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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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The study of biological activity of transformation products of diclofenac and its interaction with chlorogenic acid

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ARTICLE INFO

Article history:
Received 1 December 2019
Received in revised form
26 January 2020
Accepted 27 January 2020
Available online 7 February 2020

Keywords:
Diclofenac
Biodegradation
Chlorogenic acid
Advances oxidation processes (AOPs)
Wastewaters
Microbial biosensors

ABSTRACT

In the present work we compared the biological activity of DCF, 4′-OHDCF and 5-OHDCF as molecules of most biodegradation pathways of DCF and selected transformation products (2-hydroxyphenylacetic acid; 2,5-dihydroxyphenylacetic acid and 2,6-dichloroaniline) which are produced during AOPs, such as ozonation and UV/H_2O_2. We also examined the interaction of DCF with chlorogenic acid (CGA). CGA is commonly used in human diet and entering the environment along with waste mainly from the processing and brewing of coffee and it can be toxic for microorganisms included in activated sludge. In the present experiment the evaluation of following parameters was performed: *E. coli* K-12 cells viability, growth inhibition of *E. coli* K-12 culture, LC_{50} and mortality of *Chironomus aprilinus*, genotoxicity, *sodA* promoter induction and ROS generation. In addition the reactivity of *E. coli* SM *recA:luxCDABE* biosensor strain in wastewater matrices was measured. The results showed the influence of DCF, 4′-OHDCF and 5-OHDCF on *E. coli* K-12 cells viability and bacteria growth, comparable to AOPs by-products. The highest toxicity was observed for selected, tested AOPs by-products, in comparison to the DCF, 4′-OHDCF and 5-OHDCF. Genotoxicity assay indicated that 2,6-dichloroaniline (AOPs by-product) had the highest toxic effect. The oxidative stress assays revealed that the highest level of ROS generation and *sodA* promoter induction were obtained for DCF, 4′-OHDCF and 5-OHDCF, compared to other tested compounds. We have also found that there is an interaction between chlorogenic acid and DCF, which resulted in increased toxicity of the mixture of the both compounds to *E. coli* K-12, comparable to parent chemicals. The strongest response of *E. coli* SM biosensor strain with *recA:luxCDABE* genetic construct in filtered treated wastewaters, comparable to control sample was noticed. It indicates, that *E. coli* SM *recA:luxCDABE* biosensor strains is a good tool for bacteria monitoring in wastewater environment. Due to toxicity and biological activity of tested DCF transformation products, there is a need to use additional wastewater treatment systems for wastewater contaminated with pharmaceutical residues.

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https://doi.org/10.1016/j.jes.2020.01.022

Introduction

The group of non-steroidal anti-inflammatory (NSAID) and pain relieving drugs is one of the most frequently detected in environmental martices. Pharmaceutical residues belong to chemicals of emerging concern (CECs) pollutants which are defined as nonregulated unstudied compounds posing a risk to aquatic ecosystems. It includes drug abuse substances, personal care products, hormones, surfactants, industrial additives, and pharmaceutical agents (Islas-Flores et al., 2013). Diclofenac (DCF) 2-(2,6-dichloroanilino)phenylacetic acid is widely prescribed and it is one of the main representatives of the NSAID group. It was estimated that annual worldwide consumption of DCF as a human and veterinary pharmaceutical drug is > 1000 tons/year (Memmert et al., 2013). DCF as anthropogenic micropollutant is frequently detected in wastewater and natural waters and it is one of the most serious problem worldwide (Sathishkumar et al., 2020). DCF is even proposed as a suitable marker for anthropogenic pollution. DCF and its metabolites together with human excreta get into the wastewater treatment plants (WWTPs). Currently, DCF is found in groundwater, surface (including marine) water, wastewater and even drinking waters at a concentrations from 0.02 ng/L to 20.00 μg/L worldwide. Moreover, DCF metabolites as 4′-hydroxy-DCF, 5-hydroxy-DCF and *p*-benzoquinone imine of 5-hydroxy-DCF have been detected in sewage water and river sediments (Liu and Wong, 2013; Ivshina et al., 2019). To date, the highest DCF concentrations have been recorded in hospital and pharmaceutical manufacturer's wastewater in South Korea and were 6.88 and 203 μg/L, respectively. DCF is resistant to biodegradation. The removal efficiencies by WWTPs ranges from 21 to 40%. From WWTPs DCF and its metabolites are released to aquatic environment (Moreira et al., 2018).

Ecotoxicologists in the study of micropollutant toxicity, including pharmaceuticals, point out that pharmaceutical micropollutants, do not always cause lethal effects on living organisms at environmental concentrations. However, they can induce changes in their life cycle, sex ratios, growth or varying degrees of anatomical deformities. These developmental abnormalities of living organisms can lead to a diminished biological performance of wild populations and even to their extinction (Feito et al., 2012). Several ecotoxicological studies emphasised that exposure to DCF affects biochemical processes of living organisms. It was showed that DCF at concentration of 0.1 μg/L and 0.2 μg/kg had a negative impact in duckweed plants and freshwater fish, respectively. It was also evidenced that DCF had a poisonous effect on Indian subcontinent Gyps vultures (Moreira et al., 2018). In connection to mentioned observations, DCF has been suggested to be added into the list of priority substances in EU's Water Framework Directive (Directive, 2013/39/EU) (Moreira et al., 2018). Biodegradation is one of the most important processes in the removal of the majority of xenobiotics, including pharmaceuticals in wastewater and surface waters (Bessa et al., 2017; Langenhoff et al., 2013). To date, it has been shown that both bacteria and eukaryotic organisms have the ability to degrade DCF. Bacterial DCF degradation mainly concerns Gram-positive bacteria such as *Actinoplanes* and *Brevibacterium* as well as by microorganisms of activated sludge. Eukaryotic organisms involved in DCF degradation are mainly basidiomycetes (*Bjerkandera*, *Trametes*, *Phanerohaete*), zigomycetes (*Cunninghamella*) and entomopathogenic (*Beauveria*) fungi (Ivshina et al., 2019). In the work of Moreira et al. (2018) authors showed the potency of alphaproteobacterial strain *Labrys portucalensis* F11 for the complete DCF degradation. The ability for DCF biodegradation was also documented for *Klebsiella* sp., and *Enterobacter hormaechei* (Aissaoui et al., 2017a, 2017b; Stylianou et al., 2018). Recently, Palyzová et al. (2019) performed bacterial *Raoultella* sp., mediated degradation of DCF and identified hydroxylated, methylhydroxylated, hydroxylated, oxidized, and decarboxylated diclofenac metabolites. Navrozidou et al. (2019) installed and operated an immobilized cell biofilter to select the diclofenac-degrading microbiota that are present in the activated sludge. During this experiment some bacterial and fungal taxa involved in diclofenac degradation in activated sludge systems were identified, from bacteria were members of the species *Granulicella pectinivorans* and *Rhodanobacter terrae*, followed by members of the species *Castellaniella denitrificans*, *Parvibaculum lavamentivorans*, *Bordetella petrii*, *Bryocella elongata* and *Rhodopseudomonas palustris* and from fungal taxon was *Wickerhamiella* (Navrozidou et al., 2019). Previous experimental works have shown that 4′-hydroxy-DCF and 5-hydroxy-DCF are metabolites of most metabolic pathways of microbial degradation of diclofenac (Marco-Urrea et al., 2010; Doruk et al., 2018; Moreira et al., 2018). Additionally, knowledge about biological changes occurring in the bacterial cells under the influence of DCF and its metabolites is limited (Ivshina et al., 2019).

Pharmaceuticals, due to their chemical structure are often resistant to biodegradation. As conventional water and wastewater treatment processes are ineffective thus leading to an increased presence of the parent drugs and their metabolites in aquatic environments. Therefore, it is necessary to introduce additional advanced treatment technologies to solve a persistent pollution problem. A wide variety of advanced treatment technologies have been evaluated in recent years to remove pharmaceutical residues, including chemical oxidation using ozone and ozone/hydrogen peroxide, membrane filtration such as nanofiltration and reverse osmosis. Numerous works indicate that, advanced oxidation processes (AOPs) such as photo-Fenton, sonolysis, electrochemical oxidation, radiation and ozonation etc., have

a great potential for removal of pharmaceuticals from real and synthetic wastewater matrices (Ikehata et al., 2006; Esplugas et al., 2007). AOPs are based on reactive oxygene (ROS) generation such as hydroxyl radicals, H_2O_2, O_3 and superoxide anion radicals, which leads to mineralization of pollutants to CO_2, H_2O and inorganic ions or acids. Over the past decade, number of works it has proven that AOPs are efficient in removing DCF. However, numerous experimental works have shown that the main limitation of AOPs use is the generation of toxic intermediates (by-products). Literature describes DCF environmental occurrence and degradation, but very little is known about the toxicity and biological effects of its AOPs by-products (Esplugas et al., 2007; Ikehata et al., 2006; Kanakaraju et al., 2018; Scheurer et al., 2012).

The drugs are designed to already release therapy effects in human and animal organisms in relevant low doses. They belong to biologically active compounds and after disposal to wastewater can react with other pollutants and create more toxic complexes. Both in wastewater and surface water they also meet with chemical compounds produced by plants and microorganisms (Lonappan et al., 2016).

Chlorogenic acid (CGA) belongs to the group of phenolic acids and it is very popular in human diet. CGA is one of the most available phenolic acid compounds in foods, such as coffee and tea. Numerous experimental works have shown strong antimicrobial, anti-inflammatory and antioxidant properties of CGA. Due to these properties, CGA is suitable as an ideal preservative and food additive (Naveeda et al., 2018). CGA is present mainly in food-processing wastewaters (Yardim, 2012). Spent coffee ground (SCG) is the waste that is accumulated after coffee consumption, or it can be described as a by-product of brewing proces. It was estimated that, due to high coffee consumption worldwide around 6 million tons of spent coffee grounds are produced each year. The release of spent coffee grounds (SCG) to the environment due to increasing coffee consumption causes environmental contamination. Chlorogenic acid is main phenol presented in SCG. Chlorogenic acid contained in SCG can be toxic for microorganisms included in activated sludge and may negatively affect the proper functioning of the sludge and *wastewater* treatment processes. In addition, chlorogenic acid may interact with pharmaceutical residues in wastewater to form more toxic complexes (Vítezová et al., 2019).

In the present work, the comparison of toxicity of DCF, including molecules from DCF biodegradation pathways and intermediate products formed during selected AOPs was presented. The following parameters were analyzed in the work: *E. coli* K-12 cells viability, growth inhibition of *E. coli* K-12 culture, LC_{50} and mortality of *Chironomus aprilinus*, oxidative stress: ROS generation and superoxide dismutase protein (*sodA*) promoter induction and genotoxicity test. The influence of DCF in the mixture with chlorogenic acid on *E. coli* K-12 strain was also studied. We also monitored the response pattern of *E. coli* SM *recA*:*luxCDABE* in artificially contaminated raw and treated, filtered and unfiltered wastewater. For genotoxicity and oxidative stress assessment two *E. coli* SM biosensor strains with plasmid fusion of *recA* (genotoxicity assay) and *sodA* (oxidative stress assay) promoters to *luxCDABE* reporter gene were used. In previous works it was shown, that *E. coli* SM *luxCDABE* strain with *recA* and *sodA* promoters were successfully used for detection of genotoxic and oxidative stress generation after bacteria exposure to antibiotics, genotoxins and environmental pollutants (Kessler et al., 2012; Melamed et al., 2012).

1. Materials and methods

1.1. *DCF transformation products*

In order to select transformation products - biodegradation metabolites and AOPs by-products of DCF, a literature review in the PubMed database on this subject was conducted. Only chemical compounds which were detected and identified by scientists as transformation products of DCF in several scientific publications were selected (Bouju et al., 2016; Doruk et al., 2018; Haiba et al., 2017; Kawase et al., 2017; Langenhoff et al., 2013; Lonappan et al., 2016; Marco-Urrea et al., 2010; Moreira et al., 2018; Schmidt et al., 2018; Vieno and Sillanpää, 2014; Vogna et al., 2004; Yu et al., 2013). DCF (Diclofenac sodium salt), 4′-OHDCF, 5-OHDCF, 2-hydroxyphenylacetic acid, 2,5-dihydroksyphenylacetic acid, 2,6-dichloroaniline were commercially obtained (Sigma Aldrich, UK). While, chlorogenic acid was purchased from Cayman Chemical company. Chemical structures, molecular formulas of tested chemicals and classification of molecules from DCF biodegradation pathways and intermediate products formed during selected AOPs were presented in Table 1.

1.2. *Chemical treatment of* E. coli *strains and* Chironomus aprilinus

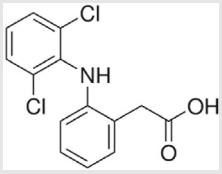
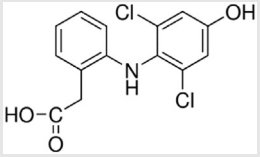
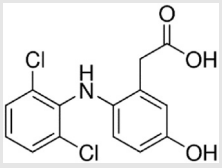
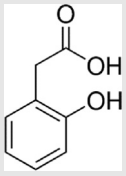
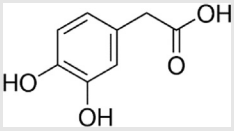
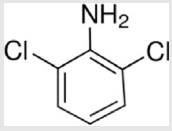
To determine the impact of compounds tested in the work on evolutionarily different organisms (determination of species sensitivity) to *E. coli* strains and *Chironomus aprilinus* larvae were selected for toxicity studies. Both *E. coli* strains and *Chironomus aprilinus* larvae occur in the wild environment and they are often used by ecotoxicologists for toxicity testing.

The same concentrations of DCF and its transformation products were used to compare the toxicity and biological effects toward *E. coli* strains and *Chironomus aprilinus*. All 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 DCF and its transformation products were added to the *E. coli* cultures and *Chironomus aprilinus* for a final concentrations of 10 μg/mL. Chlorogenic acid was also applied at the concentration of 10 μg/mL. Mixtures of DCF with chlorogenic acid were prepared in the following proportions: a) 10 μg/mL of DCF+10 μg/mL of chlorogenic acid. The *E. coli* control cultures and *Chironomus aprilinus* control sample were incubated without the tested compounds.

1.3. E. coli cell *viability assay*

The *E. coli* K-12 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

Table 1 – Chemical structures and molecular formulas of tested chemicals.

Chemicals	Chemical structure	Molecular formula
Diclofenac		$C_{14}H_{11}Cl_2NO_2$.
Biodegradation metabolite: 4′-hydroxydiclofenac		$C_{14}H_{11}Cl_2NO_3$
Biodegradation metabolite: 5-hydroxydiclofenac		$C_{14}H_{11}Cl_2NO_3$
AOPs by-product: **Process: UV/H_2O_2** 2-hydroxyphenylacetic acid		$C_8H_8O_3$
AOPs by-product: **Process: ozonation** 2,5-dihydroxyphenylacetic acid		$C_8H_8O_4$
AOPs by-product: **Process: UV/H_2O_2** 2,6-dichloroaniline		$C_6H_5Cl_2N$

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. The method allows evaluating the toxicity of the selected chemicals to the bacteria (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 hr. 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 hr incubation with tested chemicals and it was calculated as a percentage of control cells, incubated without tested compound. Three independent experiments were done.

1. 4. *E. coli* K-12 culture growth inhibition effect

The growth inhibition effect of DCF, its transformation products and the mixtures of DCF with chlorogenic acid were 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 hr. Non-treated *E. coli* cultures were used as the control. The values of bacterial growth inhibition (GI) after 24 hr 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. Experiments were conducted in triplicate.

1.5. The toxicity of the tested chemicals to Chironomus aprilinus

Toxic potential of chemical compounds tested in the work was based on the calculation of percentage of mortality and LC_{50} (LC_{50}–Lethal Concentration, the concentration of the toxic substance causing the death of a 50% of organisms in the population) values of *Chironomus aprilinus* larvae after treatment for 24 and 48 hr. Toxicity tests with diclofenac and its transformation products were performed on 11 days old *Chironomus aprilinus*, which were obtained from a Saxon Shop in Bialystok. LC_{50} values were calculated according to Reed method (Erhirhie et al., 2018). Identical individuals in terms of size and viability were selected for testing. In the experiment, diclofenac and its transformation products were tested in a series of 5 different concentrations 250, 62.5, 15.625, 3.906, 0.976 mg/L. The experiment was conducted in sterile Petri dishes with water prepared from the city water supply system. Water was taken two days before the experiment and left at room temperature, around 24 ± 4°C. 10 individuals of *Chironomus aprilinus* were added to each Petri dishes with 20 mL of water with the appropriate concentration of the tested chemicals. All tested compounds were dissolved in DMSO. To reduce the impact of DMSO on the tested organisms, prepared, solutions of chemicals in DMSO were diluted in the water in a ratio of 1: 9 (1 mL DMSO and 9 mL medium) and then added at appropriate concentrations to Petri dishes. The experiment was conducted for 24 and 48 hr at room temperature, around 24 ± 4°C. The control sample did not contain any chemical compounds. After 24 and 48 hr of incubation, the number of live and dead individuals was counted and the percentage of mortality and LC_{50} values were calculated. Experiment was done in triplicate.

1.6. Genotoxic effect estimation

Possible genotoxic effects of the DCF and its transformation products were investigated by analysis of the SOS-response by means of inducible *recA-luxCDABE* fusion-gene products and the luminescence assay. 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 was used. *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. Recently, 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. These sensors are based on light production resulting from the action of the enzyme, luciferase (Kostrzyńska et al., 2002; Melamed et al., 2012; 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 (Kessler et al., 2012; Kostrzyńska et al., 2002; Lee et al., 2013; Melamed et al., 2012). *E. coli* strain used in this study was presented in Table 2. *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 like 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/min) 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 hr 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

Table 2 – *E. coli* SM biosensor strains used in this study.

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

positive control. Chemicals-free bacterial culture was used as the control. The results are showed as Fold Induction (FI) values in comparison to the control. Experiment was carried out in three independent series.

1.7. *Assessment of luminescence values*

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

$$L = IL/OD \quad (1)$$

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

1.8. *The fold induction values calculations*

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

$$FI = L_{TS}/L_{CS}; \quad (2)$$

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

1.9. *Oxidative stress studies*

1.9.1. *ROS generation*

Generation of ROS is induced by both endogenous and exogenous stimuli. ROS are formed in aerobic organisms as by-products of normal metabolism, as a consequence of partial reduction of molecular oxygen to superoxide anion, hydrogen peroxide, and hydroxyl radical (Islas-Flores et al., 2013). Bacteria have been preferably used for the rapid detection of ROS due to their fast response, high growth rate, and low cost. The production of ROS by *E. coli* K-12 after treatment with DCF and its transformation products was evaluated using a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, UK) and according to the method like 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 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 μmol/L 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. All the experiments were done in triplicates.

1.9.2. SodA *promoter induction*

Reactive species are destructive to DNA, proteins, and lipids. To protect the cells of aerobic organisms from damage by reactive species, evolution has equipped them with detoxification and repair systems. 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). Oxidative stress after bacteria treatment with tested chemicals and the mixture was investigated by analysis of the induction of *sodA-luxCDABE* fusion-gene products and the luminescence assay. *SodA* promoter is a promoter of superoxide dismutase gene. 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 2. *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 like 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/min) 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 hr 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. Experiment was carried out in three independent series.

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

Raw and treated municipal wastewaters from the Stawisk Wastewaters Treatment Plant (WWTP) were filtered using a bacteriological filter with a diameter of 0.45 μm (Sartorius). A sample of the raw wastewater used during the laboratory studies was collected after mechanical treatment processes (grids and sand separator). The aim of this approach was to use wastewater containing the smallest possible amounts of suspensions and solid contaminants naturally occurring in wastewater flowing into wastewater treatment plant. A sample of treated wastewater was taken at the outflow from the secondary settling tank. This was dictated by the fact that biological transformations occur in the secondary settling tank due to the presence of activated sludge. Therefore, collection of wastewater sample directly from the secondary settling tank might not fully reflect the characteristics of treated wastewater outflowing to environment.

These studies are preliminary studies because the reactivity of the *E. coli* SM *recA:luxCDABE* biosensor strain has not been previously studied in the wastewaters environment.

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 a DCF at concentration of 10 μg/mL and 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 hr of incubation. The results are showed as Fold induction, (FI) values in comparison to the control, non treated with DCF *E. coli* SM *recA:luxCDABE* culture inoculated in raw and treated filtered and no filtered wastewaters. Experiment was conducted in triplicate.

1.11. *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.

2. Results

2.1. E. coli *cells viability*

Obtained in our experiments results showed the strongest inhibition of *E. coli* cells viability in the case of DCF and 4′-OHDCF and 5-OHDCF (Fig. 1). Bacterial cells viability inhibition values were above 39% for DCF, 35% for 4′-OHDCF and 34% for 5-OHDCF. Compared to 4′-OHDCF and 5-OHDCF, tested molecules from selected AOPs had less effect on bacteria cells viability. It was detected that chlorogenic acid caused inhibition of bacteria cells viability of 11%. Chlorogenic acid mixed with DCF worked synergistically and significantly enhanced the values of inhibition of cells viability, even to 52% compared to control sample. The results are presented as a percentage of cell viability of treated *E. coli* culture in comparison to the control sample, bacteria culture not treated with chemicals. The character of the particular compounds was similar, which resulted in the lack of statistically significant differences between them. However, statistically significant differences ($\alpha = 0.05$) were observed between chlorogenic acid and nalidixic acid and DCF + chlorogenic acid. Considering the fact that the initial concentration of individual compounds was 10 μg/mL, statistically observed significant differences may indicate a different character of the individual compounds in relation to *E. coli*.

2.2. E. coli *growth inhibition*

The growth inhibition effect of DCF and its transformation products 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 56% was obtained after 24 hr treatment with the mixtures of DCF with chlorogenic acid. There is an interaction between chlorogenic acid and DCF, which resulted in increased toxicity of the mixture of the both compounds to *E. coli* K-12, comparable to parent chemicals. A clear decrease in *E. coli* K-12 growth was also noted after bacteria incubation with DCF and 4′-OHDCF and 5-OHDCF. Between the remaining tested

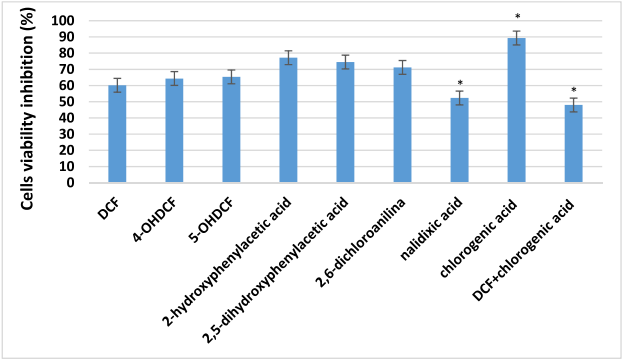


Fig. 1 – The inhibition of *E. coli* K-12 cells viability by DCF, 4′-OHDCF, 5-OHDCF, 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, 2,6-dichloroaniline, NA, chlorogenic acid and the mixture of chlorogenic acid with DCF. Bacteria cultures were incubated 24 hr with 10 μg/mL of each compound and the mixture of DCF and chlorogenic acid in the proportions of 10 μg/mL DCF and 10 μg/mL chlorogenic acid. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

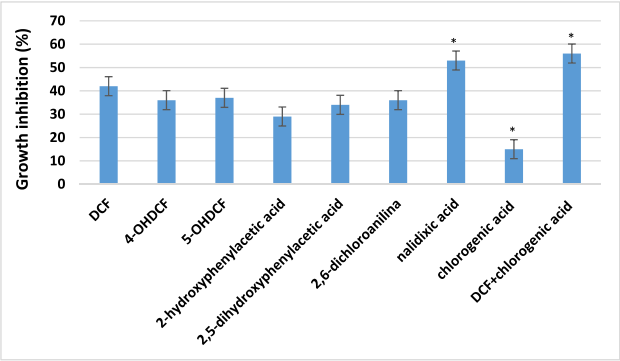


Fig. 2 – The growth inhibition of *E. coli* K-12 culture after 24 h incubation with DCF, 4′-OHDCF, 5-OHDCF, 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, 2,6-dichloroaniline, NA, chlorogenic acid and the mixture of chlorogenic acid with DCF. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

transformation products, the strongest inhibition of bacterial growth was caused by 2,6-dichloroaniline. The results are showed as a percentage of growth inhibition of treated *E. coli* culture in comparison to the control sample. The described observations were partially confirmed by statistical analysis, where statistically significant differences between chlorogenic acid and nalidixic acid and DCF + chlorogenic acid were observed. Taking into account the obtained values of growth inhibition it can be stated that chlorogenic acid in comparison with nalidixic acid and DCF + chlorogenic acid mixture shows lower growth inhibition of *E. coli* K-12, but in comparison with other compounds its influence on growth inhibition is similar. It should be emphasized here that there were no statistically significant differences between the other studied compounds.

2.3. *The toxicity estimation to* Chironomus aprilinus

Toxicity tests with diclofenac and its transformation products were performed on 11 days old *Chironomus aprilinus* larvae for 24 and 48 hr. After 24 and 48 hr of incubation, the number of live and dead individuals was counted and the percentage of mortality and LC_{50} values were calculated. Toxicity tests showed significantly higher toxicity of 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid and 2,6-dichloroaniline (especially after 48 hr incubation) comparable to DCF, 4′-OHDCF and 5-OHDCF. The highest toxicity was recorded for 2,6-dichloroaniline after both 24 and 48 hr of incubation. The calculation of the LC_{50} value for 5-OHDCF, NA and 2,5-dihydroxyphenylacetic acid after 24 hr of incubation was not possible, because 50% of the mortality of the tested individuals was not obtained. For these chemicals the mortality has reached values of 30%, 40% and 40%, respectively. Based on the results of the toxicological analysis in relation to the Directive of the Council of the European Community (Directive of the Council of the European Community 92/32/EEC, OJ L 154, 5.6.1992, p. 1), 2,6-dichloroaniline was classified as toxic. Other tested compounds were harmful and slightly harmful. The results are showed as percentage of mortality (Fig. 3) and LC_{50} (Table 3). Experiment was done in triplicate. Along with a decrease in the concentration of individual compounds, a decrease in mortality of *Chironomus aprilinus* larvae was observed. Each of the observed decreases in mortality was statistically significant, both at the time of 24 and 48 hr. Therefore, it was statistically confirmed that the initial concentration of individual compounds has a significant effect on the mortality of organisms. Additionally, at the same concentrations, the mortality of organisms was higher after 48 hr compared to 24 hr of inhibition. In this case, all the results showed statistically significant differences. On this basis, it can be concluded that the inhibition time is a factor determining the mortality of *Chironomus aprilinus* larvae in the case of individual studied compounds.

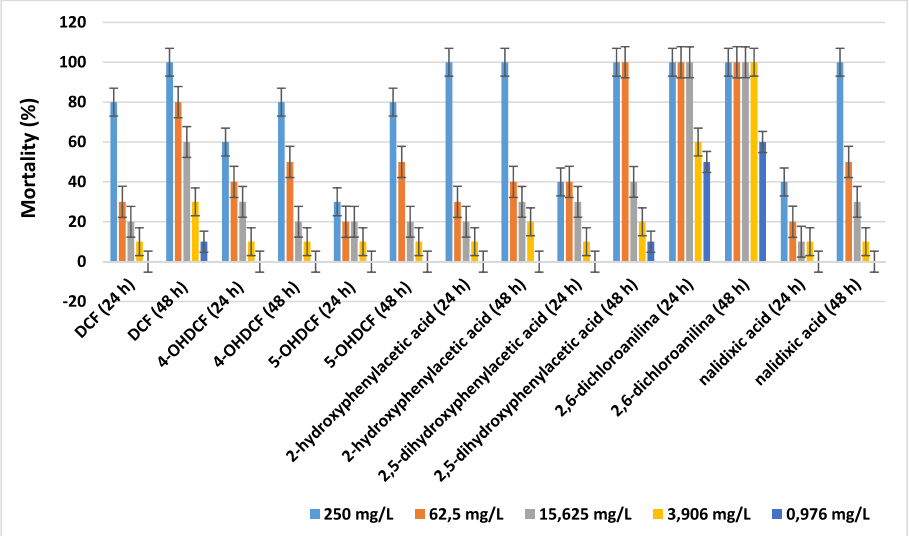


Fig. 3 – The mortality of *Chironomus aprilinus* after 24 and 48 hr incubation with DCF, 4′-OHDCF, 5-OHDCF, 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, 2,6-dichloroaniline and NA. Mean values from three independent experiments ± SD are shown, * all results are statistically significant at $p \leq 0.05$.

Table 3 – LC_{50} values after 24 and 48 h incubation of *Chironomus aprilinus* with DCF and its transformation products and NA.

Chemicals	Process	LC_{50} after 24 hr (mg/L)	LC_{50} after 48 hr (mg/L)
DCF	Drug	156.25	105.12
4′-OHDCF	Biodegradation metabolite	208.33	79.35
5-OHDCF	Biodegradation metabolite	–	63
2-hydroxyphenylacetic acid	AOPs by-product, UV/H_2O_2	104.16	78.125
2,5-dihydroxyphenylacetic acid	AOPs by-product, ozonation	–	19.53
2,6-dichloroaniline	AOPs by-product, UV/H_2O_2	0.976	0.813
Nalidixic acid (NA)	–	–	62.5

Explanations: - it was not possible to calculate the LC_{50} value for 5-OHDCF, NA and 2,5-dihydroxyphenylacetic acid after 24 hr of incubation, because 50% of the mortality of the tested individuals was not obtained.

2.4. *Genotoxic effect estimation*

The possible genotoxic effects of the DCF and its transformation products 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 in our experiments results showed that all tested chemicals induced *recA* promoter, exhibiting several response patterns. DCF and its transformation products were applied at the same concentration of 10 μg/mL. It has been noticed the strongest induction of reporter strain *recA:luxCDABE* in response to NA treatment (Fig. 4). The strong induction of *recA:luxCDABE* by DCF and 2,6-dichloroaniline was observed with the highest values of FI for 2,6-dichloroaniline (5.2) and 4.65 for DCF. Presented studies also showed the strongest sensitivity of *recA:luxCDABE* reporter strain to 4′-OHDCF and 5-OHDCF than to 2-hydroxyphenylacetic acid and 2,5-dihydroxyphenylacetic acid. The genotoxic potency of tested compounds is directly proportional to the induction of *recA* promoter and luminescence signal emission. Obtained results indicated that among the tested DCF transformation products, 2,6-dichloroaniline exhibits the strongest genotoxic activity. The results are presented at Fig. 4 as Fold Induction (FI) value of treated bacteria culture in comparison to the contol sample. In the case of FI values, statistically significant differences were observed between nalidixic acid and the other studied compounds. The remaining compounds, despite visible differences in FI values, did not show statistically significant differences. Taking into account the fact that the tests were conducted at a concentration of 10 μg/mL of each of the compounds, it can be stated that the obtained result indicates that the action of nalidixic acid is different from that of the other studied compounds.

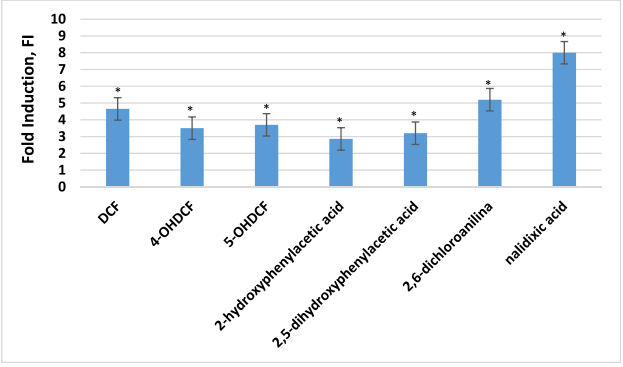


Fig. 4 – The genotoxic response pattern of *E. coli* SM *recA:luxCDABE* after 5 h incubation with 10 μg/mL of DCF, 4′-OHDCF, 5-OHDCF, 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, 2,6-dichloroaniline and NA. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

2.5. *Oxidative stress assay*

2.5.1. *ROS generation*

Oxidative stress occurs when excess oxygen radicals are produced in cells, which could overwhelm the normal antioxidant capacity. Previous experimental studies 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 and its transformation products. Considering the comparison of the activity of tested molecules, our research showed that DCF, 4′-OHDCF and 5-OHDCF generate more ROS compared to selected tested compounds that are produced during AOPs (Fig. 5). It indicated that oxidative stress is a possible underlying mechanism of DCF, 4′-OHDCF and 5-OHDCF action. The results are presented at Fig. 5 as percentage of ROS increase of treated sample comparable to the control. The described regularities in relation to cell viability and growth inhibition are partially reflected in the statistical analysis. The ROS value obtained for nalidixic acid differed statistically significantly from the values obtained for other compounds. Similarly, statistically significant differences between DCF and DCF + chlorogenic acid occur only in the case of ROS.

2.5.2. SodA *promoter induction*

Superoxide dismutase is a key enzyme involved in oxygen radical scavenging. We investigated the induction of *sodA* promoter of superoxide dismutase gene in *sodA-luxCDABE* genetic construct in *E. coli* SM biosensor strain. *SodA* is a promoter of superoxide dismutase gene. 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. All tested chemicals were applied at the same concentration of 10 μg/mL

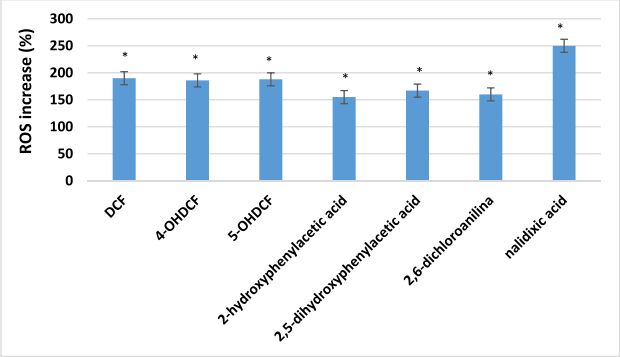


Fig. 5 – Reactive oxygen species (ROS) increase induced in *E. coli* K-12 cultures incubated with 10 μg/mL of DCF, 4′-OHDCF, 5-OHDCF, 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, 2,6-dichloroaniline and NA. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

and were incubated 5 hr with *E. coli* SM *sodA:luxCDABE*. Our results presented that DCF and its transformation products induced *sodA* promoter with different efficiency. The strongest induction of *sodA* promoter was obtained for DCF with FI values 6.85.4′-OHDCF and 5-OHDCF had a stronger impact on *sodA* promoter induction than chemicals that are produced during AOPs. Of these, 2,6-dichloroaniline stimulated the *sodA* promoter most strongly. The results are presented at Fig. 6 as Fold Induction (FI) value of treated bacteria culture comparable to the contol sample.

No statistically significant correlations were observed between Cells viability (CV), Growth inhibition (GI), Reactive oxygene species (ROS) and *sodA* promoter induction values obtained for DCF. Nevertheless, correlation coefficients between these parameters belonged to the group of moderate and strong correlations, and the correlation coefficient for individual pairs of CV and *sodA* promoter induction variables was 0.50, for GI and *sodA* promoter induction 0.76, while for ROS and *sodA* promoter induction 0.87. This means that with an increase or decrease in CV, GI or ROS values *sodA* promoter induction decreased simultaneously. For 4′-OHDCF the correlation coefficient between CV and *sodA* promoter induction was 0.57, between GI and *sodA* promoter induction was 0.11, while between ROS and *sodA* promoter induction the correlation coefficient was 0.18. For 5-OHDCF the correlation coefficient between CV and *sodA* promoter induction was −0.47, between GI and *sodA* promoter induction the correlation coefficient was 0.39, while between ROS and *sodA* promoter induction the correlation coefficient was 0.37. In turn, taking into account all measurement results without dividing the values into individual compounds, the values of correlation coefficients between CV and *sodA* promoter induction were −0.36, between GI and *sodA* promoter induction and between ROS and *sodA* promoter induction statistically significant correlations were observed, whose values of coefficients were equal to 0.64 and 0.79 respectively.

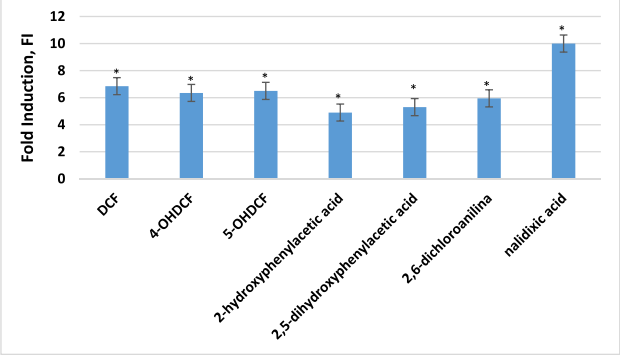


Fig. 6 – The response pattern of *E. coli* SM *sodA:luxCDABE* after 5 h incubation with 10 μg/mL of DCF, 4′-OHDCF, 5-OHDCF, 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, 2,6-dichloroaniline and NA. Mean values from three independent experiments ± SD are shown, * statistically significant result $p \leq 0.05$.

2.6. recA *promoter induction in the matrice of wastewaters*

On the base of our results we noticed a different pattern of response of *recA* promoter in the DCF artificially contaminated matrice of raw and treated, filtered and no filtered wastewaters (Fig. 7). We applied DCF at concentration of 10 μg/mL. For each sample we measured luminescence and OD values at time 0 and after 2, 4, 24 and 48 hr of incubation. We obtained the strongest *recA* promoter induction with the FI values 1.63 and 3.06 in the case of raw, filtered wastewaters, contaminated with DCF and treated, filtered wastewaters contaminated with DCF, comparable to the control sample. The weak luminescence signal from *recA* promoter in *E. coli* SM was noticed in raw and treated wastewaters contaminated with DCF. Generally, we noted the strongest luminescence response of *E. coli recA* cultures in filtered raw

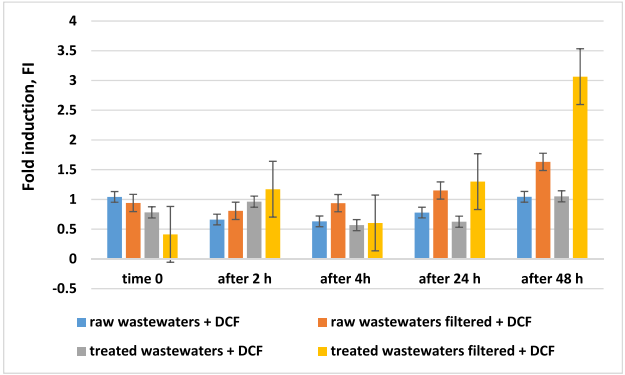


Fig. 7 – The response pattern of *E. coli* SM *recA:luxCDABE* biosensor strain to raw and treated, filtered and no filtered wastewaters contaminated with 10 μg/mL of DCF. The results are presented as Fold induction (FI) of treated sample in comparison to control, not treated bacteria cultures. Mean values from three independent experiments ± SD are shown, * all results are statistically significant at $p \leq 0.05$.

and treated wastewaters than in no filtered samples. This indicates, that the composition of microflora the filtered and no filtered wastewaters matrices affects the sensitivity of the *recA* promoter to the added DCF. Considering the raw and treated unfiltered wastewater, slight changes in the level of the luminescent signal induction from the *recA* promoter were observed after 4 and 24 hr incubation. The results are presented at Fig. 7 as the Fold induction (FI) of treated sample in comparison to the control. Changes in luminescence of the *recA:luxCDABE* biosensor observed in the conducted studies were reflected in the statistical analysis. Slight changes in luminescence during the considered incubation time resulted in the lack of statistically significant differences. The obtained results indicate that incubation time up to 24 hr has a significant influence on luminescence of the biosensor. Additionally, the value of luminescence of the studied biosensor in different types of wastewater in particular incubation times was compared. Comparison of this type showed that all luminescence values differ statistically significant. On the basis of this observation it can be stated that depending on the degree of wastewater contamination with other substances, the value of luminescence varies.

3. Discussion

Diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid) belongs to the group of non-steroidal anti-inflammatory drug (NSAID). This group of medicines is known to be largely used throughout the world as inflammatory reducer and painkiller. DCF is one of the most frequently detected micropollutants of anthropogenic origin in wastewater and water. Numerous studies have shown DCF high reactivity towards living organisms, especially in chronic tests (Vieno and Sillanpää, 2014). Currently, intensive research is conducted on the use of AOPs in the DCF degradation process (Felis et al., 2009). AOPs are based on reactive oxidant species generation and destruction of micropollutants. So far, AOPs have been shown to be effective in removing DCF (Celiz et al., 2009; Le Corre et al., 2012). However, the formation of toxic by-products is a significant limitation of these processes (Scheurer et al., 2012). Over the past decade, many scientific papers have been published on the occurrence and degradation of DCF. However, there is little work on the toxicity and biological effects of biodegradation metabolites and AOPs by-products. In the present work the toxicity and effects on selected biological parameters of DCF, its selected biodegradation metabolites and by-products, formed after ozonation and UV/H_2O_2 treatment were compared (Kanakaraju et al., 2018).

Microbial biodegradation is the main process of DCF removal in environment (Kümmerer, 2009). Several studies revealed that 4'-OHDCF and 5-OHDCF are the main microbial biotransformation products during DCF degradation by indigenous microflora of river sediments and in a laboratory scale pilot wastewater treatment plant (Fatta-Kassinos et al., 2011; Memmert et al., 2013).

Among AOPs, the ozonation and UV/H_2O_2 have demonstrated good prospective at lab-scale level for DCF removal. Due to high oxidant power of ozone molecule (O_3) ozone based processes are applied in the oxidation of organic/inorganic compounds, disinfection, wastewater and drinking water treatment. During the ozonation oxidation of micropollutants occurs. This leads to a change in the biological activity of micropollutants and in most cases with the formation of more readily biodegradable compounds. Previous studies showed that in aqueous solutions, micropollutants could be attached in two modes, either by direct reaction of the O_3 molecule or after decomposition of O_3 by OH radicals (Scheurer et al., 2012). UV/H_2O_2 is the next oxidation system that significantly improves the removal of resistant pharmaceuticals. During this process UV photolysis of H_2O_2 causes formation of hydroxyl radical (OH), which is strong oxidant and can attack organic micropollutants causing degradation.

Experimental studies indicated that during the application of two widely used oxidation systems ozonation and UV/H_2O_2 undesired by-products are formed. The generation of persistent by-products during ozonation raises the need for evaluation the toxicity before and after treatment by ozonation (Kanakaraju et al., 2018). Vogna et al. (2004) in their work provided the by-products analysis during DCF oxidation with ozone and UV/H_2O_2. 2,5-dihydroxyphenylacetic acid was identified as main by-product of DCF ozonation. Product analysis suggested that 2-hydroxyphenylacetic acid and 2,6-dichloroaniline are intermediates of DCF UV/H_2O_2 treatment (Vogna et al., 2004).

In the present experimental studies the toxicity of DCF, its selected biodegradation metabolites and ozonation and UV/H_2O_2 by-products was compared. Obtained results showed that 2,6-dichloroaniline as DCF UV/H_2O_2 treatment by-product was classified as toxic compound to *Chironomus aprilinus*. Generally, tested transformation products created during AOPs strongly influenced viability of *Chironomus aprilinus* than DCF and 4'-OHDCF and 5-OHDCF. Different results appeared in *E. coli* cells viability tests, where the strongest inhibition of bacteria cells viability after their treatment with DCF and 4'-OHDCF and 5-OHDCF was found. Our results indicate the varied species sensitivity of *E. coli* and *Chironomus aprilinus* to the tested chemical compounds. Presented results are in agreement with previous studies where authors demonstrated the diverse sensitivity of bacteria and various species and developmental stages of the tested organisms to DCF (Lonappan et al., 2016; Praskova et al., 2014). There is no comparative study of toxicity for DCF and its transformation products on *Chironomus aprilinus*. Therefore, the obtained results can only refer to experiments conducted on aquatic organisms. The lethal concentration values for DCF obtained in presented study are in agreement with the values of lethal concentrations of mentioned authors. The toxicity tests with use of *Daphnia magna* sp., presented that DCF at concentration mg/L induced high mortality rate. Similarly, tests with *Ceniodaphnia dubnia* sp., showed mortality effect of DCF at concentrations of mg/L (Praskova et al., 2014). Earlier studies presented that DCF had a significant impact on community structure and function of river biofilm at even lower concentrations of 100 ng/L (Lonappan et al., 2016). Until now, the molecular mechanism of DCF activity in fish organisms has been well understood. Histopathological investigation of rainbow trout tissues revealed the accumulation of DCF in liver, kidney and gills. Protein accumulation, structural

alterations and necrosis in these tissues were observed (Praskova et al., 2014). Aissaoui et al. in their work examined the toxicity of DCF and its selected microbial biodegradation metabolites toward mice. They noticed toxic effect of DCF, but no effect was observed after treatment of animals with 4′-OHDCF and 5-OHDCF (Aissaoui et al., 2017a, 2017b).

The antibacterial effect of DCF was presented against both gram-positive and gram-negative bacteria (Praskova et al., 2014). In this study stronger *E. coli* K-12 growth inhibition effect for DCF and 4′-OHDCF and 5-OHDCF than selected AOPs by-products was observed. The proposed mechanism of this antibacterial activity of DCF is inhibition of bacterial DNA synthesis or impairment of membrane activity. It has also been shown that DCF works synergistically when combined with antibiotics (Salem-Milani et al., 2013).

Pharmaceuticals have the ability to form complexes with other chemical compounds found in wastewaters and surface waters. These complexes could be more toxic than parent molecules (Lonappan et al., 2016).

In this paper the antibacterial potency of the mixture of DCF with chlorogenic acid was studied. Chlorogenic acid (CGA) is popular in human diet. CGA is used in the human food in the form of drinks. Chlorogenic acid belongs to the group of secondary phenolic metabolites that are produced by certain plant species including tea, green roasted bean, coffee, berry fruits, cocoa, citrus fruits, apples and pears (Naveeda et al., 2018). CGA is present in wastewater where it can react with different chemicals. Conducted studies showed that there is an interaction between CGA and DCF that increased the toxicity of the mixture of CGA + DCF to *E. coli* K-12. Presented results are in line with the insights of earlier authors, who claim that pharmaceuticals as biologically active substances in wastewater and surface water can interact with other chemicals to form more active complexes (Lonappan et al., 2016). Studies have indicated that CGA has antimicrobial effects against *Klebsiella pneumoniae*, *Helicobacter pylori*, *Escherichia coli*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*. The antifungal effect against *Candida albicans* of CGA was also detected. Moreover, the antiviral activities against HSV-1 and HSV-2, HIV and adenovirus were also reported (Naveeda et al., 2018). In our earlier works we also detected the genotoxic and cytotoxic activity of caffeic and rosmarinic acids and their complexes with Li, Na and K (Matejczyk et al., 2018).

Recently, microbial biosensors technology is a very useful device for stresses and toxicity, such as oxidative and genotoxic stress and protein-denaturing compounds measurements (Kostrzyńska et al., 2002; Melamed et al., 2012; Hassan et al., 2016; He et al., 2016). In presented experiment genotoxic effect and oxidative stress with use of *E. coli* SM biosensor strains with *recA* (genotoxic potential assay) and *sodA* (oxidative stress) after 5 hr bacteria exposure to DCF and its transformation products applied at concentration of 10 μg/mL was examinated. Eariel authors showed 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 (Kostrzyńska et al., 2002; Melamed et al., 2012). We revealed that DCF and 2,6-dichloroaniline (UV/H_2O_2 by-product) had the strongest genotoxic effect. Other DCF transformation products also showed genotoxic effects in *E. coli*. Similar results were obtained by Melamed et al. (2012), where authors observed a high level of *recA* induction in response to selected drugs and other genotoxic compounds.

A wide variety of environmental stressors can alter the intracellular redox status by means of oxidative damage ultimately leading to cell death (Carlos et al., 2010). Recently, in toxicological studies in aquatic systems for the evaluation of the damage induced by pharmaceutical residues oxidative stress biomarkers are used. Oxidative stress parameters analysis are very important tools to assess the damage impact of environmental pollutants to living cells. These biomarkers are defined as any measureable biochemical, physiological or morphologic changes associated with exposure to a toxic agent. In many cases, the toxic effects of xenobiotics, including pharmaceuticals are mediated by reactive oxygen species (ROS) formation. The superoxide dismutase (SOD) assay is also important biomarker for monitoring environmental pollution (Islas-Flores et al., 2013). In this work with application of *sodA* promoter in fusion with *luxCDABE* in *E. coli* the potency of DCF and its transformation products to oxidative stress generation was examinated. Obtained results indicated that DCF and its biodegradation metabolites 4′-OHDCF and 5-OHDCF caused stronger induction of *sodA* promoter than UV/H_2O_2 and ozonation by-products. The results of genotoxic and oxidative stress studies are supported by the results of other authors, such as Islas-Flores et al., 2017, that showed that DCF and DCF with the mixture with ibuprofen (IBP) induced free radical production, oxidative stress and cyto-genotoxicity in tissues of *Cyprinus carpio*. Similar results were noted by Gómez-Oliván et al. (2014), where a genotoxic potency and oxidative stress induction by DCF and IBP in *Daphnia magna* were observed.

In the present study with use of DCF at concentration of 10 μg/mL, artificially contaminated raw and treated, filtered and unfiltered wastewater, the induction of *recA* promoter was detected. As mentioned above, *recA* promoter is sensitive to DNA-damage compounds. 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. During the treated wastewaters filtration process, competitive microflora that inhibits the growth of *E. coli* SM *recA:luxCDABE* culture was reduced. Furthermore, treated wastewater compared to raw wastewaters may contain less organic compounds and other inhibitors that inhibit the expression of the *recA:luxCDABE* gene construct in *E. coli* SM (Vieno and Sillanpää, 2014; Lonappan et al., 2016). In our results we also observed the weaker induction of *recA* promoter in the environment of wastewaters than in pure DCF solutions. It is estimated that around 300 bacterial species are involved in the biological treatment of municipal wastewaters. The presence of pharmaceuticals in wastewaters, including DCF, may disturb the qualitative and quantitative composition of the activated sludge microflora, leading to a reduction in the efficiency of the biodegradation process (Lonappan et al., 2016). In addition, the presence of other chemical compounds in wastewaters could have an effect on reducing the level of *recA* promoter induction in *E. coli*. Moreover, in wastewaters DCF can interact with other inorganic and organic contaminants leading to the creation of

toxic compounds. These chemicals may influence the expression of *luxCDABE* in *recA:luxCDABE* genetic construct in *E. coli* SM.

4. Conclusion

In the present work with use of *E. coli* and *Chironomus aprilinus*, we compared the toxicity of DCF, its selected most biodegradation pathways metabolites and selected AOPs (such as ozonation and UV/H_2O_2) by-products. (1) The obtained results indicate greater toxicity, especially in 48-hr tests of by-products formed during UV/H_2O_2 treatment and ozonation than 4′-OHDCF, 5-OHDCF and DCF. (2) We also found that DCF has ability to interact with chlorogenic acid and increase the toxicity of the mixture of these compounds to *E. coli*.

The results of the present studies as well as the observations of earlier authors (Vogna et al., 2004; Vieno and Sillanpää, 2014; Lonappan et al., 2016; Kanakaraju et al., 2018) points out the need to broaden research into the toxicity and environmental effects of drug metabolites and selected AOPs by-products. Regarding to pharmaceutical residues elimination from wastewaters, broader measures should be taken to increase public awareness of the proper handling of pharmaceutical waste. However, from the side of technological solutions, the optimalization of biological processes is demanding. In the case of wastewaters loaded with a pharmaceuticals and its metabolites (hospital wastewater and wastewater from the pharmaceutical industry), the obligatory introduction of additional treatment methods. Then, taking into account the use of technology based on AOPs for pharmaceutical degradation, the formation of toxic by-products during these processes must be kept in mind. This raises the need for additional systems designed to remove toxic intermediates after AOPs.

Acknowledgements

This work was financially supported by National Science Centre, Poland, under the research project number 2018/29/B/NZ9/01 997. We would like to thank Professor Joanna Surmacz-Górska from the Silesian University of Technology, Department of Environmental Biotechnology for scientific discussions 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.

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