it was found in *Escherichia coli* and related bacteria, such as *Salmonella spp*., *Klebsiella pneumoniae* and *Serratia marcescens*. The *micF* gene encodes a non-translated 93 nt antisense RNA. In response to stress conditions *micF* as an antisense RNA gene post-transcriptionally controls outer membrane porin protein F (*ompF*) expression. Up to date, it has been revealed that various environmental factors, such as: oxidative stress, hydrogen peroxide, osmolarity increase, temperature increase, nutrients, weak acids, antibiotics, and other toxic compounds stimulate *micF* expression (Delihas et al., 2001). With the application of *katG, sodA* and *inaA* promoters we obtained a weaker response to the tested chemicals in comparison to *recA* and *micF* promoters. The three promoters *katG, sodA* and *inaA* reacted to the tested chemicals with a similar efficiency. The *katG, sodA* and *inaA* promoters are involved in stress response. *KatG* promoter responds to hydrogen peroxide stress. The *katG* gene codes bifunctional hydroperoxidase I, having both catalase and peroxidase activity. The next, *sodA* promoter is sensitive to superoxide stress. *SodA* gene codes superoxide dismutase (SOD). These enzymes play a key role in the first line of antioxidant defense. Reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide (H_2O_2) are thought to be by-products of aerobic respiration (Ninganagouda et al., 2014; Ong et al., 2017; Ajiboye 2019; Diaz-Garcia et al., 2019). ROS have damage effects on DNA, cellular proteins, and lipids. To protect cells from cellular damage aerobic organisms have evolved detoxification and repair systems. SOD is a key enzyme in oxygen defense systems. SOD catalyzes the dismutation of $O_2^{\cdot-}$ into oxygen and H_2O_2, the latter being broken in turn to water by catalase or peroxidase (Bae et al., 2011; Ighodaroa et al., 2018). The *inaA* promoter reacts to pH changes. *InaA* gene encodes for a protein of unknown function but is induced in a pH-dependent manner and by the presence of chemical stressors, such as benzoic and salicylic acids. The *inaA* gene expression is under the control of MarA protein and it is part of the *mar* regulon (Leea and Mitchell 2012). Among the promoters studied, DNA-damage inducible *recA* promoter was used for genotoxic potency assessment of the tested compounds. On the base of our results we detected the strongest induction of *recA* promoter after *E. coli recA:luxCDABE* strain treatment with NA and the mixtures of DCF with metabolites in comparison to the other tested chemicals. NA is a first-generation quinolone-based antibiotic. The main molecular mechanism of NA action is its ability to increase in bacteria cells the concentration of enzyme--DNA cleavage complexes leading to the DNA replication inhibition and cell death (Aldred et al., 2014). As mentioned above, in previous studies the antimicrobial activity of DCF was also presented. Additionally, DCF in combination with other antibiotics enhanced their antimicrobial activity. The inhibition of DNA replication and negative effects on cell membranes are the main DCF cytotoxic mechanisms (Dastidar et al., 2000; Aldred et al., 2014).

Diclofenac belongs to a group of a nonsteroidal anti-inflammatory drugs (NSAID) with anti-inflammatory, analgesic, and antipyretic properties. In mammals, DCF is biotransformed to 4'-OHDCF and 5-OHDCF which have been shown to be highly reactive and induce ROS formation. Additionally, the main metabolite, 4'-OHDCF shows weak anti-inflammatory and analgesic activities. Diclofenac after metabolism in the human body is excreted in the urine and bile. Approximately, 35% of the DCF dose is excreted in the bile as biologically active conjugates of unchanged diclofenac and its metabolites (Altman et al., 2015). DCF and its metabolites get directly into wastewaters and enter aqueous environment. Our experiments presented stronger impact of DCF mixtures with its metabolites on *E. coli* SM biosensor strains in relation to parent drug. Similar results were obtained in *E. coli* K-12 cell viability test and estimation of growth inhibition.

NSAIDs, including DCF, have strong potency to induce toxicity on aquatic organisms. In natural ecosystems, organisms are exposed to a mixture of toxic agents. Previous studies indicated that NSAIDs are associated with increased ROS production. Moreover, during the biotransformation of DCF, metabolites and reactive oxygen species (ROS) are produced which induces oxidative stress (Dastidar et al., 2000; Dutta et al., 2007; Ghosh et al., 2015). Figure 7 presents the proposed oxidative stress-based mechanism of action of DCF, its metabolites and mixtures. This mechanism was also proposed by previous authors for antibiotics, phenolic acids and 2-(2-nitrovinyl) furan (Ghosh et al., 2015; Ajiboye et al., 2019). According to this mechanism the tested compounds stimulate electron transport chain activity leading to electron leakage as well as •O2− and H_2O_2 production. This induces oxidative stress. Moreover, these conditions decrease GSH level in *E. coli* cells, resulting in the reduced ROS detoxification capability. Finally, H_2O_2 is accumulated and in the presence of Fe_2^+ is converted to •OH that damages DNA (Ajiboye 2019). Our results also indicate that the oxidative stress coud be a most probable mechanism of DCF, its metabolites and their mixtures action. In our experiment we obtained the highest level of ROS generation after bacteria treatment with the mixtures of DCF and its metabolites. Moreover, we observed the strongest induction of *recA* promoter in *E. coli* biosensor strain. This promoter is very sensitive to oxidative-DNA damage. Similarly, the high level of induction of oxidative stress-sensitive *micF*, *katG*, *sodA* promoters was noted.

Glutathione, a low molecular weight thiol antioxidant is found in gram-negative bacteria including *E. coli*. In most gram--positive bacteria, with the exception of some *Streptococcus* and *Enterococcus* species, GSH is not found. Due to its antioxidant character, GSH maintains the redox potential of the cell and it has ability to prevent cellular damages caused by reactive oxygen species or toxic heavy metals (Schmacht et al., 2017). In our experiment, we noted a marked reduction in GSH content after *E. coli* K-12 exposure to DCF, its metabolites and their mixtures. These results indicated that the tested compounds significantly decreased the GSH-dependent antioxidant protection of *E. coli* culture.

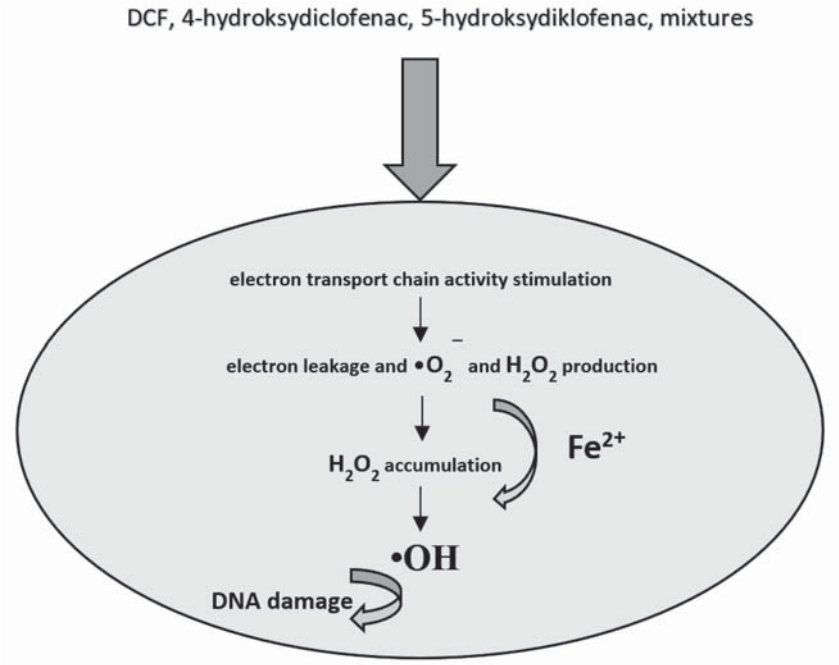

Fig. 7. Proposed mechanism of action of DCF, its metabolites and mixtures. Tested chemicals and their mixtures stimulate electron transport chain activity leading to electron leakage and •O2− and H_2O_2 production. This causes oxidative stress. Moreover, these conditions decrease GSH level in *E. coli* cells, resulting into reduced ROS detoxification capability. Finally, H_2O_2 is accumulated and in the presence of Fe^{2+} is converted to •OH that damage DNA.

Conclusion

Our results indicate that DCF metabolites 4'-OHDCF and 5-OHDCF have the ability to interact with DCF. We observed that DCF mixtures with metabolites have a stronger impact on the viability and development of *E. coli* culture. In addition, our results indicate the possibility of metabolite interaction with each other and the formation of mixtures that also strongly affect bacteria viability and growth. Similar results were noticed with use of *E. coli* biosensor strains with the fusion of bacteria promoters with *luxCDABE* gene where we found that metabolite mixtures with DCF had the influence on *recA*, *katG*, *sodA*, *micF* and *inaA* promoters induction. The results obtained indicate that the metabolites enhance the biological effect of DCF on *E. coli* strains.

We have also found a synergistic effect of CA in combination with DCF. This mixture had a stronger impact on *E. coli* cells viability than DCF alone. It suggest that DCF can potentially interact with different chemicals which are present in wastewater matrices and it can create more toxic complexes. Our results are in line with the observations of other authors, who warn against the danger of biological effect and toxicity of mixtures of pharmaceutical residues and their metabolites in the environmental matrices. In addition, the results we receive will help to focus more attention on the need of the estimation of the acute and chronic potential effects of pharmaceutical residues and their metabolites in the environment. Moreover, further investigations on the ecotoxic potential of their mixtures are required (Fatta-Kassinos and Nikolaou 2011). Regarding to DCF elimination from wastewaters it is not enough to optimize the existing biological processes only. There is a need to develop effective and cost-efficient methods based on oxidation or adsorption, such as AOPs technologies (e.g. ozonation) or adsorption on activated carbon. We should remember that during pharmaceutical degradation with AOPs technologies the formation of toxic by-products takes place. This raises the need for additional systems designed to remove toxic intermediates after AOPs. Therefore, use of reverse osmosis after AOPs based technologies to remove toxic by-products could be a justified solution (Vieno and Sillanpää 2014). Our research also showed that used in this work five *E. coli* biosensor strains are useful devices for the assessment of the biological effect of DCF residues and its mixtures with metabolites on living bacteria cells.

Conflict of interest

No conflict of interest have been declared.

Funding

This work was financially supported by National Science Centre, Poland, under the research project number 2018/29/B/NZ9/01997.

Acknowledgements

We would like to thank Professor Joanna Surmacz-Górska from the Silesian University of Technology, Department of Environmental Biotechnology for very helpful scientific discussions and assistance on the topic of pharmaceutical residues in the environment.

Authors are very grateful to Professor Shimshon Belkin, Dr Tal Elad and Dr Sharon Yagur-Kroll, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel for providing bacteria strains and scientific assistance.

References

Acuña, V.A., Ginebreda, J.R., Mor, M., Petrovic, S., Sabater, J., Sumpter, D. & Barceló, D. (2015). Balancing the health benefits and environmental risks of pharmaceuticals: diclofenac as an example. *Environment International*, 85, pp. 327–333.

Ajiboye, T.O. (2019). Contributions of reactive oxygen species, oxidative DNA damage and glutathione depletion to the sensitivity of *Acinetobacter baumannii* to 2-(2nitrovinyl) furan. *Microbial Pathogenesis*, 128, pp. 342–346.

Aldred, K., Kerns, R.J. & Osheroff, N. (2014). Mechanism of Quinolone Action and Resistance. *Biochemistry*, 53, pp. 1565−1574.

Altman, R., Bosch, B., Brune, K., Patrignani P. & Young, C. (2015). Advances in NSAID development: evolution of diclofenac products using pharmaceutical technology. *Drugs*, 75, 859–877.

Bae, Y.S., Oh, H., Rhee, S.G. & Yoo Y.D. (2011). Regulation of reactive oxygen species generation in cell signaling. *Molecules and Cells*, 32, pp. 491–509.

Behera, S.H., Kim, H.W., Oh, J.E. & Park H.-S. (2011). Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. *Science of Total Environment*, 409, pp. 4351–4360.

Beltran, F.J., Pocostales, P., Alvarez P. & Oropesa A. (2009). Diclofenac removal from water with ozone and activated carbon. *Journal of Hazardous Materials*, 163, pp. 768–776.

Bouju, H., Nastold, P., Beck, B., Hollender, J., Corvini, P.F. & Wintgens, T. (2016). Elucidation of biotransformation of diclofenac and 4-hydroxydiclofenac during biological wastewater treatment. *Journal of Hazardous Materials*, 15, pp. 443–52.

Cunha, S.C., Pena, A. & Fernandes, J.O. (2017). Mussels as bioindicators of diclofenac contamination in coastal environments. *Environmental Pollution*, 225, pp. 354–360.

Dastidar, S.G., Ganguly, K., Chaudhuri K. & Chakrabarty, A.N. (2000). The anti-bacterial action of diclofenac shown by inhibition of DNA synthesis. *International Journal of Antimicrobial Agents*, 14, pp. 249–51.

Delihas, N. & Forst, S. (2001). *MicF*: an antisense RNA gene involved in response of *Escherichia coli* to globall stress factors. *Journal of Molecular Biology*, 313, pp. 1–12.

Desbiolles, F., Malleret, L., Tiliacos, C., Wong-Wah-Chung, P. & Laffont-Schwob, I. (2018). Occurrence and ecotoxicological assessment of pharmaceuticals: Is there a risk for the Mediterranean aquatic environment? *Science of Total Environment*, 15, 639, pp. 1334–1348.

Doruk Aracagök, Y., Göker H. & Cihangir, N. (2018). Biodegradation of diclofenac with fungal strains. *Archives of Environmental Protection*, 44, pp. 55–62.

Dutta, N.K., Annadurai, S., Mazumdar, K., Dastidar, S.G., Kristiansen J.E., Molnar J., Martins, M. & Amaral, L. (2007). Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. *International Journal of Antimicrobial Agents*, 30, pp. 242–9.

Díaz-García, D., Ardiles, P.R., Prashar, S., Rodríguez-Diéguez, A., Páez P.L. & Gómez-Ruiz, S. (2019). Preparation and study of the antibacterial applications and oxidative stress induction of copper maleamate-functionalized mesoporous silica nanoparticles. *Pharmaceutics*, 14, pp. 11–23.

Fatta-Kassinos, D., Meric, S. & Nikolaou, A. (2011). Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. *Analitical and Bioanalitical Chemistry*, 399, pp. 251–275.

Felis, E., Surmacz-Górska, J., Miksch K. & Ternes, T. (2005). Presence of pharmaceutics in wastewater from wastewater treatment plant "Zabrze-Śródmieście" in Poland. *Archiwum Ochrony Środowiska*, 31, 3, pp. 49–58.

Felis, E., Wiszniowski J. & Miksch, K. (2009). Advanced oxidation of diclofenac in various aquatic environments. *Archives of Environmental Protection*, 35, 2, pp. 15–25.

Gernjak, W., Maldonado, M.I., Malato, S., Caceres, J,, Krutzler, T., Glaser, A. & Bauer, R. (2003). Degradation of polyphenolic content of olive mill wastewater (OMW) by solar photocatalysis. In: Vogelpohl A, [Ed.] 3rd International conference on oxidation technologies for water and wastewater treatment, pp. 879–84.

Ghosh, R., Alajbegovic, A. & Gomes, A. V. (2015). NSAIDs and cardiovascular diseases: role of reactive oxygen species. *Oxidative Medicine and Cellular Longevity*, 3, pp. 67–78.

Haiba, E., Nei, L., Kutti, S., Lillenberg, M., Herodes, K., Ivaski, M., Kipper, K., Aro, R. & Laaniste, A. (2017). Degradation of diclofenac and triclosan residues in sewage sludge compost. *Agronomy Research*, 15(2), pp. 395–405.

Hassan, S.H.A., Van Ginkel, S.W., Hussein, M.A.M., Abskharon, R. & Oh, S.E. (2016). Toxicity assessment using different bioassays and microbial biosensors. *Environment International*, 92–93, pp. 106–118.

Ighodaroa, O.M. & Akinloye, O.A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase(CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, 54, pp. 287–293.

Janion, A. (2008). Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. *International Journal of Biological Sciences*, 4, 338–344.

Kawase, A., Hashimoto, R., Shibata, M., Shimada, H. & Iwaki, M. (2017). Involvement of reactive metabolites of diclofenac in cytotoxicity in sandwich-cultured rat hepatocytes. *International Journal of Toxicology,* 36, pp. 260–267.

Kessler, N., Schauer, J.J., Yagur-Kroll, S., Melamed, S., Tirosh, O., Belkin S. & Erel, Y. (2012). A bacterial bioreporter panel to assay the cytotoxicity of atmospheric particulate matter. *Atmospheric Environment*, 63, pp. 94–101.

Kim, S.-H., Lee, H.-S., Ryu, D.-S., Choi, S.-J. & Lee, D.-S. (2011). Antibacterial activity of silver-nanoparticles against *Staphylococcus aureus* and *Escherichia coli*. *Korean Journal of Microbiology and Biotechnology*, 39, pp.77–85.

Klopčič, I., Markovič, T., Mlinarič-Raščan, I. & Sollner Dolenc, M. (2018). Endocrine disrupting activities and immunomodulatory effects in lymphoblastoid cell lines of diclofenac, 4-hydroxydiclofenac and paracetamol. *Toxicology Letters*, 294, pp. 95–104.

Kristiansen, J.E., Hendricks, O., Delvin, T., Butterworth, T.S., Aagaard, L., Christensen, J.B., Flores V.C. & Keyzer H. (2007). Reversal of resistance in microorganisms by help of non-antibiotics. *Journal of Antimicrobial Chemotherapy*, 59, pp. 1271–9

Kümmerer, K. (2009). The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. *Journal of Environmental Management*, 90, pp. 2354–2366.

Langenhoff, A., Inderfurth, N., Veuskens, T., Schraa, G., Blokland, M., Kujawa-Roeleveld, K. & Rijnaarts, H. (2013). Microbial removal of the pharmaceutical compounds ibuprofen and diclofenac from wastewater. *Biomed Research International*, 325, pp. 806–830.

Leea, S. & Mitchell, R.J. (2012). Detection of toxic lignin hydrolysate-related compounds using an *inaA::luxCDABE* fusion strain. *Journal of Biotechnology*, 20, pp. 598–604.

Lonappan, L., Brar, S.K., Das, R.K., Verma, M. & Surampalli, R.Y. (2016). Diclofenac and its transformation products: environmental occurrence and toxicity – a review. *Environment International*, 96, pp. 127–138.

Lusetti, S.L. & Cox, M.M. (2002). The bacterial RecA protein and therecombinational DNA repair of stalled replication forks. *Annual Review of Biochemistry*, 71, pp. 71–100.

Magnani, C.,Isaac, V.L.B., Correa, M.A. & Salgado, H.R.N. (2014). Caffeic acid: a review of its potential use in medications and cosmetics. *Analytical Methods*, 6, pp. 3203–3210.

Mantzavinos, D. & Kalogerakis, N. (2005). Treatment of olive mill effluents, Part I. Organic matter degradation by chemical and biological processes – an overview. *Environmental International*, 31, pp. 289–295.

Marciocha, D., Kalka, J., Turek-Szytow, J. & Surmacz-Górska, J. (2013). A Pretreatment Method for Analysing Albendazole by HPLC in Plant Material. *Water of Air and Soil Pollution*, 224, pp. 1646–1658.

Marco-Urrea, E., Pérez-Trujillo, M., Cruz-Morató, C., Caminal, G. & Vicent, T. (2010). Degradation of the drug sodium diclofenac by *Trametes versicolor* pellets and identification of some intermediates by NMR. *Journal of Hazardous Material*, 176, pp. 836–842.

Maul, R.W. & Sutton, M.D. (2005). Roles of the *Escherichia coli* RecA protein and the global SOS response in effecting DNA polymerase selection in vivo. *Journal of Bacteriology*, pp. 7607–7618.

Melamed, S., Lalush, C., Elad, T., Yagur-Krol, S., Belkin, S. & Pedahzur, R. (2012). A bacterial reporter panel for the detection and classification of antibiotic substances. *Microbiology and Biotechnology*, 5, pp. 536–548.

Memmert, U., Peither, A., Burri, R., Weber, K., Schmidt, T., Sumpter, J.P. & Hartmann, A. (2013). Diclofenac: new data on chronic toxicity and bioconcentration in fish. *Environmental Toxicological Chemistry*, 32, pp. 442–452.

Moreira, I.S., Bessa, V.S., Murgolo, S., Piccirillo, C., Mascolo, G. & Castro, P.M.L.(2018). Biodegradation of diclofenac by the bacterial strain *Labrys portucalensis* F11. *Ecotoxicology and Environmental Safety*, 15, 152, pp. 104–113.

Nava-Álvarez, R., Razo-Estrada, A.C., García-Medina, S., Gómez--Olivan, L.M. & Galar-Martínez, M. (2014). Oxidative stress induced by mixture of diclofenac and acetaminophen on Common Carp (*Cyprinus carpio*). *Water, Air & Soil Pollution*, 225, pp. 1873–1885.

Ninganagouda, S., Rathod, V., Singh, D., Hiremath, J., Singh, A.K., Mathew, J. & Manzoorul-Haq, J. (2014). Growth kinetics and mechanistic action of reactive oxygen species released by silver nanoparticles from *Aspergillus niger* on *Escherichia coli*. *BioMed Research International*, 13, pp. 1–9.

Nowrotek, M., Sochacki, A., Felis, E. & Miksch, K. (2016). Removal of diclofenac and sulfamethoxazole from synthetic municipal wastewater in microcosm downflow constructed wetlands: Start-up results. *International Journal of Phytoremediation,* 18, 2, pp. 157–63.

Ong, K.S., Cheow, Y.L. & Lee, S.M. (2017). The role of reactive oxygen species in the antimicrobial activity of pyochelin. *Journal of Advanced Research*, 8, pp. 393–398.

Osorio, V., Sanchís, J., Abad, J.L., Ginebreda, A., Farré, M., Pérez, S. & Barceló, D. (2016). Investigating the formation and toxicity of nitrogen transformation products of diclofenac and sulfamethoxazole in wastewater treatment plants. *Journal of Hazardous Material*, 309, pp. 157–164.

Praveena, S.M., Shaifuddin, S.N.M., Sukiman, S., Nasir, F.A.M., Hanafi, Z., Kamarudin, N., Ismail T.H.T. & Aris, A.Z. (2018). Pharmaceuticals residues in selected tropical surface water bodies from Selangor (Malaysia): Occurrence and potential risk assessments. *Science of Total Environment*, 15, 642, pp. 230–240.

Schmacht M., Lorenz E. & Senz, M. (2017). Microbial production of glutathione. *World Journal of Microbiology and Biotechnology*, 33, pp. 106–118.

Schmidt, S., Hoffmann, H., Garbe L.A. & Schneider, R.J. (2018). Liquid chromatography-tandem mass spectrometry detection of diclofenac and related compounds in water samples. *Journal of Chromatography* A, 23, 1538, pp. 112–116.

Simazaki, D., Kubota, R., Suzuki, T., Akiba, M., Nishimura, T. & Shoichi, K. (2015). Occurrence of selected pharmaceuticals at drinking water purification plants in Japan and implications for human health. *Water research* 76, pp.187 – 200.

Sochacki, A., Felis, E., Bajkacz, S., Nowrotek M. & Miksch, K. (2018). Removal and transformations of diclofenac and sulfamethoxazole in a two-stage constructed wetland system. *Ecological Enginnering*,122, 159–168.

Vieno, N. & Sillanpää, M. (2014). Fate of diclofenac in municipal wastewater treatment plant – a review. *Environment International*, 69, pp. 28–39.

Yagur-Kroll, S. & Belkin, S.(2011). Upgrading bioluminescent bacterial bioreporter performance by splitting the *lux* operon. *Analitical and Bioanalitical Chemistry*, 400, pp. 1071–1082.

Yagur-Kroll, S., Bilic, B. & Belkin S. (2010). Strategies for enhancing bioluminescent bacterial sensor performance by promoter region manipulation. *Microbiology and Biotechnology*, 3, pp. 300–310.

Ocena oddziaływania diclofenaku i jego metabolitów biodegradacji 4'-hydroksydiklofenaku i 5-hydroksydiklofenaku na *Escherichia coli*. Synergistyczny efekt diklofenaku z kwasem kawowym

Streszczenie: Celem pracy było określenie oddziaływania diklofenaku, jego metabolitów biodegradacji 4'-OHDCF i 5-OHDCF oraz ich mieszanin na szczepy *E. coli*. Efekt biologiczny i stres oksydacyjny wywołany działaniem badanych w pracy związków chemicznych oceniono, poddając analizie następujące biomarkery: żywotność komórek *E. coli* K-12, hamowanie wzrostu kultury bakterii, wytwarzanie ROS i ocena zawartości glutationu (GSH). Ponadto zbadaliśmy wpływ mieszaniny DCF z CA na żywotność komórek *E. coli*. Monitorowaliśmy także reaktywność szczepu biosensora *E. coli* SM recA: luxCDABE w ściekach. Otrzymane wyniki wykazały najsilniejszy wpływ mieszanin DCF z 4'-OHDCF i 5-OHDCF na szczepy *E. coli*. Mieszanki diclofenaku z metabolitami działały inhibująco na rozwój kultury *E. coli* K-12 i żywotność komórek. Zaobserwowano także synergistyczne, inhibitorowe działanie kwasu kawowego w połączeniu z DCF na żywotność komórek *E. coli*. Najintensywniejszą generację ROS oraz redukcję GSH zaobserwowano po potraktowaniu bakterii mieszaniną DCF i jej metabolitów. Sugeruje to, że stres oksydacyjny jest najważniejszym mechanizmem leżącym u podstaw działania DCF i jego metabolitów. Ponadto, w przeprowadzonym eksperymencie wykazano użyteczność mikrobiologicznego biosensora *E. coli* SM recA w monitorowaniu ścieków zanieczyszczonych DCF. Uzyskane wyniki wskazują, że metabolity DCF 4'-OHDCF i 5-OHDCF mają zdolność interakcji z DCF. Zaobserwowaliśmy, że mieszaniny DCF z metabolitami mają większy wpływ na żywotność i rozwój kultury *E. coli* oraz indukcję promotorów w biosensorowych szczepach *E. coli*.