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RESEARCH ARTICLE

Non-antimicrobial pharmaceuticals can affect the development of antibiotic resistance in hospital wastewater

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Abstract

Within this study, we were interested in the effects of wastewater on the development of antimicrobial resistance. Microorganisms can relatively promptly adapt to evolutionary pressures of the environment, including antibiotics. Therefore, we tested how the adaptability of the model microorganism *Salmonella enterica* is affected by wastewater full of pharmaceuticals, illicit drugs, and other micropollutants. Wastewater samples had been taken from effluent of hospitals and from wastewater treatment plant (WWTP) Petržalka influent and effluent. In these samples, presence of 38 substances was monitored. The highest concentration was observed in case of tramadol, citalopram, venlafaxine, cotinine, atenolol, valsartan, carbamazepine, azithromycin, and ciprofloxacin. According to this data, we focused also on individual pharmaceutical compounds presented in wastewater samples in elevated concentrations. Effect on resistance development of two pain relief medications (carbamazepine, tramadol), hypotensive medications (atenolol, valsartan), and the nicotine metabolite (cotinine) was also investigated. For this study, we employed concentrations presented in wastewater as well as in urine of patients and/or users. To determine the frequency of mutations leading to ciprofloxacin resistance, we applied the modified Ames test employing the strain *Salmonella* Typhimurium. Resistance index increased in the case of all wastewater samples from conventional hospitals where we observed a 1.22–1.69-fold increase of mutations leading to ciprofloxacin resistance. Tested compounds caused rise of resistance index in lower concentrations found in wastewater. The most significant increase of resistance index was detected after carbamazepine treatment.

Keywords *Salmonella enterica* · Resistance · Cotinine · Tramadol · Carbamazepine · Atenolol · Valsartan · Wastewater

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Introduction

There is no doubt that the human activity represents the main source of licit and illicit drugs as well as different sensitive and antibiotic-resistant microorganisms in the environment. Many pharmaceuticals are not fully degraded in the body and consequently are excreted in urine or stool and enter wastewater. The main producers of wastewater with broad spectrum of pharmaceuticals in high concentrations are hospitals and pharmaceutical industry itself (Berto et al. 2009; Fick et al. 2009). Due to these substances, these wastewaters represent a chemical, biological, and physical risk for public and environmental health. Beside this, many countries have no legal requirements for hospital effluent treatment prior to its discharge into the municipal sewerage (Carraro et al. 2017). This aquatic environment is full of essential substrates for microorganisms, as well as antibiotics, other pharmaceuticals and their metabolites and provides suitable conditions for the development of antibiotic-resistant strains. Part of these compounds is degraded by environmental factors (UV radiation, light, pH), but

considerable concentrations (of the order of ng/L-mg/L) of pharmaceuticals are disseminated in the environment around the world (Birošová et al. 2014). Contact of pharmaceuticals and metabolites with bacteria represent an inevitable situation which can lead to the development of antibiotic resistance (Bouki et al. 2013).

Main and well-studied agents responsible for induction of antibiotic resistance are personal care products containing bactericide agents and antibiotics itself (Russel 2003; Birošová and Mikulášová 2009; Capita et al. 2019). These compounds act directly on bacteria and in certain concentrations are lethal for them. After direct application, however, metabolic changes and dilution occur. This effect allows bacteria present in wastewater to survive besides these compounds and further gives opportunity for induction of unwanted resistance. Continuous research showed that other xenobiotics can also have adverse effects on bacteria. It is well known that all factors affecting mutability of chromosomal genes confer resistance among different bacteria (Birošová and Mikulášová 2014). Martinez and Baquero (2000) suggest that frequency of genome mutations in vivo is much higher compared to that observed in vitro due to environmental stress. This environmental stress represents challenge for bacteria to survive in hostility conditions thanks to increased adaptability and compensatory mutations (Maisnier-Patin and Andersson 2004; Rodríguez-Verdugo et al. 2013).

Carbamazepine is an antiepileptic pharmaceutical which is used to treat a variety of seizures, the right trigeminal neuralgia, schizophrenia, bipolar disorder, and other psychiatric and neurological disorders. Carbamazepine undergoes metabolic transformation and only about 1% leaves the body intact. Although small part of carbamazepine continues to WWTP, common activation was found to be of low efficiency in further degradation of these residue concentrations (Kaiser et al. 2014; Al Khalili and Jain 2019). Because of its persistence, this substance belongs among the most frequently found substances together with its toxic metabolites in the aquatic environment (Ekpeghere et al. 2018). Tramadol is one of the widely prescribed analgesics for treatment of moderate-severe pain in Europe. It is considerably addictive and approximately 30% is excreted in unchanged form via urine by users. This psychoactive compound is only partially removed from sewage water by WWTPs and thus is continuously discharged in respective recipient rivers (Mackuľak et al. 2016). Atenolol and valsartan are frequently prescribed antihypertensives worldwide. Atenolol is widely used β-blocker clinically (Akbar and Alorainy 2014). This cardioselective β1-blocker is not metabolized in patients and about 50% is excreted urinary in its active form. According to its frequent use, it was detected in waste and surface waters in many European countries (Küster et al. 2010). Valsartan is angiotensin II agonist used for treatment of hypertension, for reduction the risk of cardiovascular mortality in patients with heart failure. It has also positive renal and metabolic effects in patients with type 2 diabetes (Ecder 2014). Valsartan can be detected in wastewater in high concentrations (more than 20 μg/L). In WWTP and in the environment, this compound undergoes biotransformation to valsartan acid which is more resistant to degradation (Nödler et al. 2016) Cotinine is tobacco plants alkaloid and predominant metabolite of nicotine (Triggle 1996; Dwoskin et al. 1999). Cotinine is the best indicator of tobacco smoke exposure. Its occurrence in the surface and groundwater systems is a very good indicator of the human presence. Urine cotinine concentration is used as a marker of smoking, including passive (Raja et al. 2016). In contrast to the nicotine is cotinine molecule of stable awareness, and thus, it is suitable for the monitoring of smoking in a population by means of analysis of wastewater (Mackuľak et al. 2015).

The aim of this study was to investigate effect of hospital and WWTP wastewaters on the development of ciprofloxacin resistance in *Salmonella enterica subsp. enterica* serotype Typhimurium. According to high levels of carbamazepine, tramadol, atenolol, valsartan and cotinine in wastewater, the influence on the mutation frequency and rate leading to antibiotic resistance of these non-antimicrobial compounds was studied. This work also contains data related to presence of 38 pharmaceuticals, illicit drugs, and their metabolites in WWTP influent and hospital effluent wastewater.

Materials and methods

Bacterial strains, pharmaceuticals, solvents, chemicals

Bacterial strain of *S. enterica*, subs. *enterica* serotype Typhimurium CCM 4763 used in this study was received from the Collection of Microorganisms, Masaryk University, Brno (Czech Republic). Ciprofloxacin, carbamazepine, cotinine, tramadol, atenolol, and valsartan were purchased from Sigma-Aldrich (Germany). LC-MS grade methanol and acetonitrile (LiChrosolv, Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid, used to acidify mobile phases, was purchased from Labicom (Olomouc, Czech Republic). Ultrapure water for HPLC analysis was obtained from an Aqua-MAX-Ultra system (Younglin, Kyounggi-do, Korea). Standard origin and preparation were described in details in article of Fedorova et al. (2013). In samples of wastewater, presence of codeine, tramadol, citalopram, oxazepam, risperidone, venlafaxine, 2-oxy-3-hydroxy-LSD (O-H-LSD), 6-acetylmorphine, amphetamine, benzoylecgonine, cathinone, cocaine, cotinine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), ketamine, LSD, 1,3-benzodioxolyl-N-methylbutanamine (MBDB), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylendioxy-N-ethylamphetamine (MDEA), methamphetamine (MDMA), mephedrone, methadone, methamphetamine,

methylphenidate, oxycodone, midazolam, norbuprenorphine, norketamine, THC-COOH (11-nor-9-carboxy-delta-9-tetrahydrocannabinol), carbamazepine, azithromycin, ciprofloxacin, erythromycin, penicillin V, oxytetracycline, tetracycline, atenolol, and valsartan was monitored.

Characterization of sampling sites

WWTP Petržalka

WWTP Petržalka treats wastewater from the largest housing estate in Bratislava. This WWTP currently serves for 125,000 inhabitants and treats up to 33,000 m^3/day of municipal wastewater. The treatment process includes mechanical pretreatment and an activated sludge system which is followed by a secondary clarifier. Sludge is anaerobically stabilized and the generated biogas is processed to energy. Sampling points were situated on the influent and effluent stream. Samples were taken every 15 min by an automatic sampling device and mixed as 24-h composite samples.

Characterization of the investigated hospitals

In Slovakia, more than 1.3 million Slovaks are hospitalized every year (the number of permanent hospital beds is around 35,000), of which more than 20% is hospitalized in the capital of Slovakia in Bratislava (NHIC 2017). The National Register of Hospitalized patients in Czech Republic registered more than 2.3 million completed hospitalization cases. Hospitals accounted for 94.7% of all hospitalizations (UZIS 2017). This is one of the main reasons why we have drawn attention to these types of healthcare facilities as a point continuous pollutant of municipal wastewater with drugs and antibiotic-resistant microorganisms. In this study, we investigated the wastewater effluents of four selected health care institutions from the Slovak and the Czech Republic. Oncology institute (203 permanent beds) and hospital Malacky (220 permanent beds) were selected in Bratislava, the capital city of the Slovak republic. Wastewater effluent from the hospital in Vsetín (350 permanent beds) and Valašské Meziříčí (190 permanent beds) were selected in the Czech Republic. The Slovak health care institutions usually dispose of mechanical pre-treatment and classic nitrification sometimes in combination with chlorination (more details in Table 1). A hospital in Vsetín and Valašské Meziříčí does not actually have any treatment of the wastewater (Table 1). For all hospitals, the wastewater for analyses was sampled after the in situ treatment, prior the inlet to the public sewer.

Sample collection

Analyses of pharmaceuticals, illicit drugs, and their metabolites in influent and effluent wastewaters of WWTP and effluent from healthcare institutions were realized during a time period from February to August 2015. Sampling was performed according to Birošová et al. (2014) and Mackuľak et al. (2014). Effluent's samples were collected using an automatic sampler device in 15-min intervals during 24 h (from 7:00 AM until 7:00 AM the next day). Samples were collected in sterile plastic bottles. For resistance development assay, these samples were filtrated through 0.2-μm Supor membrane (Pall Corporation, USA) and applied immediately. For other analysis, samples were frozen (− 20 °C) maximally 2 h after the sampling.

In line solid phase extraction-liquid chromatography tandem-mass spectrometry analysis

Frozen sewage samples were let to thaw at room temperature, homogenized, and split to aliquots for analysis and degradation experiments. The mixture of isotope labeled internal standards (D6-amitriptyline, D10-carbamazepine, 13C3,15N-ciprofloxacin, 13C6-sulfamethoxazole, 13C,D3-tramadol, 13C3-trimethoprim, D5-oxazepam, D5-methamphetamine, D5-amphetamine, D8-benzoylecgonine, D3-cocaine, D5-MDA, D5-MDMA, D9-methadone, and D9-THC-COOH) was added to 10 mL of homogenized and filtered sample (regenerated cellulose syringe filter 0.45 μm pore size) prior to analysis. The extraction and analysis were performed in one step using in line SPE liquid chromatography coupled with either hybrid mass analyzer Q-Exactive (hybrid quadrupole orbital trap high-resolution MS, Thermo Scientific) for psychoactive compounds, or the sample LC step but with triple quadrupole MS/MS detection for other pharmaceutical classes. Isotope dilution and internal standard methods together with matrix-matched standards were used to eliminate matrix effects. Detailed description of the method and its performance are given in paper of Fedorova et al. (2013) and Lindberg et al. (2014). Briefly, methods showed good linearity up to 2000 ng L^{-1}, acceptable repeatability at all validation concentration level exceeded 20% of RSD only for a few compounds at the lowest concentration in influent. Limits of quantification (LOQ) are highly dependent on matrix effect in given matrix, LOQs in every sample are calculated based on instrumental LOQ corrected to corresponding internal standard in the sample and matrix matching standard. This procedure resulted to different LOQ for each sample, which are reported in the result table as values <.

Determination of mutation frequencies to antibiotic resistance

Effect of wastewater samples and studied compounds on the development of mutations leading to antibiotic resistance was performed according to Birošová et al. (2007). Samples of wastewater were first pretreated with filtration through

0.2 μm Supor membrane (Pall Corporation, USA). *S. enterica* ser. Typhimurium strain has grown overnight in antibiotic-free Luria-Bertani (LB) broth with the viable cell number being around 10^9 mL^{-1}. This culture was divided into 0.1-mL aliquots to which 0.1 mL of tested compound or filtrated wastewater and 0.5 mL of phosphate buffer of pH 7.4 was added. Cultures were treated for 30 min at 37 °C. Then, the fresh LB medium was added and cultures were incubated for 3 h at 37 °C to allow a few cell divisions and protein expression to occur. The number of ciprofloxacin-resistant mutants that emerged in each culture was determined by plating the entire culture on LB agar plates containing a selective ciprofloxacin concentration (0.06 μg/mL), which represents clinical resistance breakpoint (EUCAST 2019). The total number of viable cells was determined by plating an appropriate dilution of cultures on nonselective medium. Colony-forming units (CFU) were counted after incubation for 24 h and resistant colonies on selective plates for 72 h at 37 °C.

Statistical analysis

The influence of tested samples of wastewater and compounds to mutation frequencies leading to antibiotic resistance was expressed as resistance factor (RF) which represents ratio of frequency of resistant mutants affected by compound or sample and frequency of spontaneous resistant mutants. Mutation frequency was calculated as a mean number of resistant cells divided by the total number of viable cells per culture. Mutation rate factor represents ratio of mutation rate (MR) of resistant mutants affected by compound or sample and MR of spontaneous resistant mutants. Mutation rate was calculated according to study by Rosche and Foster (2000).

$$MR = \frac{m}{1.44N} \quad (1)$$

where m represents the number of mutations per culture (Eq. 2) and N is the final number of cells in a culture.

$$\frac{r}{m} - \ln m = 1.24 \quad (2)$$

where r is the mean number of mutants (resistants) in a culture.

Data shown in this study represent the mean of three independent experiments; each experiment was made in ten parallels and statistically evaluated by Student's t test.

Results and discussion

Pharmaceuticals in studied wastewater samples

We have focused on the analysis of 38 pharmaceuticals, illicit drugs, and their metabolites in wastewater from WWTP Petržalka and four hospitals (Table 2). Obtained data show that main determined micropollutants such cotinine, carbamazepine, azithromycin, ciprofloxacin, methamphetamine, tramadol, citalopram, and venlafaxine were found in wastewater in concentration reached levels above 1000 ng/L (Table 2). Levels of cotinine from the Slovak and the Czech healthcare facilities ranged from 740 to 3580 ng/L (Table 2). These values correspond with results obtained in Switzerland, Australia, Italy, or Portugal (Chen et al. 2013; Lopes et al. 2014; Senta et al. 2015). Concentration of methamphetamine in WWTP influent was about 440 ng/L. This illicit drug belongs to the most widespread compound in the Slovak and the Czech Republic, because of its relatively cheap production (Mackuľak et al. 2014). However, addicted consumers are treated in hospitals; this illicit drug is often present also in hospital effluents. Tramadol is very frequently prescribed psychoactive pharmaceutical in the Czech and the Slovak Republic (NHIC 2014). It is highly addictive and can lead to abuse. During monitoring, its concentration ranged from 442 to 12,640 ng/L (Table 2). Levels of psychoactive substance oxazepam reached the maximum at 310 ng/L (Table 2). Codeine belongs to opiates for cough and diarrhea treatment and is active substance of several pharmaceuticals (NHIC 2014). Its concentration in studied wastewaters reached up to a maximum value of 170 ng/L (Table 2). Citalopram and venlafaxine are the most frequently prescribed antidepressants in the Slovak and the Czech Republic (NHIC 2014). Citalopram concentration in hospital wastewaters ranged from

Table 1 Characteristics of selected healthcare institutions

Parameter	Hospital Malacky	Hospital Valašské Meziříčí	Hospital Vsetín	Oncologic institute Bratislava
Date of analysis	28-08-2015	20-02-2015	20-07-2015	17-04-2015
COD	1225 mg/L	1460 mg/L	1350 mg/L	1160 mg/L
pH	7.1	7.4	7.3	7.0
Number of beds	110	300	350	200
Flow	62 m^3/day	110 m^3/day	125 m^3/day	92 m^3/day
Method of wastewater treatment	Nitrification/chloration	No treatment	No treatment	Nitrification/chloration

COD chemical oxygen demand

Table 2 Occurrence of selected pharmaceuticals, drugs, and metabolites in healthcare facilities effluents and WWTP influent

	Hospital Vsetín	Hospital Valašské Meziříčí	Hospital Malacky	Oncologic institute Bratislava	WWTP Petržalka	
	Effluent	Effluent	Effluent	Effluent	Influent	Effluent
Group of studied compounds	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L
Analgesics						
Codeine	132	105	170	44	26	20
Tramadol	442	12,600	1240	9560	476	324
Antidepressants/psychiatric						
Citalopram	90	1320	107	720	57	55
Oxazepam	27	310	60	66	29	34
Risperidone	< 2.4	< 7.2	< 7.9	< 6.5	< 3.2	< 4.4
Venlafaxine	332	2440	424	566	196	200
Psychoactive compounds and metabolites						
2-oxy-3-hydroxy-LSD	< 9.0	< 16	< 6.6	< 4.6	< 1.6	< 3
6-acethylmorphine	< 5.5	< 6.5	< 6.2	< 3.5	< 2.6	< 2.1
Amphetamine	< 4.5	30	15	< 4.8	< 2.5	< 3.7
Benzoylecgonine	< 5.2	< 13	< 13	< 4.5	88	32
Cathinone	< 2.4	< 4.4	< 4.1	< 4.5	< 1.1	< 2.8
Cocaine	< 4.1	< 8.6	< 11	< 4.5	66	14
Cotinine	740	832	3580	3040	2440	91
EDDP	< 10	< 38	< 4	< 4.0	< 11	< 2.7
Ketamine	< 9.5	< 14	< 5.6	< 6.4	< 6.4	< 4.2
LSD	< 4.5	< 8.5	< 4.9	< 6.5	< 6.5	< 4
MBDB	< 4.4	< 12	< 6.8	< 4.8	< 2.8	< 2.6
MDA	< 3.2	< 3.7	< 6.8	< 5.8	< 5.5	< 4.2
MDEA	< 3.5	< 9.4	< 7.2	< 5.3	< 3.3	< 4.9
MDMA	< 7.0	< 10	< 9	< 8.5	< 5.5	< 4.7
Mephedrone	< 6.1	< 9.6	< 7.4	< 9.2	< 4.8	< 4.4
Methadone	< 10	< 40	< 5	< 2.8	< 8.8	< 3.8
Methamphetamine	< 3.1	285	93	18	440	68
Methylphenidate	< 3.3	< 9	< 24	< 4.9	< 4.2	< 2.3
Oxycodone	< 4.2	116	< 1.8	< 2.8	< 2.4	< 3.3
Midazolam	< 4.7	132	< 4.9	< 1.8	< 1.4	< 2.3
Norbuprenorphine_gluc	< 5.4	< 8.3	< 3.1	< 6.4	< 2.8	< 1.7
Norketamine	< 12	< 14	< 6.5	< 7.9	< 2.9	< 2.6
THC-COOH	< 2.2	14	16	15	66	< 6.5
Carbamazepine	480	1220	880	45	348	265
Antibiotics						
Azithromycin	1420	984	1140	322	230	58
Ciprofloxacin	2104	1780	2550	5360	346	322
Erythromycin	46	65	44	15	20	17
Penicillin_V	< 9	< 8	< 7	< 7	< 10	< 5
Oxytetracycline	< 4.3	< 7.3	< 8.9	< 4.8	< 7.5	< 5
Tetracycline	< 5.2	< 8.1	< 9.1	< 8.4	< 9.4	< 4
Antihypertensive						
Atenolol	6.0	11,700	215	71	410	210
Valsartan	416	4400	7080	700	4600	1210

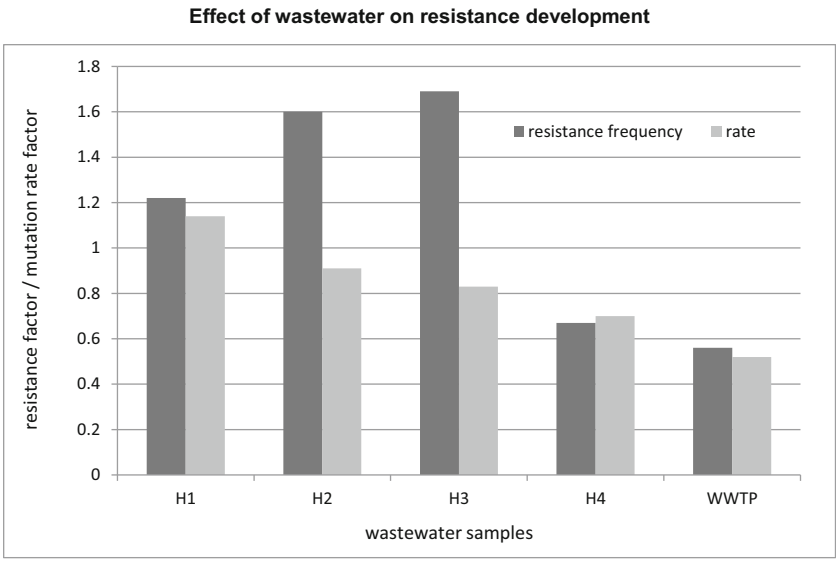

Fig. 1 Effect of wastewater on mutation frequency and rate leading to ciprofloxacin resistance in *S.* Typhimurium H1-hospital Valašské Meziričí, H2-hospital Vsetín, H3-hospital Malacky, H4-oncological hospital, WWTP-Petržalka

90 to 1320 ng/L and venlafaxine from 332 to 2440 ng/L (Table 2). Levels of carbamazepine in wastewater from monitored healthcare facilities were in range 45–1220 ng/L and in WWTP influent water up to 348 ng/L. The most frequent prescribed antibiotics in both countries are β-lactams, fluoroquinolones, and macrolides (NHIC 2014), what correlates with their occurrence in hospital wastewater (azithromycin 322–1420 ng/L and ciprofloxacin 1780–5360 ng/L). The most concentrated antibiotic in WWTP influent was ciprofloxacin (346 ng/L). Domination of ciprofloxacin and azithromycin in Slovak wastewaters was described also in our previous study (Birošová et al. 2014). Concentration of valsartan was higher compared to atenolol in almost all tested samples, except hospital effluent in Valašské Meziříčí. In this sample, level of atenolol was the highest (11,700 ng/L). In contrast, the lowest atenolol level was observed in second hospital effluent in Czech Republic (6 ng/L). This reflects also high difference in prescription of antihypertensives in one region. In this hospital effluent also, valsartan level was the lowest (Table 2).

Effect of wastewater on the development of antibiotic resistance

Several thousands of active ingredients are used for pharmaceuticals in even more products. In hospitals as well as in surgeries, a variety of substances besides pharmaceuticals are in use for medical purposes as diagnostics and disinfectants. After administration, pharmaceuticals are excreted by the patients into wastewater. Unused medications are sometimes disposed of in drains (Kümmerer 2001). Indeed, hospital wastewaters are complex mixtures of chemical and

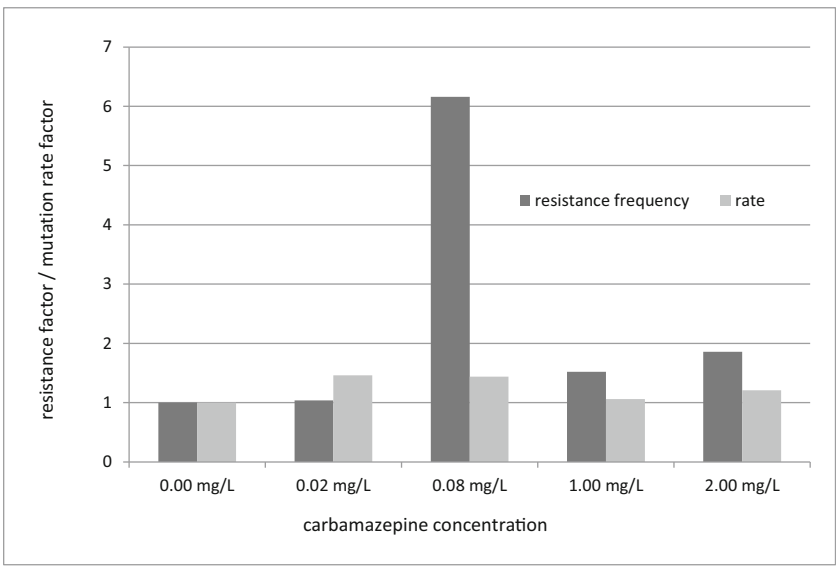

Fig. 2 Effect of carbamazepine on mutation frequency and rate leading to ciprofloxacin resistance in *S.* Typhimurium

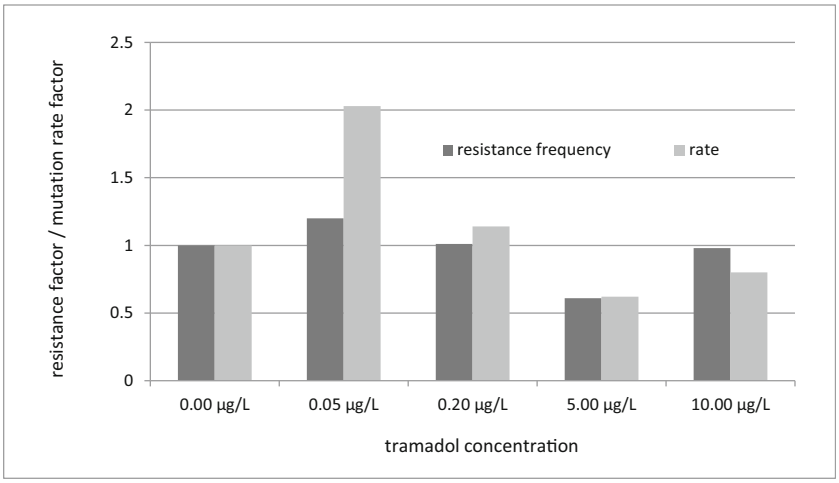

Fig. 3 Effect of tramadol on mutation frequency and rate leading to ciprofloxacin resistance in *S.* Typhimurium

biological substances which are continually discharged (Laffite et al. 2016). According to this fact, in this part of this work, we have focused on the effects of five wastewater samples on the development of ciprofloxacin resistance in model strain *S. enterica* subsp. *enterica* serotype Typhimurium. However, hospitals belong to main contributor of pharmaceuticals to wastewater, four samples were taken from different hospital effluent in the Slovak and the Czech Republic. One sample represented influent wastewater from WWTP in the capital of Slovakia. We have investigated also effect of WWTP effluent water. However, result was the same as in case of WWTP influent; in the figure, it is stated only WWTP-Petržalka. From Fig. 1, it is evident that hospital wastewater contributes to increase of mutation frequency leading to ciprofloxacin resistance. Resistance factor of three tested hospitals was above 1, so frequency of mutations leading to ciprofloxacin resistance after treatment with hospital water (H1, H2, H3) was higher compared to frequency of spontaneous mutations in control samples. The highest increase was detected in case of the Slovak hospital in Malacky where mutation frequency was almost 1.8-fold higher compared to control with spontaneous resistants. In case of specialized oncological hospital and WWTP, moderate decrease in mutation frequency was determined. Decrease was observed also in mutation rate which gives more information about biological processes than mutation rate does. Only slight increase was detected in case of hospital effluent water in Valašské Meziříčí. Samples have not decreased number of cells in culture compared to control. It is evident that wastewater from both hospitals (Malacky, Valašské Meziříčí) possesses the highest levels of majority pharmaceuticals, metabolites, and antibiotics (Table 2). These high concentrations can generate higher selective pressure leading to higher mutation frequency in bacteria.

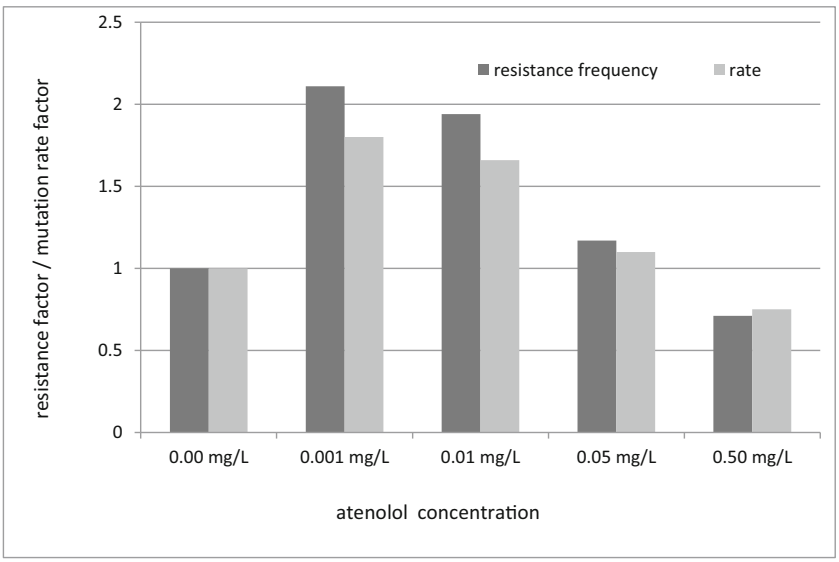

Fig. 4 Effect of atenolol on mutation frequency and rate leading to ciprofloxacin resistance in *S.* Typhimurium

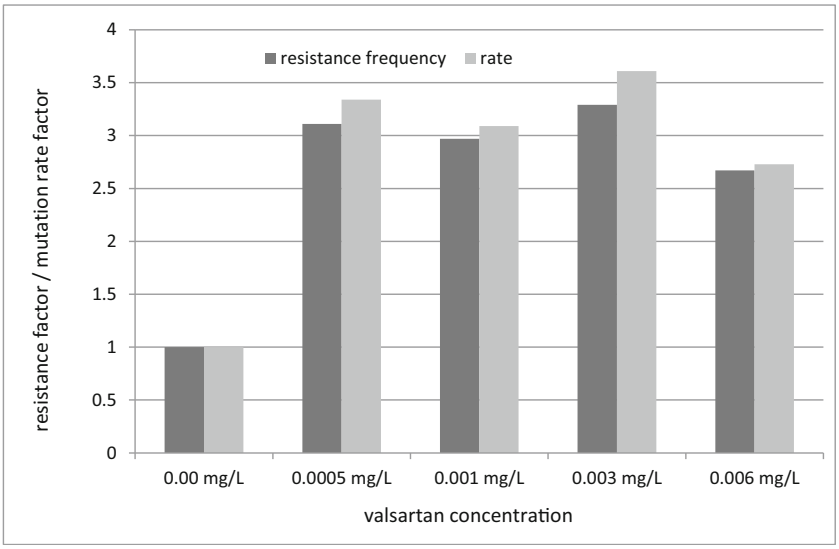

Fig. 5 Effect of valsartan on mutation frequency and rate leading to ciprofloxacin resistance in *S.* Typhimurium

Effect of selected non-antimicrobial substances on development of antibiotic resistance

It is well known that antibiotics in the human and animal husbandry and the relevant sustainable industries may promote the emergence of antibiotic-resistant bacteria (Xu et al. 2016), but effect of non-antimicrobials on the development of antibiotic resistance is still very poorly understood area. Many studies worldwide showed that carbamazepine, tramadol, atenolol, and valsartan belong to the most relevant pharmaceuticals with the highest median concentration levels in wastewater but also in surface water (Kasprzyk-Hordern et al. 2009; Rúa-Gómez et al. 2012; Loos et al. 2013; Mackuľak et al. 2016). Beside these pharmaceuticals, stable metabolite of nicotine (cotinine) is also in wastewater represented at higher levels (Hedgespeth et al. 2012; Mackuľak et al. 2015). According to high wastewater levels of these non-antimicrobial compounds in wastewater in last part of this work, we were interested whether these agents can affect development of mutations leading to antibiotic resistance in bacteria. Concentrations applied in study ranged from values detected in user's urine to values observed in wastewater. Figure 2 shows influence of carbamazepine on the development of ciprofloxacin resistance. Only 3% of carbamazepine is excreted in unchanged form. Urine concentration of this aromatic anticonvulsant ranges from 0.05 to 24 mg/L (Beltran et al. 2007), while its concentrations in Slovak and Czech wastewaters reach 2–800 μg/L (Fedorova et al. 2016; Mackuľak et al. 2016). The lowest applied concentration has no effect on mutation frequency. Surprisingly, carbamazepine in concentration 0.08 mg/L caused 6-fold increase of mutation frequency leading to ciprofloxacin resistance. This

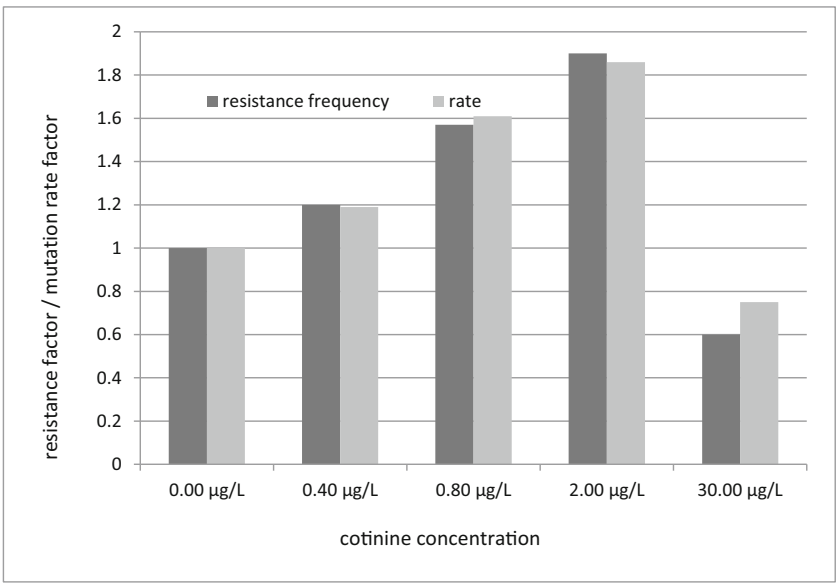

Fig. 6 Effect of cotinine on mutation frequency and rate leading to ciprofloxacin resistance in *S.* Typhimurium

concentration reflects the highest levels of this compound in wastewaters. Higher concentrations designed according to urine levels have caused only moderate increase of resistance factor. Mutation rate was slightly increased after exposition to lower, wastewater concentrations of carbamazepine. Wang et al. (2019) showed that carbamazepine also promotes horizontal gene transfer as well as SOS response, which can play role in increased mutation frequency.

In case of tramadol, higher percentage (15–35%) is excreted in unchanged form, and in some cases, urine levels of this analgesic pharmaceutical can reach 3081 μg/L (Cheng et al. 2008; Kasprzyk-Hordern et al. 2009). Wastewater concentration in Slovakia ranged from 413 to 85 ng/L (Mackuľak et al. 2016). From Fig. 3, it is evident that wastewater concentrations can cause 2-fold increase of resistance factor and a moderate increase of mutation rate. Although tramadol belongs to painkillers, it has also bactericidal effect (Tamanai-Shacoori et al. 2007). This could be also a reason why human targeted drug such as tramadol with antibiotic effect could lead to antibiotic resistance in low concentrations (Maier et al. 2018).

Up to 50% of atenolol excreted in urine and the same amount in stool (Clarke 2004). Figure 4 shows that the lower concentrations which are frequently found in aquatic environment caused the highest increase of mutation frequency leading to ciprofloxacin resistance. These concentrations doubled resistance index. Mutation rate has also raised compared to spontaneous mutation rate in control. This could be linked to ability of β-blockers such as atenolol to change microbial metabolic activity (Pino-Ótin et al. 2017) and thus contribute to the development of antibiotic resistance.

Second tested antihypertensive compound valsartan showed effect in all tested concentrations (Fig. 5). Applied concentrations reflect levels of valsartan in wastewater. Fáberová et al. (2017) stated that wastewater valsartan concentration in Slovakia ranged from 0.09 to 32.98 μg/L. Frequency and rate of mutations leading to ciprofloxacin resistance in *S.* Typhimurium were higher compared to atenolol. This correlates with conclusion of Godoy et al. (2015) that antihypertensives can affect also non-target organism and that there is a lack of ecotoxicity data, especially the chronic ones.

Last tested compound was cotinine. Cogo et al. (2008) showed that cotinine can affect virulence factors of some bacteria, so it possibly may also influence antibiotic resistance. Detection of this metabolite in wastewater serves to estimation of nicotine consumption in cities. Concentration of cotinine in heavy smokers can reach 7179 μg/L (Vine et al. 1993). According to Mackuľak et al. (2015), wastewater levels of cotinine in Slovakia range from 165 to 4100 ng/L. From Fig. 6, it is evident that with increasing concentration of this metabolite is increasing also resistance factor and mutation rate. Concentration 2 μg/L which reflects average wastewater level in the capital city of Slovakia caused twofold rise of mutation frequency as well as rate leading to ciprofloxacin resistance. On the other hand, in this case, the same trend was observed as in case of tramadol, carbamazepine, and atenolol. Exposure of *S.* Typhimurium to higher concentration has led to slight decrease of resistance factor. Similar increase of mutations leading to antibiotic resistance after exposure to studied compounds was observed in our previous study (Birošová and Mikulášová 2014). This work showed that lower concentrations of environmental pollutants and compounds generated during food processing may contribute to the development of mutations leading to antibiotic resistance.

Conclusion

Data in this study point out on presence of selected analgesics (tramadol, carbamazepine), antihypertensives (atenolol,valsartan), antidepressant (citalopram, venlafaxine) as well as synthetic antibiotics (ciprofloxacin, azithromycin) and cotinine in hospital effluents in higher concentration. Our results also show that such wastewater can increase mutation frequency leading to antibiotic resistance. Experiments with non-antimicrobial pharmaceuticals such as tramadol and carbamazepine showed that this phenomenon may be caused not only by subinhibitory concentrations of antibiotics. Carbamazepine, tramadol, atenolol, valsartan, and cotinine in very low concentrations detected in wastewater can increase frequency and rate of mutations leading to ciprofloxacin resistance in *S.* Typhimurium. Although concentrations of these compounds in WWTP influent and effluent water are much lower compared to hospital effluent, this type of wastewater has not affected mutation frequency and rate leading to antibiotic resistance.

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