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ORIGINAL ARTICLE

Toxicity of wastewater from health care facilities assessed by different bioassays

Gabriela Jírová [1,2], Alena Vlková [1,3], Martina Wittlerová [2], Markéta Dvořáková [3,5], Lucie Kašparová [2], Jan Chrz [3,4], Kristina Kejlová [3], Zdeňka Wittlingerová [1], Magdaléna Zimová [1,2], Barbora Hošíková [4], Jana Jiravová [4], Hana Kolářová [4]

1 Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Praha - Suchdol, 165 00, Czech Republic
2 National Institute of Public Health, Centre of Health and Environment, Šrobárova 48, Praha 10, 100 42, Czech Republic
3 National Institute of Public Health, Centre of Toxicology and Health Safety, Šrobárova 48, Praha 10, 100 42, Czech Republic
4 Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, Olomouc, 775 15, Czech Republic
5 Charles University in Prague, Third Faculty of Medicine, Ruská 87, Praha 10, 100 00, Czech Republic

Correspondence to: Gabriela Jírová, M.Sc.
Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Praha - Suchdol, 165 00, Czech Republic.
TEL: + 420 267082564; E-MAIL: jirovag@fzp.czu.cz

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Abstract

OBJECTIVES: The purpose of this study was to determine toxicity of wastewater from hospitals in the Czech Republic using traditional and alternative toxicological methods. The pilot study comprised weekly dynamics of sewage ecotoxicity of treated wastewater from one hospital in two different seasons. A detailed investigation of wastewater ecotoxicity, genotoxicity and reprotoxicity followed in five different hospitals.

METHODS: The seven following bioassays were used in this study: algal growth inhibition test (ISO 8692), *Vibrio fischeri* test (ISO 11348-2), *Daphnia magna* acute toxicity test (ISO 6341), *Allium cepa* assay, Ames test (OECD TG 471), Comet assay and YES/YAS assay.

RESULTS: The wastewater ecotoxicity during one week showed no differences in separate working days, however, higher toxicity values were recorded in May compared to November. In the following study, samples from two of the five hospitals were classified as toxic, the others as non toxic. Genotoxicity has not been confirmed in any sample. In several cases, wastewater samples exhibited agonist activity to the estrogen and androgen receptors.

CONCLUSION: The study demonstrated different levels of toxicity of treated hospital wastewater. Variable sensitivity of individual bioassays for tested wastewater samples was recognized. A more extensive study including proposal for improvement of hospital wastewater treatment within the Czech Republic can be recommended with the aim to decrease the discharge of toxic chemicals into the local sewage system and the environment.

Abbreviations:

WW	- wastewater
HWW	- hospital wastewater
UWW	- urban wastewater
WWTP	- wastewater treatment plant
PHs	- pharmaceuticals
EDs	- endocrine disruptors
EC_{50}	- effective concentration of the tested substance that causes negative effect (inhibition, immobilization) in 50% of the organisms
EC20	- effective concentration of the tested substance that causes negative effect (inhibition, immobilization) in 20% of the organisms
TU	- toxic unit

INTRODUCTION

Wastewater from health care facilities (HWW) differs from classical urban wastewater (UWW) mainly due to the content of wider spectrum and higher quantity of pharmaceuticals (PHs) and chemicals. The main substances that can be found in HWW are antibiotics, analgesics and anti-inflammatories, psychiatric drugs, β-blockers, anaesthetics, disinfectants, chemicals from laboratory activities, developer and fixer solutions from photographic film processing and X-ray contrast media (WHO 2013).

The occurrence of PHs in wastewater is limited by the amount of drugs and chemicals used (varying in different countries and evolving over time), entry into sewage, degradation in sewage treatment plants and occurrence in surface and groundwater (Váňa *et al.* 2010). If these substances are not sufficiently removed, the contamination of the aquatic environment will inevitably increase and affect all the relevant ecosystems. Hence, a better understanding of the effect of PHs in the environment is required.

In Europe, no specific directive or guideline for the management of hospital effluents has been adopted yet. Liquid waste must not be discharged into a foul sewer but treated as a waste and collected and disposed as such. For the effluents from the hospital foul sewer there is no specific regulation issued and so each member state of the European Union has its own distinct legislation (EU 2000).

The principles of drainage and subsequent purification of HWW in the Czech Republic are indicated in the standard ČSN 75 6406. The methods of WW and sludge treatment are further regulated with regard to the occurrence, character and amount of germs, radioactive substances and local conditions. Health care facilities are obliged to disinfect WW if the facilities are designed to isolate and treat transmissible diseases or to manipulate infectious material (ČSN 75 6406).

PHs and personal care products are considerably resistant to current procedures of WW treatment. It has been demonstrated that the majority of these substances and mixtures are not totally eliminated from the liquid phase during WW treatment, especially substances with low lipophilicity (Suarez *et al.* 2009). If sewage treatment takes place only at the point of origin (in the hospital sewage treatment plant), the cleaning efficiency is around 90%. The maximum cleaning effect is achieved with the double cleaning of HWW, i.e. at the place of origin of the hospital treatment plant and subsequent purification by a municipal cleaning plant (Pauwels *et al.* 2006).

In most cases, HWW is diluted with municipal sewage, and this usually leads to a reduction in pharmaceutical compounds amount in the final WW (Verlicchi *et al.* 2012). However, distinct drugs, even in small concentrations, may be still toxic to the environment. In their review, Orias and Perrodin (2013) summarized data on observed concentrations of 297 pollutants measured in the HWW including pharmaceutical and non-pharmaceutical compounds (disinfectants, alcohols, detergents, heavy metals). Metals as elements associated with medical care are non-negligible components of sewage water with a great variability of possible concentrations. Therefore, in our study, selected metals were determined in order to extend the characteristics of the WW samples. Mercury is used in manometers for measuring and controlling pressure, in thermometers, in dental amalgam fillings, esophageal dilators and gastrointestinal tubes. Chemical compounds of mercury are used as antiseptics in pharmaceuticals, as reagents in laboratories, and as catalysts. Health care facilities are one of the main sources of mercury release into the environment (Rustagi & Singh 2010). They release 5% of the mercury to water bodies through untreated WW, and e.g. in the United Kingdom, more than 50% of total mercury emissions come from mercury contained in dental amalgam and laboratory and medical devices (WHO 2013). In spite of successfully reduced emissions into the aquatic environment in the past, mercury continues to be one of the heavy metals whose discharged volume is still high. Mercury ultimately accumulates at the bottom of water bodies, where it is transformed into its more toxic organic form, methyl mercury, which accumulates in fish tissue. Platinum-based cytotoxic drugs are among the most used for the treatment of testicular, prostate, colon and breast tumors. Most of the platinum series cytotoxic agents are excreted via the urine and thus enter the HWW (Kümmerer *et al.* 1999). Gadolinium complexes are used in magnetic resonance. The concentrations measured in hospital effluents are in the range of a few μg/l to 100 μg/l (Kümmerer *et al.* 2000). For its antimicrobial properties, silver is a frequent ingredient of creams, wound dressings and antimicrobial coatings on medical devices. Silver is also used in bone prostheses, reconstructive orthopedic surgery and cardiac devices (Lansdown, 2006). Aluminum and alum are also used in medicine, they have contraction and anti-inflammatory effects (e.g. aluminum acetate for swelling). They are used in dentistry, PHs industry, and manufacture of surgical instruments (Goullé *et al.* 2012).

The purpose of this study was to determine toxicity of HWW in the Czech Republic using conventional and alternative toxicological methods. Our study represents the first study in the Czech Republic investigating HWW by means of a wide range of biological methods comprising not only tests of ecotoxicity, but also genotoxicity and reprotoxicity.

Standard ecotoxicity assays are a way to determine some PHs and personal care products effects, such as acute or chronic ecotoxicity, on organisms of different trophic levels. Different species of fish, crustaceans, algae are often used for this purpose; however, other microorganisms, such as bacteria, have also been used in these studies. Fish test has not been used in our study with respect to the EU directive (EU 2010) on the protection of animals used for experimental purposes requiring the reduction of animal tests and their replacement by alternative methods. In our study, the preferred ecotoxicity method was luminescence bacteria test. The luminescence inhibition bioassay with marine photobacteria *Vibrio fischeri* has been confirmed as a useful tool for estimation of acute toxicity of numerous chemicals (Rosal *et al.* 2010b; Białk-Bielińska *et al.* 2017; Välitalo *et al.* 2017). Green algae (e.g. *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata*) comprise an essential component of aquatic ecosystems and they are often considered as a good indicator for anthropogenic pollution and water quality (Ma *et al.* 2006) with high sensitivity in toxicity testing (Magdaleno *et al.* 2014b; Russo *et al.* 2017; Vasconcelos *et al.* 2017). *Daphnia magna* is a fresh water cladoceran crustacean that is very sensitive to chemicals or pollutants (Flaherty & Dodson 2005; Boillot & Perrodin 2007) and it is widely used to evaluate the ecotoxicity of WW (Erbe *et al.* 2011; Kern *et al.* 2014). If it is exposed to stress factors, its life, morphology, behavior, and physiological properties may change (Jiang *et al.* 2018). Among several methods using higher plants, the *Allium cepa* test is frequently used in the biomonitoring of wide range of compounds (Herrero *et al.* 2012) or for testing toxicity of WW (Firbas & Amon 2013). The risks associated with the discharge of PHs and chemicals into the environment are based not only on their acute and chronic ecotoxicity, but also their genotoxicity and endocrine disruption (Rosal *et al.* 2010a).

Genotoxicity was studied by a combination of two tests: Ames test and Comet assay. The Ames test (OECD 1997) has been widely used to assess the genotoxic effect of various types of water, such as drinking water (Shen *et al.* 2003), water after sewage treatment (Morisawa *et al*. 2003), or water from municipal or hospital wastewater treatment plants (Jolibois & Guerbet 2006; Ferk *et al.* 2009; Sharma *et al.* 2015). *Salmonella typhimurium* TA100 and TA98 strains are generally used in these assays. The Comet Assay, also known as single cell gel electrophoresis (SCRE), enables to determine whether there has been deoxyribonucleic acid (DNA) damage to a single cell from apoptosis (cell death) or cytotoxicity (toxicity to cells) and the extent of this damage (Singh *et al.* 1988; Tice *et al.* 2000).

Endocrine disruption (ED) endpoints for testing of biotic systems are of great concern since EDs are recent common contaminants of aquatic ecosystems. Important sources of EDs are effluents from sewage treatment plants including those in health care facilities. Certain EDs, such as natural and synthetic hormones are not completely removed with the use of conventional wastewater treatment systems. With regard of these concerns, there is increasing pressure to develop advanced wastewater treatment methods and also an appropriate battery of tests that will include endocrine disruption endpoints (Hecker & Hollert 2011). Certain *in vitro* methods based on transfected cell lines have been already included in the OECD concept and *in vitro* methods based on yeast strains have been standardized in the ISO standard system, e.g. Draft ISO 19040 (OECD, 2012; ISO, 2017). Both biological systems are effective to be used for hazard identification within (eco)toxicological purposes. In our study, the yeast-based microplate assay YES/YAS was used for determination of estrogenic and androgenic potential of concentrated WW samples.

Due to relative simplicity, sensitivity , low cost of experimentation and small amount of sample required all implemented short-term bioassays have proved to be an important tool in genotoxic and reprotoxic studies.

MATERIAL AND METHODS

Wastewater samples

This study involved investigation of WW from five hospitals (H1–H5) located in the central region of the Czech Republic. Table 1 shows the characteristics of the selected hospitals. In order to monitor the weekly variation of HWW ecotoxicity, the samples from hospital H1 were collected in two different seasons. The first sampling series was done in November 2016 (Nov. 21–Nov. 25, 2016), the second sampling in May 2017 (May 22–May 26, 2017). Five composite samples were taken in separate working days during the week in both of the series. The sampling scheme was designed in accordance with literature data (Goullé *et al.* 2012) documenting decreasing amount of toxic substances in WW on Saturdays and Sundays because of the absence of typical medical activities.

The sampling of effluent from five different hospitals was performed in February 2018 (Feb. 13–Feb. 22, 2018) with the aim of detailed evaluation of ecotoxicity, genotoxicity and reprotoxicity. One composite sample was taken from each hospital.

Our composite samples were collected in the course of the maximal WW flow, i.e. from 9 a.m. to 1 p.m., taking a partial sample every hour. This scheme was in concordance with findings of Boillot *et al.* (2008) reporting in their study of daily ecotoxicological fluctuations of HWW that toxicity peak occurred from

Tab. 1. Characteristics of the selected hospitals.

	H1	H2	H3	H4	H5
Type of hospital	university	general	oncology	general	university
Total capacity (number of beds)	2189	996	245	476	1 375
Wastewater generation (m^3/day)	50–100*	51*	124	10*	250
Wastewater treatment process	mechanical - biological	mechanical - biological	mechanical - biological	mechanical - biological	mechanical - biological
Disinfection process	NaOCl	NaOCl	NaOCl	Cl_2	Cl_2
Wastewater discharges	urban sewer system	urban sewer system	water flow	urban sewer system	urban sewer system

* WW only from one part of the hospital.

9 a.m. to 1 p.m. during the period of the maximum flow rate and the highest frequency of care activities. Our samples were taken after treatment activities in the discharge site either into the urban sewer system or into the water flow. The samples were transferred immediately to the laboratory in cooling boxes and stored at ≤–18 °C prior to analysis. With the exception of Ames test and Comet assay, which methodically require sterile samples, the analyses were performed on non filtered samples. To ensure sterile samples, filtration was performed using DURAPORE membrane filter (MILLIPORE) – hydrophilic, porosity 0.22 μm. WW samples tested for estrogenic and androgenic potential using the YES/YAS assay were 250× concentrated in compliance with Draft ISO/DIS 19040-1:2017(E) standard (ISO 2017).

The values of physical and chemical parameters of the samples are displayed in Table 2 (a) samples from hospital H1 collected in November 2016 and May 2017, (b) samples from hospitals H1–H5 collected in February 2018. The physicochemical characteristics were determined according to standard methods: temperature (ČSN 75 7342), pH (ISO 10523), conductivity (ISO 7888), dissolved substances (ČSN 75 7346), free and total chlorine (ISO 7393-2). The measurements of the temperature and the free chlorine were performed in-the-field in order to monitor their values during sampling. The analysis of metals and iodine was performed as follows: Total mercury concentration was determined using an atomic absorption spectrophotometer AMA 254 Trace Mercury Analyzer (Altec). The samples were analysed without sample pre-treatment. Total gadolinium, platinum, lead, silver, aluminium and iodine concentrations were determined using an inductively coupled plasma mass spectrometer (ICP-MS Elan DRC-e, Perkin Elmer). For determination of gadolinium, platinum, lead, silver and aluminuim, the water samples were diluted 1 to 100 times using 1% (v/v) solution of nitric acid and germanium, indium and rhenium were used as internal standards. For iodine determination, the water samples were diluted 10 to 100 times using 1% tetramethylammonium hydroxide (TMAH), 0,02% TRITON X-100 and tellurium was used as an internal standard. The reference material (drinking water) from the interlaboratory comparison and reference material Seronorm™ Trace Elements Urine L-2 was used for the laboratory quality control.

Toxicological bioassays

This study represents the first study exploring HWW in the Czech Republic by means of a wide range of biological methods. Seven different traditional and alternative toxicological bioassays were employed. Their characteristics are listed in Table 3.

Algal growth inhibition test

The test was carried out using freshwater algae *Desmodesmus subspicatus* (BRINKMANN 1953/SAG 86.81) obtained from the Culture Collection of Autotrophic Organisms (CCALA). $K_2Cr_2O_7$ was used as positive control for monitoring the sensitivity of algae culture. Five test sample dilutions in triplicates were prepared in every test run. The test flasks were inoculated by algal cells to obtain 10^4 cells.ml^{-1} and incubated under 23±2 °C with constant illumination intensity of 6 000–10 000 lx and color temperature 4 300 K. After 72 h exposure, direct cell count measurement was performed using microscope OLYMPUS CH30. The probit method was used for the calculation of EC_{50}. Inhibition of specific growth rate was calculated in relation to negative control samples (test growth medium) growing under the same standard conditions.

Luminescent bacteria test

Liquid-dried luminescent marine bacteria *Vibrio fischeri* NRRL- B-11177 (HACH LANGE) were used. Bacteria sensitivity was monitored using positive controls ($ZnSO_4.7H_2O$ and $K_2Cr_2O_7$). Bacteria were reconstituted by adding reactivation solution. Samples salinity was corrected by NaCl. The suspensions of diluted WW samples and bacteria were maintained at 15±1 °C.

Tab. 2. Physicochemical characteristics of the samples.
a) samples from hospital H1 collected in November 2016 and May 2017.

Parameter	Temperature* °C		pH		Conductivity µS.cm^{-1}		Free chlorine* mg.l^{-1}	
	X/2016	V/2017	X/2016	V/2017	X/2016	V/2017	X/2016	V/2017
Monday	10.0	13.5	8.05	8.01	2010	1125	0.04	0.25
Thuesday	10.4	13.5	8.01	8.03	2130	1186	0.05	0.32
Wednesday	11.0	13.0	8.12	8.07	2150	1205	0.07	0.15
Thursday	10.6	13.4	8.02	8.07	2090	1206	0.07	0.17
Friday	11.0	13.0	8.03	8.09	2160	1232	0.05	0.20

* in-the-field measurements-average values of five partial samples.

b) samples from hospitals H1-H5 collected in February 2018.

Parameter	Unit	H1	H2	H3	H4	H5
Temperature *	°C	6.5	6.5	13.0	4.0	6.0
pH		7.91	7.51	7.88	7.65	7.81
Conductivity	µS.cm^{-1}	1163	869	979	811	23800
Dissolved substances	mg.l^{-1}	580	532	707	465	1970
Free chlorine *	mg.l^{-1}	0.04	0.08	0.20	0.08	0.14
Total chlorine	mg.l^{-1}	0.06	0.53	0.42	>6.00	2.09
I	µg.l^{-1}	130	1577	86	1390	183
Hg	µg.l^{-1}	1.48	0.50	<0.30	0.52	0.47
Ag	µg.l^{-1}	0.58	0.09	0.04	0.15	0.92
Gd	µg.l^{-1}	1.55	2.75	0.15	5.01	1.51
Pt	µg.l^{-1}	0.17	0.05	0.45	0.11	0.13
Pb	µg.l^{-1}	0.35	0.58	0.19	0.70	0.42
Al	mg.l^{-1}	3.71	1.82	1.33	2.00	2.37

* in-the-field measurements-average values of five partial samples.

Tab. 3. Characteristics of bioassays used in the study.

Method	Organism	Standard	Sample preparation	Biological parameter and exposure time	Endpoint
Algal growth inhibition test	*Desmodesmus subspicatus*	ISO 8692	non filtered	growth inhibition 72 h	EC_{50} [%] / TU
Luminescent bacteria test	*Vibrio fischeri*	ISO 11348-2	non filtered	bioluminescence inhibition 15 min, 30 min	EC_{50} [%] / TU
Crustacean immobilization test	*Daphnia magna*	ISO 6341	non filtered	mobility inhibition 24 h, 48 h	EC_{50} [%] / TU
Allium cepa assay	*Allium cepa*	----	non filtered	inhibition of bulb root elongation 72 h	EC_{50} [%] / TU
Bacterial reverse mutation test (Ames agar plate test)	*Salmonella typhimurium*	OECD TG 471	filtered	number of revertants 72 h	qualitative determination
Comet assay single-cell gel electrophoresis	NIH 3T3 mouse fibroblasts	----	filtered	% DNA in tail 24 h	qualitative determination
YES/YAS-Yeast based reporter gene assays	*Saccharomyces cerevisiae*	in compliance with Draft ISO 190 40	non filtered concentrated	β-gal expression 48 h	qualitative determination

Solution of 2% NaCl was used as negative control. Bioluminescence was recorded after 15 min and 30 min of exposure to eight increasing sample concentrations. Every concentration was measured in two replicates using luminometer Sirius (Berthold Detection Systems) and thermostat LUMIStherm (HACH LANGE). EC_{50} which causes 50% inhibition of the bacteria light emission with respect to the negative control was calculated for each sample.

Crustacean immobilization test

The test was performed using less than 24 h old specimens of *Daphnia magna* Straus. Neonates of at least third generation originated from the laboratory culture. The sensitivity of crustaceans was controlled by regular tests with $K_2Cr_2O_7$ and test medium was used as a negative control in all runs. Six sample concentrations were used per test. Five organisms in four replicates (for a total of twenty organisms) were exposed to each concentration for 48 h with 16:8 light:dark cycle without feeding. Temperature during the test was maintained at 20±2 °C. For the test validity, the oxygen concentration had to be ≥2 $mg.l^{-1}$. The percentage of immobilization in the control group had to be ≤10%. *Daphnia* immobility was the test endpoint and the EC_{50} after 24 h and 48 h exposure was determined.

Allium cepa assay

The experiment was performed using small onion (*Allium cepa* L.) bulbs of the size 16–18 mm, free from any chemical treatment. The sensitivity of the test onion bulbs was controlled by 1% MMS (methylmethansulfonate) as positive control. The bulbs were exposed for 72 h to undiluted samples and negative control (tap water) in six replicates. The test temperature was maintained at 22±2 °C with protection against direct sunlight. At the end of the experiment the root length was measured with a precision of 1 mm and inhibition of root elongation relative to negative control was calculated.

Data evaluation of ecotoxicity tests

The EC_{50} values calculated for each species were transformed to toxic units (TU) using the formula:

$$TU=(1/EC_{50}[\%])\times 100$$

Tab. 4. Toxicity classification system by Persoone et al. 2003.

Toxic unit	Toxicity class	Toxicity
TU < 0.4	I	non toxic
0.4 ≤ TU < 1.0	II	low toxic
1.0 ≤ TU < 10.0	III	toxic
10.0 ≤ TU < 100.0	IV	very toxic
TU > 100	V	extremely toxic

High TU value indicated high toxic effect on the organism.

Many authors applied TU to evaluate ecotoxicity of industrial, urban and hospital WW (*e.g.* Manusadžianas *et al.* 2002; Zgórska *et al.* 2011; Vasquez & Fatta-Kassinos 2013; Maselli *et al.* 2015; Hamjinda *et al. 2015;* Laquaz *et al.* 2017). Our samples were ranked by toxicity classification system (Table 4) based on the calculation of TU as suggested by Persoone *et al.* (2003). The samples were classified into five classes on the basis of the highest TU value shown by one of the organisms applied.

Statistical analysis of ecotoxicity tests

To evaluate the results of the pilot study of the weekly and seasonal variability of ecotoxicity, three-way analysis of variance (ANOVA) was used to assess the difference between factors (method, day and month). When statistically significant effects were identified, comparisons of means were further examined by Bonferroni correction to ascertain which specific means differed. Two-way ANOVA was used to assess the differences between individual levels of day and month factors for all ecotoxicity methods. Values of $p<0.05$ were taken as statistically significant. All statistical analyses were performed using SPSS software package for Windows (version 23).

Bacterial Reverse Mutation Test (Ames test)

Two tester strains to detect point mutations, which involve base pair substitution (TA100) and frameshift mutations (TA98), were used in the study. A cofactor-supplemented post-mitochondrial fraction (S9) prepared from the liver of rodents (Wistar rat) treated with enzyme-inducing agent (polychlorinated biphenyl Delor) was used for modeling of mammalian metabolic activation.

In each run, relevant positive and negative controls were included, both with and without metabolic activation. The samples and controls were tested in triplicates. Briefly, the mixture of 2 ml TOP agar with His/Bio solution, 100 µl of bacterial culture, 100 µl of the test sample, 500 µl S9 mix (S9+) or 500 µl PBS (S9-) was added to sterile test tubes maintained in a dry box (cca 37 °C). The contents of each tube was mixed and poured over the surface of minimal agar plates. The overlay agar was allowed to solidify and then the plates were placed upside down into the incubator (37 °C) for 72 hours of incubation. The number of revertant colonies was counted by automatic computer of bacterial colonies Schuett colony Quant HD (Schuett Biotec) for the tested samples and compared to the number of spontaneous revertant colonies on negative control plates. The dose dependence of the mutagenic effect was expressed as Mutagenic Index = MI. Generally, the sample eliciting at least twofold increase of revertants compared to the control revertants is considered to be mutagenic.

Comet assay

DNA damage was tested using NIH 3T3 cells (mouse embryonic fibroblasts) according to the protocol described in previous studies (Tomankova *et al.* 2011; Manisova *et al.* 2015). Briefly, the cells were incubated in DMEM with the tested water samples in ratio 1:1 for a period of 24 hours. Then the cells were trypsinized, centrifuged and the cell pellet was dispersed in PBS and vortexed. 1% low melting point agarose was added to this solution and this suspension was placed on the solidified agarose on the precoated microscope slides and covered by coverslips. After the agarose had solidified, the coverslips were immersed in lysis buffer at 4 °C for a period of 60 min. After lysis the slides were placed in an electrophoretic tank and dipped for 40 min in a cool electrophoretic solution. The electrophoresis was run at 350 mA and 0.8 V cm^{-1} for 20 min. Following completion of the electrophoretic separation the slides were carefully rinsed twice for 10 min with a neutralisation buffer at 4 °C, stained by means of SYBR Green and manually scored using fluorescence microscope with CCD camera CometScore 1.5 software. 80 cells from each sample were randomly chosen and median values of the amount of the Olive moment, DNA in tail, and DNA in the head, which is directly proportional to the intact DNA, were evaluated as follows:

Olive Moment = (tail mean – head mean) × % of DNA in the tail
Head % DNA = $100 \times (I_h / I_c)$
I_h = *total intensity of the head*
I_c = *total intensity of the comet (head and tail together)*
Tail % DNA = 100 – Head % DNA.

Statistical analysis of Comet assay

Mann-Whitney U-test with Bonferroni correction was performed for the statistical analysis of the % DNA in head.

YES/YAS microplate assay

Microplate assay (XenoScreen YES/YAS, Xenometrix®, Switzerland), based on genetically modified *Saccharomyces cerevisiae* strains, expressing human estrogen and androgen receptors, was performed according to the provided standard operating procedure, using the supplied standardized material and chemicals in order to study agonistic activity of WW samples to human estrogen and androgen receptors. WW samples were 250x concentrated in compliance with Draft ISO/DIS 19040-1:2017(E) standard, dissolved in DMSO and applied to the yeast culture for 48 h. The optical density of the red product resulting from conversion of the yellow substrate after secretion of β-galactosidase, indicating the endocrine activity of the tested substance, was measured on Biotec Eon™ High Performance Microplate Spectrophotometer.

RESULTS AND DISCUSSION

Weekly dynamics and seasonal variability of wastewater ecotoxicity

In the pilot study, focused on investigation of the weekly variation of sewage ecotoxicity, the samples of treated WW from one large hospital (H1) have been analysed. In addition to comparison of individual working days, two different seasons (spring vs. autumn) were compared. In their review Orias and Perrodin (2013) recommended to assess the HWW ecotoxicity during a day, a week and a year in order to gain more wealth of information.

For the purpose of our work three different species have been used: *D.subspicatus*, *V.fischeri* and *A.cepa.* The results are shown in Figure 1. No statistically significant differences were found in separate working days (from Monday to Friday) for *D.subspicatus* (p=0.601), TU values lay in the range of 2.46–3.58 in November and 4.14–5.40 in May. For *V.fischeri* (15 min and 30 min) TU values showed considerable fluctuation on Mondays that was probably related to weekend hospital activities. Therefore, subsequently only values from Tuesday through Friday were compared. The results showed no significant differences for *V.fischeri* 15 min (p=0.337) with TU values between 3.15–3.76 in November and 3.47–4.86 in May and for *V.fischeri* 30 min (p=0.359) with TU values between 2.27–2.94 in November and 4.16–4.73 in May. These findings differed from results of Magdaleno *et al.* (2014a), who discovered big differences of raw HWW samples (from stimulating effect to growth inhibition 44.5%) during a week using the green algae *P.subcapitata*.

Regarding seasonal variation, in our study higher toxicity values were recorded in May compared to November (from Tuesday to Friday). The statistical analysis of results obtained in these two months confirmed significant difference for *D.subspicatus* (p=0.039) and *V.fischeri* 30 min (p=0.002) while no significant difference was found for *V.fischeri* 15 min (p=0.085). Seasonal differences could be caused by a wide range of specific hospital therapeutic activities and their variability over time. Similar results were seen in other studies, e.g. Coutu *et al.* (2013) discovered high seasonal fluctuation in ambulatory and hospital consumption of antibiotics. Laquaz *et al.* (2017) investigated HWW and UWW and they found high variability of ecotoxicity for *P.subcapitata* during the year.They supposed that seasonal differences were due to e.g. seasonal pathologies or disinfection campaigns which may have led to the release of high quantities of toxic compounds. Magdaleno *et al.* (2014a) found that growth inhibition of *P.subcapitata* varied widely during the period from April to September. These results were also confirmed by Vasquez and Fatta-Kassinos (2013) in their study in which higher toxicity of treated UWW for *P.subcapitata, D.magna* and *V.fischeri* was observed in spring and summer in comparison with autumn and

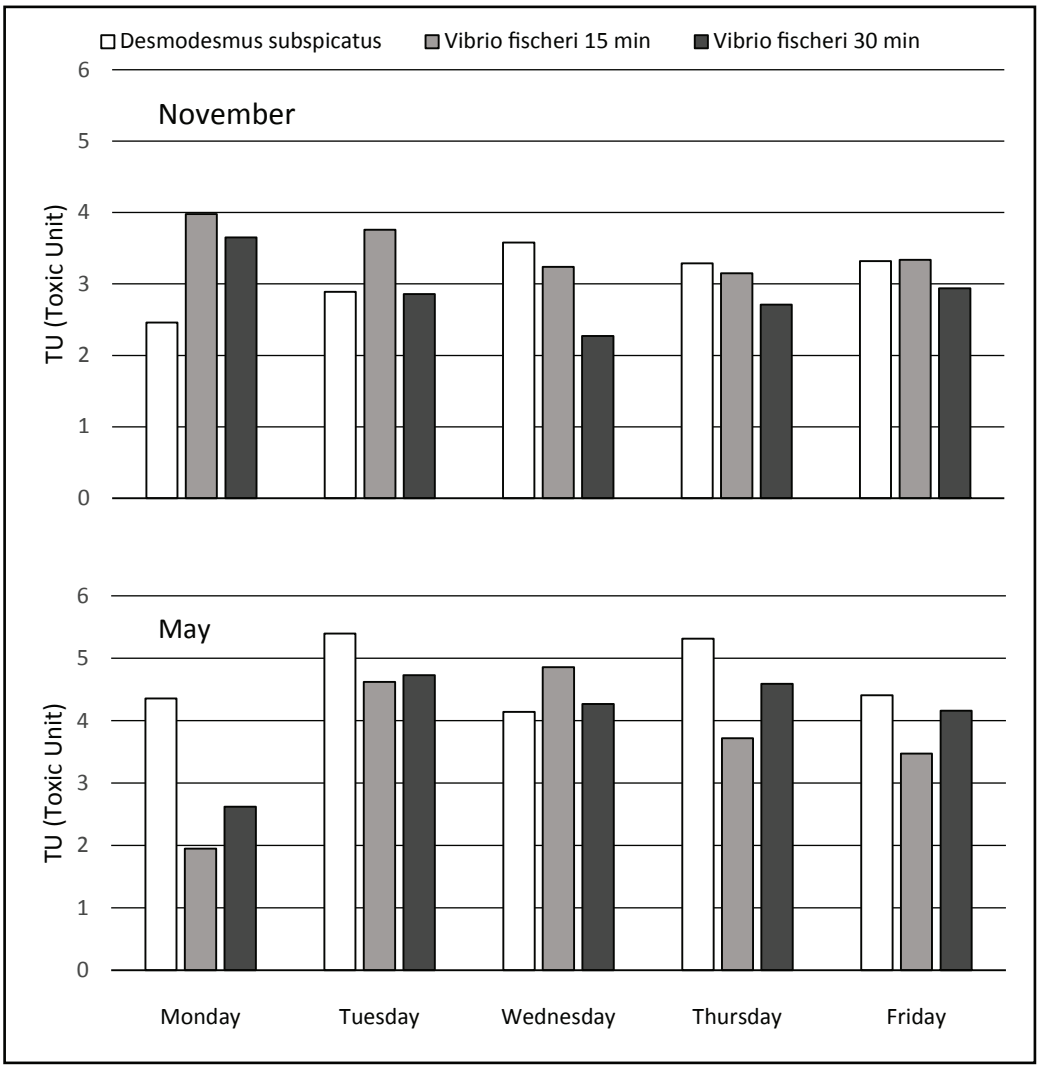

Fig. 1. Weekly dynamics of wastewater ecotoxicity from hospital H1 – comparison between November and May.

winter, potentially due to decreased dilution during the summer dry period or different composition of the WW.

In our study, *A.cepa* was less sensitive to all tested samples compared to *D.subspicatus* and *V.fischeri*. Although the data showed toxic responses of *A.cepa* with values of inhibition of bulbs roots elongation from 7.2% to 29.7% after exposure to undiluted samples, they did not achieve 50% inhibition and thus it was not possible to calculate EC_{50}. Therefore, TU was described as 0. Similar results related to sensitivity of this organism were seen in the study by Firbas and Amon (2013) who reported, that treated UWW induced equal root lenghts of *A.cepa* bulbs as the negative control.

Comparison of wastewater toxicity from different hospitals

WW samples from five hospitals with different dimensions were studied. In order to provide true information of their quality and evaluate their individual impact on the receiving UWW or directly on the water flow, a detailed investigation of ecotoxicity, genotoxicity and reprotoxicity was performed using seven conventional and alternative methods.

Ecotoxicity was determined by the use of a bioassay battery consisting of four test organisms: *D.subspicatus, V.fischeri, D.magna* and *A.cepa.* Table 5 presents the summary results for all the tested species. In order to describe and compare their ecotoxicological potential, hospitals have been classified by the toxicity classification system described in Table 4. The obtained data demonstrated different levels of ecotoxicity of samples from individual hospitals. The TU values indicated that two hospitals belong to toxicity class III as toxic and three hospitals belong to toxicity class I as non toxic.

In the study published by Hamjinda *et al.* (2015), which examined treated HWW, TU values calculated for freshwater algae *Scenedesmus quadricauda* lay in the range of 1.15–2.18 and for *Chlorella vulgaris* in the range of 1.94–2.42. Zgórska *et al.* (2011) investigated

HWW before treatment. Based on the test results estimated for *P.subcapitata* (TU = 5.32), *D.magna* (TU = 4.81), *V.fischeri* (TU = 2.16) and crustaceans *Thamnocephalus platyurus* (TU = 4.42) and *Artemia salina* (TU = 1.67), they classified their samples in toxicity class III as toxic. Numerous studies indicated that HWW are characterized by higher ecotoxicological potential than UWW. This assertion was confirmed by Laquaz *et al.* (2017). For *D.magna* 24 h the TU of UWW samples reached a maximum of 1.9. For *P.subcapitata* the TU values of raw HWW samples calculated using EC_{20} were 1.6–6.8 and TU values of UWW were up to 3.3. 21 industrial and urban WW samples before and after treatment were analysed by Manusadžianas *et* al. (2002) using six test species. Two samples were characterized as non toxic (class I), six as slightly toxic (class II), nine as toxic (class III), four as very toxic (class IV) and none of them as extremely toxic (class V).

When we drew a comparison between our tested organisms, we could observe numerous differences. As can be seen in Table 5, the rank of species´ reactions levels was for samples of hospital H1: *D.subspicatus* > *V.fischeri* > *D.magna* > *A.cepa* and of hospital H4: *D.magna* > *D.subspicatus* > *V.fischeri* > *A.cepa*. In summary, the samples of H1 and H4 were highly toxic to *D.subspicatus*, *V.fischeri* and *D.magna*, whereas *A.cepa* was affected much less with the values of inhibition of root elongation 26.3% (H1) and 19.8% (H4).

The samples of H2, H3 and H5 had low toxic effect on *D.subspicatus*, *V.fischeri* and *A.cepa*. Immobilization of *D.magna* was not observed at all. Inhibition values of these undiluted samples did not exceed 50%, therefore it was impossible to calculate EC_{50} and TU was represented as 0. The *D.subspicatus* and *A.cepa* tests showed both inhibition and stimulation of growth with the values from –12.7% to 2.7% (*D.subspicatus*) and from –9.4% to 27.9% (*A.cepa*). For *V.fischeri* EC_{20} was calculated and the values lay between 30.3%–92.0% (15 min) and 25.1%–82.1% (30 min).

Although we have found certain differences among species in sensitivity to the HWW samples, TU values of *D.subspicatus*, *V.fischeri* and *D.magna* were at the same level of classification (the same toxicity class) in all cases of samples from the selected hospitals. It implies that these three species are equally suitable for the estimation of HWW ecotoxicological potential and this bioassays battery could be used for routine HWW testing. These conclusions are in agreement with Zgórska *et al.* (2011). On the other hand, according to our findings it can be assumed that *A.cepa* test that is based only on measuring of onion bulbs roots is not sufficiently sensitive for ecotoxicity assessment of HWW but it could be suitable for detection of genotoxicity as was demonstrated in numerous studies (*e.g.* Herrero *et al.* 2012; Kerm *et al.* 2014; Magdaleno *et al.* 2014a). The test *Allium cepa* is validated by the International Program on Chemical Safety (IPCS) as an efficient test for analysis and in situ monitoring of the genotoxicity of environmental substances (Bagatini *et al.* 2009). We may consider such analysis in next studies, however, in the present study the Ames test and Comet assay for genotoxicity were employed.

The outcome of genotoxicity and reprotoxicity assays is summarized in Table 6. Genotoxicity of the

Tab. 5. Toxicity classification based on ecotoxicity tests results.

Organism	H1			H2			H3			H4			H5		
	EC_{50} [%]	TU	toxicity class	EC_{50} [%]	TU	toxicity class	EC_{50} [%]	TU	toxicity class	EC_{50} [%]	TU	toxicity class	EC_{50} [%]	TU	toxicity class
Desmodesmus subspicatus	25.3	3.95	III toxic	ND	0	I non toxic	ND	0	I non toxic	35.3	2.83	III toxic	ND	0	I non toxic
Vibrio fischeri 15 min	42.6	2.35	III toxic	ND	0	I non toxic	ND	0	I non toxic	43.1	2.32	III toxic	ND	0	I non toxic
Vibrio fischeri 30 min	28.9	3.46	III toxic	ND	0	I non toxic	ND	0	I non toxic	41.3	2.42	III toxic	ND	0	I non toxic
Daphnia magna 24 h	67.6	1.48	III toxic	ND	0	I non toxic	ND	0	I non toxic	39.3	2.54	III toxic	ND	0	I non toxic
Daphnia magna 48 h	61.3	1.63	III toxic	ND	0	I non toxic	ND	0	I non toxic	24.3	4.12	III toxic	ND	0	I non toxic
Allium cepa	ND	0	I non toxic	ND	0	I non toxic	ND	0	I non toxic	ND	0	I non toxic	ND	0	I non toxic

ND (not detected): < 50% inhibition in the undiluted sample, TU (toxic unit)=[1/EC_{50} in %]×100

Tab. 6. Genotoxicity and reprotoxicity tests results.

Method	H1	H2	H3	H4	H5
Ames test	negative	negative	negative	negative	negative
Comet assay	negative	negative	negative	negative	negative
YES	positive	positive	negative	positive	negative
YAS	positive	negative	negative	positive	negative

tested WW samples has not been confirmed by the plate Ames test, where filtered samples were tested on two strains with and without S9 activation. The number of revertants elicited by the test samples never achieved a twofold increase in numbers compared to the negative controls. Results of the Comet assay showed no significant differences in the amount of fragmented DNA in samples H1 and H4 compared to control cells. Significant differences were observed in samples H2, H3 and H5, however, the average difference higher than 5% in the amount of fragmented DNA was not observed in either of these samples, suggesting minimal genotoxic effect. Eukaryotic cells have a DNA repair mechanism that makes it possible to repair damaged DNA (Chu 2014). Based on the results of the viability tests, it can be assumed that the detected amount of the fragmented DNA did not affect the viability of NIH 3T3 cells.

A literary review showed, that most of the untreated HWW samples had a mutagenic effect (Vlkova *et al.* 2016). In contrast, the genotoxicity of treated WW samples was found significantly reduced (Sharma *et al.* 2015, Gupta *et al.* 2009). With regard to the method principles, the Ames test and Comet assay require pre-treatment of the samples and/or sterilization. The simplest and most commonly used preparation technique is filtration through a filter (e.g. cellulose nitrate, acetate cellulose filter) with a pore size of 0.45 μm (Jolibois & Guerbet 2006; Hartmann *et al.* 1999) or 0.22 μm (Paz *et al.,* 2006, Magdaleno *et al.,* 2014a). However, White *et al.* (1996) reported that during filtration some chemical substances may be captured on the filters, thus causing a loss of genotoxicity potential, and the study of Ferk *et al.* (2009) confirmed the significant effect of membrane filtration on the overall genotoxic effect, the decrease was in the range of 62 % – 77 %. In the YES/YAS assay, WW samples (250x concentrated stock samples), in 4 final concentrations (1% – 0.325% – 0.1% – 0.0325%) exhibited agonistic activity to human estrogen receptor, showing a concentration-dependent curve in two highest non-cytotoxic concentrations (1%, 0.325%), in case of samples H1, H2, and H4. Agonistic activity to human androgen receptor was confirmed in one non-cytotoxic concentration (1%), in case of samples H1 and H4. The advantage of methods based on yeasts is the absence of complex mechanisms regulating the expression of the reporter gene. Yeast based methods are not influenced by cross-cellular signaling interferences, and thus detect only a specific interaction with the receptor and are effective for screening and hazard identification.

Toxicity differences of WW from different hospitals may be caused by a number of factors. As complex mixtures of many substances, HWW are generated intermittently by different hospital services (e.g. medical care, diagnostics, disinfection, cleaning, laboratory and research activities). Therefore, HWW quality is influenced by the type and specialization of the hospital (e.g. general, oncologic, pediatric), number of inpatients, type and number of wards, season or day of the week, hospital location and also country. Orias & Perrodin (2014) recommended to continue determining the cumulative ecotoxic effects of the HWW compounds corresponding to different hospitals, size of the hospital and different locations. Hamjinda *et al.* (2015) showed a good correlation between antibiotic concentrations in HWW and amount of usage. According to Santos *et al.* (2013), the impact of hospitals to the input of PHs in UWW was in concordance with their dimensions. The contribution of great hospitals was considerably higher in comparison with smaller facilities. The variability of pharmaceutical concentrations between the WW from four hospitals were related to pharmaceutical consumption, which was connected with the number of beds, number and type of wards and units. These conclusions are in agreement with other similar studies (e.g. Al Aukidy *et al.* 2014; Verlicchi *et al.* 2012).

The quality of treatment processes is one of the crucial points which affect HWW composition before discharge into the sewage system or water flow. Although HWW is often treated before discharge into the sewage system or directly into the water flow, numerous studies confirmed a lot of residues of pharmaceutical products in HWW after treatment processes either because of deficiencies of the treatment or resistance of certain substances to the applied process. Hamjinda *et al.* (2015) investigated HWW characteristics focusing on antibiotic contamination in three hospitals, revealing the removal efficiency of different treatment processes from 0% to 99% depending on the type of drug. Similar results were reported by Santos *et al.* (2013), who discovered that removal efficiency of WWTP may vary from over 90% for PHs as acetaminophen and ibuprofen to absolutely no removal for β-blockers and salbutamol. Ketamine and its metabolites with a high ecotoxic potential to aquatic organisms cannot be removed or degraded by conventional WWTPs (Li *et al.* 2017). Wiest *et al.* (2017) found 11 of 13 monitored PHs in HWW and UWW after treatment with median concentrations from 19 ng/l to 810 ng/l and confirmed that antibiotic concentrations remained higher in HWW than in UWW. Chonova *et al.* (2015) evaluated efficiency of biological treatment with conventional activated sludge and discovered relatively high concentrations of antibiotics and analgesics in HWW after treatment, despite good removal during treatment

(antibiotics 95.1%, analgesics 99.9%), because of their high initial concentrations. The membrane bioreactor had emerged as an efficient compact technology for WW treatment. The results of the study of Albasi *et al.* (2009) proved that WW treatment using membrane reactors provides a suitable process for lowering anticancer drug cyclophosphamide concentrations before discharge into the aqueous environment. Despite this clear benefit of membrane bioreactors, removal is only partially achieved and a tertiary treatment is necessary for the complete elimination of cytostatic agents compounds. Other studies (Chiarello *et al.* 2016) showed that the membrane bioreactor also was effective in the removal of enalapril, tetracycline and paracetamol up to 94 %. The elimination efficiency of carbamazepine is very low due to the specific characteristics of the molecule such as resistance to degradation and low capacity to attach to the sludge (Ternes *et al.* 2007; Zhang *et al.* 2008).

Another important aspect that may affect living organisms is disinfection as the final stage of the treatment process performed with the aim to prevent the spread of pathogenic microorganisms. The most widely used methods of HWW treatment are disinfection with chlorine, sodium hypochlorite, chlorine dioxide, ultraviolet radiation or ozonization (Drinan & Spellman 2012; Chen *et al.* 2014). During the disinfection process, undesirable by-products such as trihalomethanes are formed by reaction of disinfectants with natural organic matters (Richardson *et al.* 2007). Sodium hypochlorite (used in H1, H2 and H3 hospitals in our study) contains about 5–20% of free chlorine. Its toxicity is lower than pure chlorine, but it can not be neglected, especially because of the amount of trihalomethanes produced. The advantages of sodium hypochlorite furthermore include greater stability, trivial handling and lower operating costs. However, it is necessary to mention its negatives, which include higher energy consumption, strong corrosivity and overall lower disinfection efficiency (Chen *et al.* 2014). Gaseous chlorine (used in hospitals H4 and H5) is a very powerful oxidizing agent and has been commonly used to disinfect HWW. The free chlorine content in Cl_2 is close to 100% (it also contains impurities), so its sterilization capability is high (Chen *et al.* 2014). According to the findings of Emmanuel *et al.* (2004) or Park *et al.* (2016) organohalogen compounds are ecotoxic and genotoxic for aquatic organisms and are considered as persistant environmental contaminants.

CONCLUSIONS

HWW is a complex mixture of many diverse compounds that have proved toxic effects on living organisms. The main problem is the insufficient knowledge of the quality of treated hospital effluent discharged to the sewage system or surface water. In our study we wanted to highlight the necessity of solving this question within the Czech Republic. For our investigation we selected seven biological methods, conventional and alternative, with the intention to compare their sensitivity and suitability for toxicological examination of HWW.

The WW ecotoxicity during one week showed no differences in separate working days, however, higher toxicity values were recorded in May compared to November. Our work demonstrated considerably different levels of toxicity of treated WW between different hospitals. The samples from two of the five hospitals have been assessed as toxic, the others as non toxic based on the evaluation by the toxicity classification system. We found that the battery of three organisms consisting of *D.subspicatus, D.magna and V.fischeri* may be appropriate for routine testing of ecotoxicological potential of HWW.

Variable sensitivity of individual bioassays for tested WW samples was determined. According to our results, *A.cepa* test based on testing the onion bulb root elongation is not sufficiently sensitive and effective for detection of HWW ecotoxicity as it has not identified any differences between the samples and classified all of them as nontoxic.

Genotoxicity has not been confirmed neither by Ames test, nor Comet assay in any sample. It can be assumed that the results of Ames test and Comet assay may be influenced by sample sterilization (by filtration) which might have caused a loss of genotoxic and reprotoxic activity as certain chemicals may be captured on the filters. The study will continue with optimization of sample preparation.

Estrogenic and androgenic potential of certain WW samples has been detected. WW is a heterogenous mixture of natural and synthetic residues and unknown hormonally active micropollutants, certain of which may be persistent or bioaccumulative. *In vitro* methods are thus effective for screening of WW treatment effectivity and for detection of potential hazard of bioaccumulative effects of endocrine disruptors from chronic exposure to low doses of these micropollutants from the aquatic environment.

Our study signalized insufficiency in the hospital sewage treatment processes. A more extensive study including proposal for improvement of HWW treatment within the Czech Republic may be recommended with the aim to decrease the discharge of toxic chemicals into the sewage system and thus to contribute to the improvement of the environment.

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