

## ***ESCHERICHIA COLI*-GFP BIOSENSORS USED TO MONITOR THE BIOLOGICAL ACTIVITY OF CARMUSTINE RESIDUES IN SURFACE WATER**

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**Abstract:** Three genetic constructs with transcriptional fusion of *recA*, *katG* and *sodA* genotoxin and genotoxin sensitive promoters with green fluorescent protein (*gfp*) reporter gene in *Escherichia coli* have been used for assessment of cytotoxic and genotoxic activity of carmustine in surface water. For experiments, the drug was used at concentrations of 0,01; 0,001; 0,0001; 0,00001 and 0,000001 mg/ml. Bacteria strains were incubated with carmustine for 3 and 24 hours. Experimental data showed different sensitivity of applied promoters for the same concentrations of carmustine. Obtained results indicated that, *recA::gfpmut2*, *katG::gfpmut2* and *sodA::gfpmut2* genetic systems were sensitive to carmustine, especially at the concentrations of 0,001; 0,0001 and 0,00001 mg/ml. The strongest reactivity was noticed for *recA* promoter ( $F_i = 14,64$ ). The results indicated that *gfp E. coli* strains with *recA*, *katG* and *sodA* could be potentially useful for monitoring of cyto- and genotoxic effect of pharmacist residues in surface water.

*Key words:* environmental monitoring, *gfp* biosensors, carmustine, cytotoxicity, genotoxicity.

### 1. Introduction

Anticancer drugs belong to the group of hazardous pharmaceuticals and require a special attention because they are mostly non-selective in their modes of action, affecting both cancerous and non-cancerous cells and often causing severe systemic side effects. Some of them interfere directly with the DNA and are recognized, even at low concentrations, as potentially fetotoxic, genotoxic, mutagenic and teratogenic substances in non-target organisms. Some investigations show an increase use of anticancer drugs, leading to increase the levels of released drugs or their metabolites to the environment (Kosjek and Heath, 2011; Kümmerer et al., 2014; Lutterbeck et al., 2015). There is still a lack of knowledge concerning the environmental fate of the drugs and its metabolites after excretion and there is a possible risks connected with their presence in the aquatic environment (Mater et. al., 2014; Climent et al., 2015).

Carmustine – 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) (Fig. 1) is an antitumour, alkylating chemotherapeutic agent used to treat brain tumors, myeloma and lymphoma. Carmustine binds nonspecifically to DNA and can produce cross-linking by different molecular mechanisms. Previous studies performed *in vitro* revealed, that BCNU inactivates some cellular proteins, such as chymotrypsin, alcohol dehydrogenase and antioxidant enzyme – such as glutathione reductase (GR). It is possible that decreased

GR activity could be responsible for the cytotoxicity of carmustine in cancer and normal tissues (El-Sayed et al., 2011; Gutenberg et al., 2013; González-González et al., 2015; Sakumari-Ramesh et al., 2015). Some ecotoxicological studies with anticancer drugs, have shown (in the case of 5-fluorouracil) that the lowest observed-effect concentration (LOEC) in alga and bacterial assays was  $10 \mu\text{g l}^{-1}$  and it was close to the concentration found in sewage effluents. Most of the recent studies report relatively high level of anticancer drugs in urban wastewaters ( $42 \text{ ng l}^{-1}$  for tamoxifen and up to  $146 \text{ ng l}^{-1}$  for cyclophosphamide) and some others studies have shown the level up to  $200 \text{ ng l}^{-1}$  of tamoxifen in natural waters. Predicted environmental concentration (PECs) for carmustine is  $0,04 \text{ ng l}^{-1}$  (Kosjek and Heath, 2011; Mater et. al., 2014; Climent et al., 2015; Lutterbeck et al., 2015).

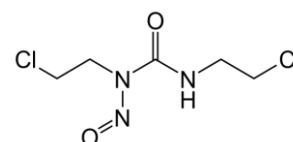


Fig. 1. Chemical structure of carmustine.

Bioassays involving genotoxin-specific recombinant bacterial biosensors have been extensively used in environmental monitoring studies over the last years. Among them, the transcriptional fusion of SOS regulon

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and bacteria stress response promoters with reporter gene is one of the most commonly used. The genotoxin-sensitive *recA*, *katG* and *sodA* promoters transcription is induced upon DNA damage, after exposition of living cells to genotoxic and mutagenic compounds. In the commonly used genetic construct the fusion of green fluorescence protein gene (*gfp*) was used as reporter in microbial biosensor creation systems (Ptitsyn et al., 1997; Kostrzyńska et al., 2002; Zaslaver et al., 2004; Alhadrami and Paton, 2013; Park et al., 2013).

In the present study the possibility of application of *E. coli* K-12 *gfp* microbial biosensor strains for cytotoxic and genotoxic effects monitoring of anticancer drug – carmustine in surface water was assessed.

## 2. Materials and methods

### 2.1. Chemicals

Carmustine was commercially obtained from Sigma Aldrich. This drug was dissolved in PBS buffer (1,44 g Na<sub>2</sub>HPO<sub>4</sub>, 0,24 g KH<sub>2</sub>PO<sub>4</sub>, 0,2 g KCl, 8 g NaCl per 1000 ml of distilled water, pH = 7) at determined experimental concentrations before they were used.

### 2.2. Bacteria strains and plasmids

In the experiments, the *Escherichia coli* K-12 MG1655 genetically modified, in stationary phase cells of: *E. coli* K-12 *recA::gfpmut2*, *E. coli* K-12 *katG::gfpmut2*, *E. coli* K-12 *sodA::gfpmut2* and *E. coli* K-12 *promoterless::gfpmut2*, were used. They contained a pUA66 plasmid-borne transcriptional fusion between DNA-damage inducible, oxidative stress *recA*, *katG* and *sodA* promoters involved in the SOS regulon and oxidative stress response and fast folding GFP variant of reporter gene-*gfpmut2*. The genetic structure of pUA66 plasmid is described in the work of Zaslaver et al., (2004). In the present work a more stable and fast folding mutant of *gfp* gene-*gfpmut2* with excitation and emission wavelengths of 485 and 507 nm was used.

### 2.3. Bacteria growth condition

All *E. coli* K-12 MG1655 strains were cultured overnight in LB agar (Merck, Germany) at 30°C supplemented with 100 µg/ml of kanamycin (Sigma-Aldrich). Colonies were carried to LB broth (10 g NaCl, 10 g tryptone and 5 g yeast extract per 1000 ml of distilled water) with 100 µg/ml of kanamycin and incubated overnight at 30°C. Afterwards the cells were centrifuged and washed with PBS buffer. The initial bacteria cells density was standardized to OD (Optical Density) value by using spectrophotometer (Multi Detection System, Promega) at the wavelength of 600 nm. The concentration of bacteria cells per ml of PBS was assessed by series dilutions system and expressed as Colony Forming Unit – CFU/ml.

Dynamic growth of bacteria strains treated with drugs was monitored by the use of standard spectrophotometer analysis of Optical Density values at the wavelength of 600 nm.

### 2.4. Bacteria cells treatment with carmustine in PBS buffer

1 ml of stationary phase bacteria cells (2×10<sup>8</sup> CFU/ml; OD = 0,2) were suspended in 4 ml of PBS buffer and tested for genotoxicity with carmustine (25 mg of carmustine in PBS buffer) at concentrations: 0,00001; 0,0001; 0,001; 0,01, and 0,1 mg/ml.

The concentration range of the drug was selected experimentally from the minimum level of *recA::gfp*, *katG::gfp* and *sodA::gfp* constructs sensitivity and according to the references' recommendations, indicated the concentrations observed in the environment (Ptitsyn et al., 1997; Kostrzyńska et al., 2002; Alhadrami and Paton, 2013; Park et al., 2013).

The bacteria incubation time with drug was estimated for monitoring of genetic constructs sensitivity for quick (3 h) and later (24 h) response. The control samples – *E. coli* K-12 *recA::gfpmut2*, *E. coli* K-12 *katG::gfpmut2* and *E. coli* K-12 *sodA::gfpmut2* strains in PBS buffer were not treated with drug. For verification of the correct activity of *recA*, *katG* and *sodA* promoters, *E. coli* K-12 strain containing pUA66 plasmid without the promoter – *E. coli* K-12 *promoterless::gfpmut2* – was used as the control. Additionally, for assessment of genotoxic sensitivity of *recA::gfp* construct, 4% acetone was used as the negative control and 50 µM methylnitronitrosoguanidine (MNNG, known genotoxin) as the positive control.

### 2.5. Bacteria cells treatment with drug in surface water

Surface water samples were collected in sterile flasks from the Białka River in Białystok. The water samples were sterilized by filtration. 1 ml of stationary phase bacteria cells (2×10<sup>8</sup> CFU/ml; OD = 0,2) were suspended in 4 ml of surface water and carmustine was used for genotoxicity testing: at concentration of 0,001 mg/ml for *recA* and *katG* promoters and 0,0001 mg/ml for *sodA* promoter. Drug concentrations were selected for the highest stimulation of the *gfp* gene expression in PBS buffer (for F<sub>1</sub> = 14,64; F<sub>1</sub> = 7,66 and F<sub>1</sub> = 3,60, respectively). The conditions of bacteria incubations and the control protocols were the same as above.

### 2.6. Analytical method for the intensity of *gfp* gene fluorescence (IF) analysis

After exposition of bacteria to tested drug, the cultures were centrifuged and washed with PBS buffer and the intensity of fluorescence of GFP in the volume of 1 ml of bacteria cells suspension (1×10<sup>4</sup> CFU/ml) in PBS buffer was measured with the use of spectrofluorometer (Multi Detection System, Promega). The measurements were done at excitation and emission wavelengths of 485 and 507 nm, respectively.

## 2.7. Assessment of Specific Fluorescence Intensity (SFI) values

The SFI value, defined as the raw fluorescence intensity (IF) divided by the optical density (OD) measured at each time point at 600 nm, was calculated according to the below formula for monitoring the dynamic of *gfp* expression after bacteria treatment with drug:  $SFI = IF / OD$ , where: SFI – Specific Fluorescence Intensity, IF – the raw fluorescence intensity of the strains at excitation and emission wavelengths of 485 and 507 nm, OD – Optical Density of the strains at 600 nm.

## 2.8. Detection of *S<sub>gfpexp.</sub>* value

For the increased SFI values with the level of *gfp* expression in comparison to the control sample, the percentage of *gfp* stimulation (*S<sub>gfpexp.</sub>*) was calculated according to the formulas:  $S_{gfpexp.}(\%) = I_{TS}(\%) - SFI_{CS}(\%)$ , where *I<sub>TS</sub>*(%) – the increase for SFI values for tested drugs sample in comparison with the control sample and *SFI<sub>CS</sub>* (%) – SFI for the control sample =100%.

## 2.9. Assessment of *F<sub>I</sub>* values

For each concentration of tested drugs the induction factors (*F<sub>I</sub>*) were calculated.  $F_I = (FI_1/OD_0)/(FI_0/OD_1)$ , where *FI<sub>1</sub>* is the raw fluorescence of the culture treated with DNA – damaging compound; *FI<sub>0</sub>* is the raw fluorescence of the control sample without genotoxin; *OD<sub>1</sub>* is the optical density at 600 nm of treated culture and *OD<sub>0</sub>* is the optical density of the control sample.

## 2.10. Classification of tested drug as genotoxins

The *F<sub>I</sub>* values were calculated for classification of tested drugs as genotoxins. According to Ptitsyn et al., (1997) and Kostrzyńska et al., (2002) a chemical was identified as a genotoxin, when its induction factor was 2 or more ( $F_I \geq 2$ ).

## 2.11. Statistical analysis

The results obtained in this study are expressed as mean ± standard deviation (SD) for n = 8. The data were analyzed by the use of standard statistical analyses, including one-way Student's test for multiple comparisons to determine the significance between different groups. The values for *P* < 0.05 were considered as significant.

## 3. Results

In the experiments, the positive fluorescence reactivity of *E. coli* K-12 was obtained for each tested genetic construct (with three different promoters *recA*, *katG* and *sodA*) and for carmustine at concentration of 0,001; 0,0001 and 0,00001 mg/ml.

*E. coli* K-12 MG1655 *recA::gfpmut2* strain treatment with carmustine caused a significant increase (*P* ≤ 0,05) in the cultures SFI, *F<sub>I</sub>* and *S<sub>gfpexp.</sub>* as measured by compared to non-treated cells (Table 1). Bacteria cells reacted with different efficiency in *gfp* expression after incubation with different concentration of carmustine. The SFI, *S<sub>gfpexp.</sub>* and *F<sub>I</sub>* values express the potency of influence of tested drugs on the sensitivity of genotoxicity and oxidative stress *recA::gfp*, *katG::gfp* and *sodA::gfp* constructs.

Table 1. SFI values for *E. coli* K-12 *recA::gfp mut2*, *E. coli* K-12 *katG::gfp mut2* and *E. coli* K-12 *sodA::gfp mut2* treated with carmustine in comparison with the control sample (bacteria strain in PBS buffer), T – time of bacteria strain incubation with drugs, *F<sub>I</sub>* – induction factor values, *S<sub>gfpexp.</sub>*(%) – the percent of stimulation of *gfp* expression after treatment of bacteria cells with drug in comparison with the control sample (100%).

C (mg/ml)	0,1		0,01		0,001		0,0001		0,00001	
T (h)	3	24	3	24	3	24	3	24	3	24
<i>recA::gfp</i>	37.40	46.90	43.70	49.76	121.00	634.00	92.00	214.0	54.32	102.20
SFI±SD	±5.20	±5.40	±4.82	±5.33	±10.15 <sup>a</sup>	±15.80 <sup>a</sup>	±9.70 <sup>a</sup>	±10.45 <sup>a</sup>	±6.23	±8.70 <sup>a</sup>
<i>F<sub>I</sub></i>	1.18	1.08	1.29	1.15	3.80	14.64	2.90	4.94	1.71	2.36
<i>S<sub>gfp exp.</sub></i> (%)	18.00	8.00	29.00	15.00	280.0	1364.0	190.0	394.0	71.0	136.0
<i>katG::gfp</i>	34.20	45.36	40.34	45.30	92.70	332.0	74.40	111.0	47.30	85.60
SFI±SD	±4.77	±5.68	±3.94	±4.76	±9.83 <sup>a</sup>	±16.43 <sup>ab</sup>	±10.02	±12.06 <sup>ab</sup>	±7.54	±9.43
<i>F<sub>I</sub></i>	1.08	1.05	1.19	1.05	2.91	7.66	2.34	2.56	1.49	1.98
<i>S<sub>gfp exp.</sub></i> (%)	7.55	5.00	19.00	5.00	191.00	666.00	134.00	156.00	49.00	98.00
<i>sodA::gfp</i>	32.33	42.50	40.76	44.20	76.40	112.00	94.60	156.00	86.70	110.00
SFI±SD	±5.33	±6.26	±4.87	±5.70	±8.40 <sup>ab</sup>	±10.66 <sup>abc</sup>	±10.12 <sup>a</sup>	±11.23 <sup>a</sup>	±9.40 <sup>a</sup>	±10.23 <sup>a</sup>
<i>F<sub>I</sub></i>	1.02	-	1.21	1.02	2.40	2.58	2.97	3.60	2.72	2.54
<i>S<sub>gfp exp.</sub></i> (%)	2.00	-	21.00	2.00	140.00	158.00	197.00	260.00	172.0	154.00
Control sample	31.80	43.30	33.80	43.30	31.80	43.30	31.80	43.30	31.80	43.30
SFI±SD	±4.50	±5.90	±4.50	±5.90	±4.50	±5.90	±4.50	±5.90	±4.50	±5.90

Mean values ± SD; n = 8; a – significantly different from control (*P* < 0.05); b – significantly different from *recA::gfp* group (*P* < 0.05); c – significantly different from *katG::gfp* group (*P* < 0.05).

Treatment of *recA::gfp* bacteria strain with carmustine up to 24 h intensified SFI,  $F_1$  and  $S_{gfpexp}$  values at concentrations of 0,001; 0,0001 and 0,00001 mg/ml. Bacteria cells incubated with drug caused the strongest stimulation of *gfp* expression for 3 h incubation at concentrations of 0,001 and 0,0001 mg/ml, compared to the control sample. A maximum point of *recA::gfp* stimulation by drug (1364% higher *gfp* stimulation as compare to control sample) was observed for *recA* at concentration of 0,001 mg/ml at 24 h incubation (Table 1).

*E. coli* K-12 *katG::gfp* treatment with carmustine resulted in a progressive significant stimulation of SFI values at concentration of 0,001 and 0,0001 mg/ml for 3 and 24 h incubation with drug compared to the control sample. The maximum point for SFI value ( $S_{gfpexp} = 666\%$ ) was at the concentration of 0,001 mg/ml and 24 h of incubation time.

*E. coli* K-12 *sodA::gfp* cells administrated with carmustine exerted some influence on SFI and the parameters with the maximum point for SFI ( $S_{gfpexp} = 260\%$ ) were for 0,0001 mg/ml and after 24 h incubation with drug.

Carmustine at concentration of 0,001; 0,0001 and 0,00001 mg/ml in 66,6% of cases significantly modulated *gfp* expression, as comparable to control sample. There were no significant differences in SFI for higher drug concentration – 0,1 and 0,01 mg/ml.

The monitoring of *E. coli recA::gfp* and *E. coli katG::gfp* and *E. coli sodA::gfp* growth at the start of bacteria treatment (time 0) with the drug indicated a significant decrease in OD (growth inhibition) values for 0,1 and 0,01 mg/ml of tested concentrations of carmustine after 24 h of incubation (Fig. 2). However there were

no statistical differences for other concentrations of the drug and for shorter time (3h) of bacteria cells incubation. Prolonged treatment (up to 24 h) of bacteria cells with anticancer drug significantly influenced the OD value of bacteria' cultures, especially at higher drug concentration. The OD inhibition values after treatment of bacteria strains with carmustine were similar for the three *E. coli* with *recA*, *katG* and *sodA* promoters. Bacteria incubated in PBS (the control sample) without any drugs, were no statistically different in OD values, from 0 to 24 hours of continuous cultivation.

The incubation of *E. coli recA::gfp*, *E. coli katG::gfp* and *E. coli sodA::gfp* biosensor in surface water enhanced the sensitivity of *recA::gfpmut2* and *katG::gfp* and *sodA::gfp* genotoxic system and increased the stimulation of *gfp* expression and SFI value in comparison to incubation in PBS. Prolonged treatment (up to 24 h) of bacteria with carmustine significantly influenced *gfp* expression with the maximum values, with  $F_1 = 16,20$  for *recA::gfp*,  $F_1 = 8,40$  for *katG::gfp* and  $F_1 = 4,80$  for *sodA::gfp* of genetic constructs as comparable to the control sample.

For assessment of genotoxic sensitivity of a *recA::gfp* genetic biosensor system, 4% acetone was tested as the negative control. In the case of this chemical there was no increased in  $F_1$  values for 3 h and 24 h of incubation. Methylnitronitrosoguanidine (MNNG) was used as the positive control at the concentration of 50  $\mu\text{M}$ . For this analyte  $F_1 = 2,8$  for 3h and  $F_1 = 8,4$  for 24 h of incubation time were obtained (data not shown). These results proved stronger sensitivity of a *recA::gfp* biosensor system for MNNG than for an acetone stressor.

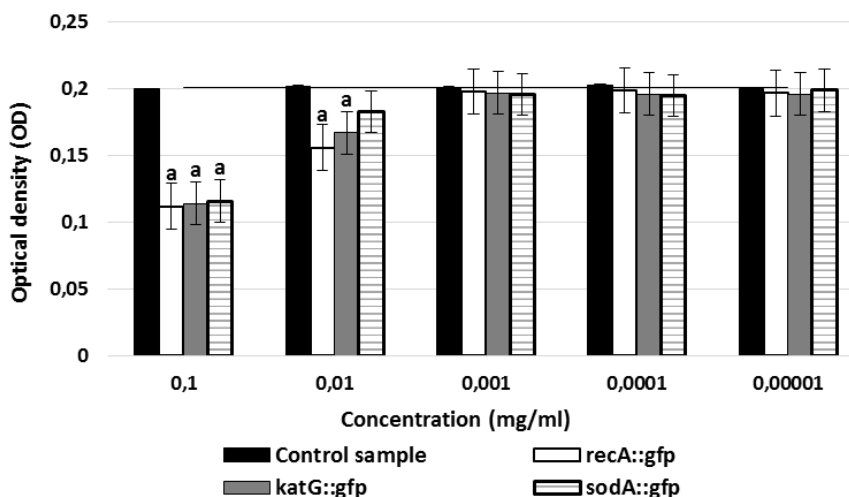


Fig. 2. *E. coli recA::gfp*, *E. coli katG::gfp* and *E. coli sodA::gfp* growth after 24 hours treatment of carmustine measured with optical density (OD). Mean values  $\pm$  SD;  $n = 8$ ; a – significantly different from control ( $P < 0.05$ ).

#### 4. Discussion

The results of this study indicate that treatment of bacteria cells with carmustine lead to over 14- fold stimulation ( $F_1 = 14,64$  in the case of *recA* promoter,  $F_1 = 7,66$  in the case of *katG* promoter and  $F_1 = 3,60$  in the case of *sodA* promoter) of bacteria genotoxin-sensitivity and *gfp* gene expression.

The results obtained in above experiment are in agreement with the studies of Ptitsyn et al., (1997), Kostrzyńska et al., (2002) and Alhadrami and Paton, (2013), who showed, that genetic constructs with *recA*, *katG* and *sodA* genes in transcriptional fusion with reporter gene systems (with *gfp* and *lux* reporters) were sensitive and useful for measurement of cyto- and genotoxicity of anticancer drugs and various chemicals in environmental studies.

According to the results obtained in this experiment carmustine – anticancer drug, modulated and influenced the reactivity of genotoxin and oxidative stress sensitive promoters of *recA*-, *katG*- and *sodA*- in relation to control sample. The results of the above experiment provided the confirmation of the possible influence of carmustine on the genes expression, as it was showed by Sukumari-Ramesh et. al., (2015). There were significant differences (about 50% for *recA* promoter, 30% for *katG* and 60% for *sodA* promoter of the cases comparable to the control sample) regarding the level of promoters sensitivity and *gfp* expression after bacteria treatment with all applied concentrations of BCNU for both short (3 h) and longer time of incubation (up to 24 h).

Previously we examined the cytotoxic and genotoxic properties of selected anticancer (cisplatin and cyclophosphamide) drugs with the use of *E. coli* K-12 *recA::gfpmut2* biosensor strain. Those studies indicated that the activity of anticancer drugs were dose- and time-dependent (Matejczyk et al., 2014; Matejczyk and Rosochacki, 2015), what was also confirmed in above experiment. Longer bacteria growth exposure (up to 24 h) to carmustine resulted in a progressive stimulation of promoters activity and the *gfp* gene expression. Higher values of  $F_1$  factor were for carmustine incubation of 24 h than for 3 h. The results obtained in this experiment showed also the different sensitivity of *recA*-, *katG*- and *sodA* promoters for applied concentrations of carmustine, similarly as it was shown by Ptitsyn et al., (1997), Kostrzyńska et al., (2002) and Alhadrami and Paton, (2013).

Our results showed that a treatment of *E. coli* with carmustine significantly inhibited bacteria growth. Bacteria incubation with that drug importantly and dose-dependently intensified its cytotoxic effect on living bacteria cells after 24 hours of incubation. Our results are in agreement with earlier empirical studies of some authors (El-Sayed et al., 2011; Gutenberg et al., 2013; Gonzáles – González et al., 2015; Sakumari-Ramesh et al., 2015), who demonstrated cytotoxic effect of carmustine on living cells. Carmustine belongs to the group of alkylating agents which directly act on/with DNA and influence the cell protein activity (Sakumari-Ramesh

et al., 2015).

The strongest reactivity of carmustine at concentration of 0,0001 and 0,001 mg/ml of *E. coli* K-12 *recA::gfp mut2*; *E. coli* K-12 *katG::gfp mut2* and *E. coli* K-12 *sodA::gfp* were obtained in surface water samples. It was possibly due to the different chemical composition and pH value of PBS buffer as compared to tested sample of surface water. Additionally the presence of some other components in surface water could influence *gfp* expression in used bacteria strains.

#### 5. Summary

In conclusion, *E. coli recA::gfpmut2*, *katG::gfpmut2* and *sodA::gfpmut2* genetic systems were sensitive to carmustine, especially at concentrations of 0,001; 0,0001 and 0,00001 mg/ml. Experimental data indicated different sensitivity of applied promoters for the same concentrations of carmustine. The strongest reactivity was noticed for *recA* promoter ( $F_1 = 14,64$ ). The results indicated that *gfp E. coli* strains with *recA*, *katG* and *sodA* could be potentially useful for monitoring of cyto- and genotoxic effect of pharmacist residues in water. The validation of used genetic systems in *E. coli* demands more experimental analysis, which should be focused on the assessment of their sensitivity to drugs with different chemical structure in relation to biological activity.

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