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Characterization and toxicity of hospital wastewaters in Turkey

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Abstract The aim of the study was to present first preliminary characterization of Turkish hospital wastewaters, their environmental risk, and a method for toxicity assessment. The hospital wastewater samples were collected from two of the largest medical faculty hospitals and a training and research hospital in Istanbul, Turkey. The samples from the selected hospitals were taken as grab samples on March 2014. Overall, 55 substances including pharmaceuticals and their metabolites, pesticides, and corrosion inhibitors were analyzed in all hospital wastewaters. Analysis of toxicity and the antibiotic resistance bacteria were investigated in addition to the chemical analysis in the wastewater of one hospital. Hazard quotients (HQs) and toxic units (TUs) were calculated as basis of the environmental risk assessment. Fourteen pharmaceuticals in hospital wastewater (HWW) were classified as "high risk" with HQ > 10. HQ_{HWW} values higher than 100 were determined for five antibiotics and one analgesic, namely, ofloxacin, clarithromycin, ciprofloxacin, sulfapyridine, trimethoprim, and diclofenac. Ofloxacin with an HQ_{HWW} of 9090 was observed to be the most hazardous compound. HQ and TU values of the wastewater treatment plant (WWTP) effluent dropped significantly due to dilution in the sewer. Further elimination by biological degradation or adsorption was observed only in some cases. However, the decreased $HQ_{WWTPeffluent}$ values do not the change environmental load significantly. Therefore, advanced treatment processes should be applied to remove the persistent compounds. In combination with the results on antibiotic resistance, we would prefer on-site treatment of hospital wastewater. Toxicological assessment was performed using cytotoxic and mutagenic screening tests. The results of the Ames assay showed that the native hospital wastewaters had strongly mutagenic activity with a ≤10-fold increase relative to negative controls. The mutagenic potentials of the samples were generally concentration and metabolic activation dependent. Multiple antibiotic resistances were demonstrated with the tested isolates to ciprofloxacin, trimethoprim, and ceftazidime. This study demonstrates that the hospital wastewaters in Istanbul exhibit strong environmental and toxicological risks, as well as high multiple drug resistance to commonly used antibiotics.

Keywords Hospital wastewater · Environmental risk assessment · Pharmaceuticals · Toxicity · Antibiotic-resistant bacteria

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Abbreviations

CLSI	Clinical and Laboratory Standards Institute
df	Dilution factor
DMEM	Dulbecco's modified Eagle medium
ECOSAR	Ecological Structure Activity Relationships
FBS	Fetal bovine serum
HWW	Hospital wastewater
HQ	Hazard quotient
IC_{50}	Half-maximal inhibitory concentration
LDH	Lactate dehydrogenase
MEC	Measured environmental concentration
MFH	Medical faculty hospital
MHA	Mueller-Hinton agar
MR	Mutagenicity ratio
NR	Neutral red
OD	Optical density
PAC	Powdered activated carbon
PEC	Predicted environmental concentration
PNEC	Predictive no effect concentration
SDA	Sabouraud dextrose agar medium
SD	Standard deviation
TRH	Training and research hospital
TSA	Trypticase soy agar medium
TU	Toxic unit
QSAR	Quantitative structure-activity relation
WWTP	Wastewater treatment plant

Introduction

Water pollution by micropollutants resulting from predominantly human activities (industry, agriculture, and urbanization) has become one of the most critical problems during recent decades. This pollution has especially been of increasing concern regarding the occurrence and toxicological risks of pharmaceuticals and other compounds in addition to pathogenic microorganisms in wastewater because of laboratory activity or medicine excretion into hospital wastewaters (Kümmerer 2001; Rahman et al. 2009; Tacconelli et al. 2009; Al Aukidy et al. 2014; Yang et al. 2015). Even if pharmaceuticals like analgesics, antidepressants, antiinflammatories, antibiotics, β-blockers, lipid regulators, hormones, antineoplastics and their metabolites, iodinated contrast agents, disinfectants, and corrosion inhibitors are commonly found in low concentrations (ng/L to μg/L range), they might cause adverse effects (Fent et al. 2006; Verlicchi et al. 2012; Verlicchi et al. 2015; Orias and Perrodin 2013; Oliveria et al. 2015). Indeed, pharmaceuticals can be excreted both partly metabolized and unchanged by conjugation to polar molecules, which are then easily released into the aquatic environment with high mobility (Halling-Sørensen et al. 1998; Heberer 2002). Various researchers have classified some pharmaceuticals according to their consumption, excretion rates, and treatability in water and wastewater treatment plants and ecological and health effects (Halling-Sørensen et al. 1998; Hernando et al. 2006; Kumar et al. 2010; Sui et al. 2012). Due to discharge of treated or untreated sewage/hospital wastewater, the occurrence of pharmaceuticals in surface waters is expected. Also, pharmaceuticals have been detected in coastal waters and tissue of aquatic species (Gaw et al. 2014; USGS 2016). The potential effects of the pharmaceuticals on humans by exposure of low doses via swimming or directly by drinking water are still unclear. The pharmaceutical concentrations in water are too low to cause an acute toxic effect. However, potential long-term effects (chronic toxicity) are still less known (Borecka et al. 2015). There are concerns due to the following two reasons: the potential combined effects of mixtures containing a wide variety of pharmaceuticals and the ability of pharmaceuticals to perform a biological effect. Bioaccumulation and undesired effects in the aquatic systems or non-target organisms are also expected (Halling-Sørensen et al. 1998; Daughton and Ternes 1999; Gaw et al. 2014). Ecotoxicological impacts on aquatic life and human exposure to pharmaceuticals via consumption of seafood are worth considering. There are limited data for the effects of the pharmaceuticals to marine organisms. Many types of alterations were found in structure and function. For example, endocrine-disrupting compounds impact growth and reproduction in fish (Gaw et al. 2014). Also, toxic effects of some pharmaceuticals (simvastatin and clofibric acid) on a marine phytoplankton species were reported by DeLorenzo and Fleming (2008). Consumption of seafood is one of the means of human exposure to pharmaceuticals linked to hospital wastewaters through sea discharge. The formation of antibiotic resistance is a serious global health problem. Antibiotic resistance has complicated the ability to treat common bacterial infections resulting in prolonged illness, disability, and death (WHO 2016). The occurrence and spread of antibiotic resistance is also an expected and important result of the wastewater discharge to the sea. Release of

hospital wastewaters with antimicrobial/antibiotic residuals into the sea may cause the development of resistant strains (Barraud et al. 2013; Harris et al. 2014; Berendonk et al. 2015; Alexander et al. 2016). Antibiotic resistance in marine bacteria, fish, marine mammals, and seabirds has been reported (Rose et al. 2009; Cabello et al. 2013). The aforementioned health effects on marine organisms and humans are remarkable.

The risks of the long-term toxicity of hospital wastewaters are not well known (Sui et al. 2012). In most countries, hospital effluents are discharged directly into public sewers and are introduced into municipal wastewater networks where they are treated with other effluents (Kovalova et al. 2012). There are currently no limits on allowed discharges, and hospitals are not obligated to specially treat their wastewaters. Only in Denmark, the discharge limits of hospital wastewater to sewer are regulated by Danish authorities. Danish municipalities finalized a guideline for municipal regulation of hospitals in December 2013. Hospital wastewater is regulated as industrial wastewater because of its high micropollutant content. Because of the complexity of the hospital wastewaters, the Danish municipalities formed a task group that finally composed a guideline. Danish municipalities have collectively set the maximum acceptable concentration for 36 pharmaceuticals in hospital wastewaters. Additionally, hospitals are ranked as major, medium, or minor sources regarding the amount of hazardous pharmaceuticals in their wastewater. In the Danish municipal guideline, the hospitals are ranked based on several criteria, pharmaceutical consumptions, exceedance of guiding limits, and antibiotic contribution to wastewater treatment plant (WWTP) (Grundfos BioBooster A/S Report 2016; KL 2013; KL 2013a; DHI 2011). To date, many waste management systems and approaches have been reported for appropriate and safe handling of hospital wastewaters (Verlicchi et al. 2015). In the case of waste management, wastewater characterization and toxicological risk assessment are two crucial approaches that should be established in all countries (Steher-Hartmann et al. 1999; Kümmerer 2001; Jolibois et al. 2003; Sharma et al. 2015). It is necessary to characterize ecotoxic potential of the hospital wastewaters as well as quantitative and qualitative characterizations. Escher et al. (2011) investigated the ecotoxicological potential of 100 pharmaceuticals in the hospital wastewaters using the quantitative structure-activity relation (QSAR) approach. QSAR modeling is based on the assumption that the physical, chemical, or biological activity of a compound is related to its molecular structure properties. Orias and Perrodin (2013) also used Ecological Structure Activity Relationships (ECOSAR) data, which help estimate the aquatic toxicity in addition to QSAR data. To prioritize the pharmaceuticals of hospital wastewaters according to their ecotoxicity, available ecotoxicological data (predicted no effect concentration (PNEC)) were used to calculate a hazard quotient (HQ) (Orias and Perrodin 2014). Mendoza et al. (2015) additionally considered the calculation of toxic units (TUs). Whereas HQ represents the environmental hazard of individual compounds, TU is used to derive the effect of a mixture. As known, pharmaceuticals are present as a cocktail of biologically active substances in the environment. The potential toxic effects of wastewater samples could possibly differ from the sum of the effects of individual components. The hospital wastewaters of Istanbul are discharged into the Sea of Marmara with pre-treatment or municipal biological wastewater treatment. The Bosphorus–Sea of Marmara strait connects it to the Black Sea and the Dardanelles strait to the Aegean. The Sea of Marmara is defined as a Geographical Sub-Area 28. Food and Agricultural Organization (FAO) has also defined the Sea of Marmara as Division 4.1 of Black Sea Sub-Area 37.4 in Area 37, which refers to the Mediterranean and Black as one of the world's major fishing areas (Devotes-Project-EU 2016). There are no studies about the occurrence of pharmaceuticals in Marmara Sea or in the marine organisms living in Marmara Sea. The studies on Marmara Sea are focused on the occurrence of heavy metals in water, sediment, and fishes (Türkmen et al. 2008; Taşkın et al. 2011; Otansev et al. 2016); organochlorines in fishes (Coelhan et al. 2006); and polycyclic aromatic hydrocarbons in water, sediment, and mussels (Taşkın et al. 2011; Balcioglu 2016). So, monitoring the occurrence, fate, and risks of the pharmaceuticals in hospital wastewaters of Istanbul is important for ecology, fishery, and human health.

The aims of this study were to characterize the wastewaters obtained from three different Turkish hospitals in Istanbul and to evaluate ecotoxic potentials of these hospital wastewaters. In this context, (i) the wastewater characterization and comparison of the different hospital wastewaters were carried out; (ii) the environmental risk assessment for the wastewater samples was determined using HQ method and TU; and (iii) a selected wastewater sample was assessed overall from the perspective of

the potentials of cytotoxicity (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyl-tetrazolium bromide (MTT) test), mutagenicity (Ames test), and antibiotic-resistant bacteria. In addition, the pharmaceuticals in the wastewaters of three hospitals were classified and categorized based on their occurrence and environmental hazards. Data obtained in this study will be an important source for sustainable management and determination of treatment requirements of hospital wastewaters in Turkey.

Materials and methods

Wastewater samples were taken from two of the largest medical faculty hospitals (MFH1 and MFH2) and a training and research hospital (TRH), all of which are located in Istanbul, Turkey. The medical faculty hospitals have a wide range of services with 1358 and 1285 beds, respectively. The training and research hospital serves as a reference hospital with 612 beds. The average daily water consumptions at MFH1, MFH2, and TRH are 1500, 1166, and 422 m^3/d, respectively.

The wastewaters from MFH1 and MFH2 are discharged to the Sea of Marmara by marine outfall after pre-treatment including screening and grit removal, whereas the wastewaters from TRH are discharged to the Sea of Marmara after passing through a municipal biological wastewater treatment plant.

A single grab sample from the selected hospitals was taken on March 2014. Wastewater characterization and environmental risk assessment were applied to all samples. In addition, a toxicological assessment was performed, and the antibiotic-resistant bacteria potential for the MFH1 wastewater was measured.

Wastewater characterization

Chemical analysis

The analytical standards, hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC and LC–MS/MS grade water and methanol were purchased from Th. Geyer (Renningen, Germany). Diluted hydrochloric acid was added to adjust a pH of 3. Samples were filtered with a 1-μm glass fiber filter (Macherey-Nagel, Düren, Germany). The sample enrichment was performed using Strata XL cartridges (200 mg, 6 mL, Phenomenex, Aschaffenburg, Germany). Conditioning of the cartridge was done by 5 mL methanol and 5 mL water at pH 3. Of the wastewater sample, 500 mL was loaded onto the cartridge. Finally, elution was performed three times with 3 mL of methanol. The extracts were dried under a gentle nitrogen stream and reconstituted with 1 mL of deionized water with 0.1% formic acid for LC–MS/MS analysis. Samples were analyzed with an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany), which was coupled to an API 3000 mass spectrometer (Sciex, Darmstadt, Germany). Data evaluation was conducted with Analyst™ 1.5 (Sciex, Darmstadt, Germany). The separation was performed on a 150 × 2-mm Synergi 4 μ Polar RP column (Phenomenex, Aschaffenburg, Germany) with a water-acetonitrile gradient of 0.1% formic acid in water (*v*/*v*) (mobile phase A) and 0.1% formic acid (*v*/*v*) in pure acetonitrile (mobile phase B) with a flow rate of 0.35 mL/min at 30 °C. The injection volume was 20 μL.

The quantification of the pharmaceuticals was carried out in multiple reaction monitoring (MRM) mode utilizing positive electrospray ionization. The calibration was weighted 1/× with a linear regression. Table 1 gives the list of compounds analyzed for the characterization of hospital wastewaters.

Environmental risk assessment

The purpose of environmental risk assessment is to determine the potential impact of individual compounds on the environment by examining both exposures resulting from the discharge and/or release of chemicals and the effects of such emissions on the structure and function of the ecosystem. The compounds are classified according to their HQ values into the following four categories: insignificant risk (HQ < 0.1), low risk (0.1 < HQ < 1), moderate risk (1 < HQ < 10), and high risk (HQ > 10) (European Commission 2003).

The risk potential of wastewaters containing pharmaceuticals from three different hospitals was estimated in the wastewater of hospital main wing before discharge to the sewer, at inlet of the WWTP (dilution in the sewer) and at discharge of the WWTP (reduction due to degradation and sorption processes during conventional biological treatment) (Escher et al. 2011). The concentration of each compound analyzed in hospital wastewaters was defined as measured environmental concentration in hospital wastewater (MEC_{HWW}). The predicted environmental concentration in the influent of

Table 1 List of compounds analyzed for the characterization of hospital wastewaters

Compound	Group	Compound	Group
1*H*–benzotriazole	Corrosion inhibitor	Metoprolol	Pharmaceutical
4 + 5-Methyl-benzotriazole	Corrosion inhibitor	Metronidazole	Pharmaceutical
Azithromycin	Pharmaceutical	^{4}N–acetyl-sulfamerazine	Metabolite
Bisoprolol	Pharmaceutical	^{4}N–acetyl-sulfamethoxazole	Metabolite
Capecitabine	Pharmaceutical	^{4}N–acetyl-sulfadiazine	Metabolite
Carbamazepine	Pharmaceutical	Naproxen	Pharmaceutical
Ceftazidime	Pharmaceutical	Norfloxacin	Pharmaceutical
Cilastatin	Pharmaceutical	Ofloxacin	Pharmaceutical
Ciprofloxacin	Pharmaceutical	Oxazepam	Pharmaceutical
Citalopram	Pharmaceutical	Oxcarbazepine	Pharmaceutical
Clarithromycin	Pharmaceutical	Paclitaxel	Pharmaceutical
Climbazole	Pesticide	Paracetamol	Pharmaceutical
Clindamycin	Pharmaceutical	Phenazone	Pharmaceutical
Cyclophosphamide	Pharmaceutical	Prophyphenazone	Pharmaceutical
Diclofenac	Pharmaceutical	Ritalinic acid	Pharmaceutical
Dimethyl-benzotriazole	Corrosion inhibitor	Roxithromycin	Pharmaceutical
Diuron	Pesticide	Simvastatin	Pharmaceutical
Docetaxel	Pharmaceutical	Sotalol	Pharmaceutical
Erythromycin	Pharmaceutical	Sulfadiazine	Pharmaceutical
Fenofibrate	Pharmaceutical	Sulfamethazine	Pharmaceutical
Furosemide	Pharmaceutical	Sulfamethoxazole	Pharmaceutical
Ibuprofen	Pharmaceutical	Sulfapyridine	Pharmaceutical
Ifosfamide	Pharmaceutical	Tamoxifen	Pharmaceutical
Isoproturon	Pesticide	Terbutryn	Pesticide
Ketoprofen	Pharmaceutical	Tramadol	Pharmaceutical
Linuron	Pesticide	Trimethoprim	Pharmaceutical
Mefanaminic acid	Pharmaceutical	Venlafaxine	Pharmaceutical
Melperon	Pharmaceutical		

wastewater treatment plant ($PEC_{WWTPinfluent}$) corresponds to the concentration of the each compounds at the inlet of the WWTP and was defined to be equivalent to the MEC_{HWW} multiplied with the dilution factor (df) in the sewer. The df is 0.0034 for MFH1, 0.0026 for MFH2, and 0.0011 for TRH. The PEC in the effluent of the WWTP ($PEC_{WWTPeffluent}$) for the wastewaters of MFH1 and MFH2 was assumed to be equal to the $PEC_{WWTPinfluent}$ because the wastewater was subjected to the pre-treatment containing the screening and grit removal, and the characterization of hospital wastewater was performed on the filtered sample. Therefore, the pharmaceutical compounds were discharged passing through the pre-treated wastewater without degradation. The $PEC_{WWTPeffluent}$ for the wastewater of TRH refers to the discharge of the WWTP, where the $PEC_{WWTPinfluent}$ was reduced by conventional biological secondary treatment with sludge age >3 days in municipal wastewater treatment, including removal of organic matter, nitrification/denitrification, and phosphorus. The fraction eliminated in the treatment plant ($f_{elimination\ in\ WWTP}$) was obtained from literature (Gros et al. 2010; Escher et al. 2011) and assumed to be 0% if no literature data were available (Escher et al. 2011).

HQ value calculated for each compound characterizes its level of involvement in the environmental hazardousness. The HQs for each individual compound were calculated according to EU guidelines (European Commission 2003) as the quotient between the environmental concentration and the PNEC. PNEC values were used by taking

the values calculated in previous studies (Table 2) (Orias and Perrodin 2013; Seidel 2013; Türk 2013; Mendoza et al. 2015; Ecotox Centre 2016). In these studies, PNEC values were obtained from the available aquatic toxicity data (Ecotox EPA, Wikipharma) using different species from different trophic levels, applying an extrapolation factor to the lowest toxicity values of a given pharmaceutical. In our study, HQ values were calculated according to the lowest

Table 2 PNEC values calculated in previous studies

	Orias and Perrodin (2013)	Mendoza et al. (2015)	Seidel et al. (2013)	Türk et al. (2013)	Ecotox Centre (2016)	
					MAC-EQS	AA-EQS
Paracetamol	6.92	2.04	583	–	–	–
Diclofenac	0.02	0.05	0.05	0.1	–	0.05
Benzotriazole	–	–	–	30	246	238
Sulfamethoxazole	0.59	–	0.59	0.15	2.7	0.6
N-acetyl-sulfamethoxazole	–	–	–	–	–	–
Cilastatin	–	–	–	–	–	–
Trimethoprim	0.0058	0.16	20	800	1100	60
Furosemide	1.0	1.56	100	–	–	–
Ciprofloxacin	0.5	–	0.036	–	0.363	0.089
Ceftazidime	–	–	0.025	–		
Carbamazepine	2.0	2.5	2.5	0.5	2550	0.5
4 + 5-Methyl-benzotriazole	–	–	–	–	–	–
Metoprolol	0.1	61.5	3.1	7.3	76	64
Ifosfamide	8.2	–	–	–	–	–
Azithromycin	0.019	–	4.8	0.44	0.09	0.09
Clarithromycin	0.04	0.02	0.062	0.31	0.11	0.06
Sulfadiazine	0.7	0.135	–	–	–	–
Venlafaxine	–	–	35.5	–	–	–
Citalopram	0.00635	–	17	–	–	–
Ofloxacin	0.022	0.1	0.113	5.3	–	–
Phenazone	0.276	–	–	–	–	–
^{4}N–acetyl-sulfadiazine	–	–	–	–	–	–
Oxazepam	0.0019	–	32	–	–	–
Cyclophosphamide	18	–	19,700	–	–	–
Clindamycin	–	–	4	–	–	–
Oxcarbazepine	–	–	–	–	–	–
Climbazole	–	–	–	–	–	–
Mefanaminic acid	–	–	–	0.1	40	4
Ritalinic acid	–	–	–	–	–	–
Prophyphenazone	–	0.8	44	–	–	–
Capecitabine	–	–	–	–	–	–
Metronidazole	100	–	36	13	–	–
Fenofibrate	–	0.78	1.6	7.2	–	–
Ibuprofen	0.2	0.01	6.6	3	23	0.3
Ketoprofen	2.0	2.0	–	7.4	–	–
Naproxen	6.6	0.33	3.3	190	370	1.7
Sulfapyridine	0.000122	–	–	–	–	–

PNEC value indicated in Table 3. TUs for each therapeutic group were calculated by simple addition of the relevant HQs (Mendoza et al. 2015).

Toxicological assessment

The wastewater samples were analyzed in terms of cytotoxic and mutagenic effects by three different cell line assays and *Salmonella* mutagenicity (Ames assay). The experiments were carried out with non-filtered and filtered wastewater samples taken from MFH1. The filtered samples were prepared by filtering through a 0.45-μm nylon syringe filter followed by filter paper.

Cytotoxicity

Cytotoxicity was determined using test kits based on different cellular mechanisms depending on the damaged region of cells. For this, NRK-52E rat kidney cells (American Type Culture Collection (ATCC) CRL-1571™, USA) were incubated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% streptomycin-penicillin at 5% CO_2, 90% humidity, and 37 °C for 24 h (60–80% confluence) in 96-well plates at 10^4 cells per well. The cells were exposed to the 0–4% of the wastewater sample based on the maximum permissible concentrations of the test conditions for 6, 12, and 24 h of incubation (Uzar et al. 2015).

In each assay, 1% DMSO and 1% Triton X-100 are used as solvent and positive controls, respectively. For Triton X-100, the studied concentrations were 0.15, 1.5, and 15 μM. For all concentrations, the tests were run in triplicate and each assay was repeated twice. The half-maximal inhibitory concentration (IC_{50}) was expressed as the concentration of sample that caused an inhibition of 50% of enzyme activity in cells.

MTT is a water-soluble, yellow-colored salt reduced by mitochondrial succinate dehydrogenase into an insoluble purple formazan product. Mitochondrial succinate dehydrogenase is active only in viable cells, so color changes by the activity of the enzyme can be used as a cytotoxicity endpoint (Van Meerloo et al. 2011). Neutral red (NR) is a weak cationic dye that accumulates in lysosomes by non-ionic passive diffusion and binds to anionic and/or phosphate groups of the lysosomal matrix by electrostatic hydrophobic bonds. In an NR uptake (NRU) assay, lysosomal integrity can be used as an indicator of cell viability by the uptake of neutral red dye into cells (Repetto et al. 2008). The lactate dehydrogenase (LDH) assay is based on the measurement of extracellular LDH activity caused by loss of membrane integrity. In damaged cells, the extracellular medium contains LDH. In the presence of LDH, NAD is reduced to NADH, whereas lactate is converted to pyruvate. Thus, the formation of NADH in damaged cells causes a change in absorbance which allows us to determine the cell viability (Fotakis and Timbrell 2006). Optical density (OD) was read at 590, 570, and 500 nm for MTT, NRU, and LDH, respectively, using a microplate spectrophotometer system (Epoch, Germany).

Mutagenicity

An Ames MPF™ 98/100 Assay Kit, a modified liquid microplate version of the conventional Ames assay, was used. The kit was obtained from Xenometrix (Allschwil, Switzerland). The strains were TA98 for frameshift mutation and TA100 for base-pair substitution strains of *Salmonella typhimurium*. The mutated TA98 and TA100 *S. typhimurium* strains are incapable of synthesizing the amino acid histidine. However, strains can produce histidine and grow if a reversion of the mutation occurs. The presence of mutagenic compounds capable of inducing reversions can cause an increase in the number of revertant colonies relative to background levels. In the assay, the catabolic activity of revertant cells causes a reduction in the pH of the solution, which results in a color change from purple to yellow. The manufacturer's protocol was used (Umbuzerio et al. 2010; Flückiger-Isler and Kamber 2012).

In some instances, chemicals themselves are not mutagenic but become mutagens after being metabolized in the liver. To mimic this in vivo activation process, the Ames assay was conducted in both the presence and absence of the S9 microsomal fraction metabolizing system, which is a mixture of mammalian liver enzymes. Lyophilized Aroclor 1254-induced rat liver S9 microsomal fractions were also purchased from Xenometrix (Allschwil, Switzerland). The mutagenic potentials of samples were assessed in the presence and absence of the S9 metabolic-activating system at a final concentration of 4.5% (*v*/*v*) in medium.

The positive controls used included 2-nitrofluorene (2 μg/mL) and 4-nitroquinoline N-oxide (0.1 μg/mL) without S9 and 2-aminoanthracene (5 μg/mL) with S9 microsomal fraction (Hakura et al. 2005). Milli-Q water was used as the solvent control. The criteria used to evaluate the Ames results were the fold increase in the

Table 3 Characterization of the selected hospital wastewater samples for pharmaceuticals, corrosion inhibitors, and pesticides, including the MEC, PNEC, and calculated HQ values for the detected compounds

Compound	Group	MEC_{HWW} (μg/L)			PNEC (μg/L)	$f_{elimination}$ in WWTP for TRH	HQ_{HWW}			$HQ_{WWTPinfluent/effluent}$		$HQ_{WWTPinfluent}$	$HQ_{WWTPeffluent}$
		MFH1	MFH2	TRH			MFH1	MFH2	TRH	MFH1	MFH2	TRH	TRH
Paracetamol	Analgesic	15	65	7.4	2.04	100	7.35	31.86	3.63	0.025	0.083	0	0
Diclofenac	Analgesic	11	0.34	0.14	0.02	34	550	17	7	1.833	0.044	0.005	<0.001
Benzotriazole	Corrosion inhibitor	9.6	1.9	0.38	30	–	0.32	0.063	0.013	0.001	<0.001	<0.001	0.0004
Sulfamethoxazole	Antibiotic	8.5	0.55	<0.01	0.15	57	56.67	3.67	NC	0.189	0.01	NC	NC
^{4}N–acetyl-sulfamethoxazole	Sulfamethoxazole metabolite	7.3	0.58	<0.01	–	–	NC	NC	NC	NC	NC	NC	NC
Cilastatin	Antibiotic	4.1	<0.01	<0.01	–	75	NC	NC	NC	NC	NC	NC	NC
Trimethoprim	Antibiotic	2.2	0.18	<0.01	0.0058	32	379.31	31.04	NC	1.264	0.080	NC	NC
Furosemide	Diuretic	1.9	<0.1	<0.1	1.0	42	1.9	NC	NC	0.006	NC	NC	NC
Ciprofloxacin	Antibiotic	1.9	24	2.1	0.036	83	52.78	666.67	58.33	0.176	1.727	0.011	<0.001
Ceftazidime	Antibiotic	1.6	NA	NA	0.025	93	64	NC	NC	0.213	NC	NC	NC
Carbamazepine	Antiepileptic	1.2	0.46	0.089	0.5	0	2.4	0.92	0.178	0.008	<0.001	<0.001	<0.001
4 + 5-Methyl-benzotriazole	Corrosion inhibitor	0.85	0.022	0.013	–	–	NC	NC	NC	NC	NC	NC	<0.001
Metoprolol	β-Blocker	0.65	5	0.2	0.1	40	6.5	50	2	0.022	0.13	0.001	<0.001
Ifosfamide	Antineoplastic	0.56	2.4	<0.01	8.2	–	0.068	0.29	NC	<0.001	<0.001	NC	NC
Azithromycin	Antibiotic	0.4	<0.01	<0.01	0.019	33	21.05	NC	NC	0.070	NC	NC	NC
Clarithromycin	Antibiotic	0.35	15	0.063	0.02	22	17.5	750	3.15	0.058	1.943	0.003	<0.001
Sulfadiazine	Antibiotic	0.16	<0.01	<0.01	0.135	69	1.19	NC	NC	0.004	NC	NC	NC
Venlafaxine	Antidepressant	0.15	0.13	0.015	35.5	0	0.004	0.004	<0.001	<0.001	<0.001	<0.001	<0.001
Citalopram	Antidepressant	0.09	0.025	0.033	0.00635	0	14.17	3.937	5.2	0.047	0.010	0.006	<0.001
Ofloxacin	Antibiotic	0.082	1.4	200	0.022	40	3.73	63.64	9090.9	0.012	0.165	5.755	0.127
Phenazone	Analgesic, antiinflammatory, antipyretic	0.062	0.023	<0.01	0.276	–	0.23	0.083	NC	<0.001	<0.001	NC	NC
^{4}N–acetyl-sulfadiazine	Sulfadiazine metabolite	0.05	<0.01	<0.01	–	–	NC	NC	NC	0.003	NC	NC	NC
Oxazepam	Antidepressant	0.04	NA	NA	0.0019	7	21.05	NC	NC	0.070	NC	NC	NC
Cyclophosphamide	Antineoplastic	0.037	0.68	<0.01	18	0	0.002	0.038	NC	<0.001	<0.001	NC	NC
Clindamycin	Antibiotic	0.036	<0.01	<0.01	4	0	0.009	NC	NC	<0.001	NC	NC	NC
Oxcarbazepine	Antiepileptic	0.029	NA	NA	–	–	NC	NC	NC	NC	NC	NC	NC
Climbazole	Antifungal	0.024	NA	NA	–	–	NC	NC	NC	NC	NC	NC	NC
Mefanaminic acid	Analgesic	0.016	NA	NA	0.1	–	0.16	NC	NC	<0.001	NC	NC	NC
Ritalinic acid	Metabolite of methylphenidate	<0.02	0.9	<0.02	–	–	NC	NC	NC	NC	NC	NC	NC
Prophyphenazone	Analgesic	0.0088	NA	NA	0.8	44	0.011	NC	NC	<0.001	NC	NC	NC

Table 3 (continued)

Compound	Group	MEC_{HWW} (μg/L)			PNEC (μg/L)	$f_{elimination}$ in WWTP for TRH		HQ_{HWW}			$HQ_{WWTPinfluent/effluent}$		$HQ_{WWTPinfluent}$	$HQ_{WWTPeffluent}$
		MFH1	MFH2	TRH				MFH1	MFH2	TRH	MFH1	MFH2	TRH	TRH
Capecitabine	Antineoplastic	<0.02	0.16	<0.02	–	–		NC	NC	NC	NC	NC	NC	NC
Metronidazole	Antibiotic	<0.03	3	0.68	13	–		NC	0.231	0.005	NC	<0.001	<0.001	<0.001
Fenofibrate	Lipid-regulating agent	NA	0.036	0.042	0.78	–		NC	0.046	0.054	NC	<0.001	<0.001	<0.001
Ibuprofen	Antiinflammatory	NA	0.086	0.62	0.01	96		NC	8.6	62	NC	0.022	0.003	<0.001
Ketoprofen	Analgesic, antiinflammatory	NA	0.081	1.5	2.0	69		NC	0.041	0.75	NC	<0.001	<0.001	<0.001
Naproxen	Antiinflammatory	NA	1.8	0.49	0.33	86		NC	5.455	1.485	NC	0.014	<0.001	<0.001
Sulfapyridine	Antibacterial-antibiotic	NA	0.047	<0.01	0.000122	–		NC	385.25	NC	NC	0.998	NC	NC
							TU	1200	2019	9235	4.0	5.2	9.7	5.7

NA not analyzed, – not available, *NC* no calculation possible

number of positive wells over the solvent control baseline and the dose dependency. The solvent control baseline was defined as the mean number of positive wells in the solvent control plus one standard deviation (SD). The fold increase of the revertants relative to the solvent control was determined by dividing the mean number of positive wells at each dose by those in the solvent control at baseline. The revertant fold increase (over baseline) was stated as the mutagenicity ratio (MR) in the study. All solvent controls from an experiment with identical conditions (same day, same bacterial culture, solvent, and incubation conditions) were combined. An MR value greater than or equal to 2 was classified as being positive for that dose. To evaluate dose-dependent responses, Student's *t* test (one-sided, unpaired) was used. Only *p* values lower than 0.05 were considered to be statistically significant. Each experiment was repeated at least twice.

Determination of antibiotic resistance bacteria

Total bacterial and fungal number

In the present study, the prevalence and antibiotic resistance patterns were investigated among bacterial and fungal strains isolated from wastewater collected from MFH1. The hospital wastewater was evaluated for bacterial and fungal loads. For this reason, total bacterial and fungal numbers were counted using trypticase soy agar (TSA) medium and sabouraud dextrose agar (SDA) medium, respectively. For detection of viable bacteria and fungus counts, 300 mL of wastewater was obtained from the hospital collector system in sterile condition. Then, 1/10, 1/100, 1/1000, and 1/10,000 dilutions of the wastewater sample containing viable microorganisms in phosphate-buffered saline (PBS) were plated onto TSA petri dishes for total viable bacterial growth and onto SDA for total viable fungal growth. These petri dishes were incubated at 37 °C for 24 h for bacterial growth and at 25 °C for 48 h for fungal growth. After incubation, the total bacterial or fungal colony counts in 1 mL of original wastewater sample were determined using the dilution factor.

Isolation and identification of clinically important bacteria

After incubation, based on colony morphology, representative colonies were picked and sub-cultured on

different selective and differential media including mannitol agar, MacConkey agar, and pseudomonas agar. After obtaining pure colonies, biochemical tests were performed via a standard procedure based on Bergey's manual (Goodfellow et al. 2012). Additionally, identification studies were performed by using API kits (bioMérieux) for the clinically significant bacterial isolates.

Antibiotic susceptibility test

Antibiotic susceptibility patterns of isolated and identified strains were investigated by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute 2009). As a reference strain, *Escherichia coli* ATCC 25922 (American Type Culture Collection, Rockville, MD, USA) was used throughout the study to verify the accuracy of the disk diffusion test procedure to ensure that inhibition zone values of the antibiotics studied were within the accuracy range stated by CLSI. Antibiotic susceptibility tests were then performed for the identified ten isolates that were associated with clinically relevant infections. Bacterial suspensions containing 1×10^8 cfu/mL were prepared for each isolates, and then, aliquots of 200 μL were plated out on Mueller Hinton agar (MHA) (Merck). The antibiotics tested in our study were chosen mainly by the conception rate in the hospital wastewater, and the antimicrobial disks impregnated with ciprofloxacin (5 μg; Oxoid Diagnostics), clindamycin (2 μg; Oxoid Diagnostics), trimethoprim (5 μg; Oxoid Diagnostics), azithromycin (15 μg; Oxoid Diagnostics), and ceftazidime (30 μg; Oxoid Diagnostics) were placed onto the MHA plates and incubated at 37 °C for 24 h. After incubation, the inhibition zone diameters were measured, and the antibiotic susceptibility pattern of each strain was classified using CLSI reference criteria.

Results and discussion

Wastewater characterization

The samples were analyzed for pharmaceuticals, corrosion inhibitors, and pesticides. Table 3 lists the detected compounds according to their concentrations in the MFH1 wastewater in descending order. Thirty-eight compounds within the 55 compounds identified were measured above the detection limits in the selected hospital wastewater samples.

As seen in Table 3, analgesics and antibiotics were the most commonly detected pharmaceuticals. In the group of analgesics and antiinflammatories, paracetamol and diclofenac were detected at high concentrations in all hospital wastewaters. The highest concentrations were observed for paracetamol (7.4–65 μg/L), whereas the highest diclofenac concentration was detected only in the wastewater of MFH1 (11 μg/L). In MFH2 and TRH, the concentrations of diclofenac were in the range of 0.14 to 0.34 μg/L. Concentrations above 1 μg/L were detected for ketoprofen and naproxen.

Several antibiotics were detected in considerable concentrations in the wastewaters of the different hospitals. Especially, ofloxacin was measured at a concentration of 200 μg/L at TRH. In the wastewater of MFH2, the concentration was 1.4 μg/L, and in the effluent of MHF1, it was 0.082 μg/L. The antibiotics sulfamethoxazole, cilastatin, ciprofloxacin, ceftazidime, and trimethoprim were measured at microgram per liter levels in the wastewater of MFH1. Ciprofloxacin, clarithromycin, and metronidazole were measured at microgram per liter levels in the wastewater of MFH2.

In the group of psychtropic drugs, carbamazepine, venlafaxine, and citalopram were detected in the wastewater of the three hospitals with the highest concentrations of carbamazepine. Oxazepam and oxcarbazepine were measured at the lowest concentrations in the wastewater of MFH1, whereas these compounds were not analyzed in the wastewaters of MFH2 and TRH.

Throughout the group of antineoplastics and immunomodulant agents, ifosfamide, cyclophosphamide, and capecitabine were detected at relatively high concentrations in the wastewater of MFH2. However, ifosfamide and cyclophosphamide were lower than the detection limits in the wastewater of TRH.

From antihypertensive pharmaceuticals, metoprolol was measured in all samples. Furosemide was detected only in the wastewater of MFH1.

The metabolite ^{4}N–acetyl sulfamethoxazole was detected at significant levels in the wastewater of MFH1. Fenofibrate, a lipid regulator, was detected at low concentrations in the wastewaters of MFH2 and TRH.

It is expected that characterization of hospital wastewaters might change among countries, hospitals, and specialties existing within the hospital seasonally, daily, or even hourly. In our study, the characterization of the hospital wastewater is based on a single grab sample,

and the characterization shows only a momentary appearance. The pharmaceutical compounds analyzed in this study were compared with hospital wastewaters in Italy (Verlicchi et al. 2012), Germany (Seidel et al. 2013), Spain (Mendoza et al. 2015), Denmark (Nielsen et al. 2013), and Portugal (Santos et al. 2013). In the group of analgesics and antiinflammatories, six compounds, namely, paracetamol, diclofenac, naproxen, ketoprofen, ibuprofen, and prophyphenazone, within the eight compounds detected in our study were reported in the hospital wastewaters of Spain, Portugal, and Italy. Paracetamol was the most commonly detected analgesic in all countries. Diclofenac was the second most detected analgesic in the studied hospitals, whereas the comparison with other countries shows a lower consumption of this substance. In the group of antibiotics, four compounds, namely, oflaxacin, clarithromycin, trimethoprim, and sulfadiazine, within the compounds detected in our study were also reported in Spain, Portugal, and Italy. The highest pharmaceutical concentration was obtained for ofloxacin in the three studied hospitals. It was also measured at high concentrations in Italy and Portugal. High concentrations of clarithromycin were detected in Turkey and Italy. In the group of the most ecotoxic compounds, trimethoprim was detected at relative high and similar concentrations in all countries.

As part of a study conducted by Seidel et al. 2013 in Germany, the concentrations of 12 pharmaceutical compounds in the hospital wastewaters of North Rhine Westphalia (Germany) were investigated and 8 of them were discussed in the context of Danish guiding limit values (DHI 2011). According to the Danish regulations for discharge to public sewer, the limit values for azithromycin, ciprofloxacin, diclofenac, ibuprofen, olanzapin, paracetamol, sulfamethoxazol, and tramadol are 0.12, 0.01, 0.01, 170, 11, 420, 0.31, and 26 μg/L, respectively (DHI 2011; KL 2013; KL 2013a). The concentrations of three pharmaceutical compounds (azithromycin, ciprofloxacin, and diclofenac) in the wastewater of the 19 hospitals in Germany were found in concentrations above the Danish guide limits in nearly every sample. The concentrations of ibuprofen, paracetamol, and sulfamethoxazole were over the Danish guide limits for various samples. The highest concentrations were found for the analgesic paracetamol (33–1057 μg/L) (Seidel et al. 2013). The results of our research showed that the concentrations of ciprofloxacin and diclofenac in the wastewater of the three hospitals were above the Danish guide limits. The concentration of azithromycin in the wastewater of MFH1 was also above the Danish guide limit, whereas the concentration of sulfamethoxazole was over the limit for MFH1 and MFH2. Although the highest analgesic concentration was found for paracetamol, it did not exceed the Danish limit.

Regarding the exceedance of guiding limits in the Danish municipal guideline, MFH1 is a major point source of diclofenac, whereas MFH2 is a major point source for ciprofloxacin and clarithromycin. The wastewater of MFH1 should be managed carefully since it was classified as a point source for four antibiotics including sulfamethoxazole, azithromycin, ciprofloxacin, and clarithromycin.

Environmental risk assessment

Table 3 shows the measured concentration of pharmaceuticals (MEC), PNEC, and HQ values calculated for each individual compound based on the hospital main wing (HQ_{HWW}), the dilution in sewer ($HQ_{WWTPinfluent}$), and the degradation and sorption in WWTP ($HQ_{WWTPeffluent}$). The PNEC values taken from six different studies are presented in Table 2 (Orias and Perrodin 2013; Seidel et al. 2013; Türk et al. 2013; Mendoza et al. 2015; Ecotox Centre 2016). In these studies, the PNEC values were derived by using the procedure suggested by the European Union in the Technical Guidance Document (European Commission 2003). Although the method based on the PNEC calculation is similar, the calculated PNEC varies owing to the consideration of the different species. For example, the difference between the PNEC values for trimethoprim, metoprolol, ibuprofen, and naproxen in these studies is more than tenfold. Moreover, most hazardous compounds were taken into account considered by Orias and Perrodin (2013), but the PNEC values for some compounds could not be calculated owing to the lack of at least acute ecotoxicity data for species from three different trophic levels. Therefore, for the HQ calculation in our study, the lowest PNEC value of each individual compound from the selected previous studies was used to perform a more realistic evaluation of its ecotoxic potential.

The calculated HQ_{HWW} of 9090.9 for ofloxacin is very high with the highest MEC_{HWW} of 200 μg/L (TRH). The calculated HQ_{HWW} for MFH2 is 63.64 and that for MFH1 3.73 (Table 3). According to Orias and Perrodin (2013), oflaxacin with HQ > 10 belonged

to the most hazardous compounds with a high-risk potential for hospital wastewaters. It should be highlighted that sulfapyridine with an extremely low concentration of 0.047 μg/L in MFH2 leads to an HQ_{HWW} of 385.25 because of its very low PNEC of 0.000122 μg/L.

In the group of analgesics and antiinflammatories, three compounds, namely, diclofenac, ibuprofen, and paracetamol, within the eight detected compounds have high-risk potentials with $HQ_{HWW} > 10$ for the examined hospital wastewaters. Diclofenac has the highest HQ_{HWW} (550) in the wastewater of MFH1 because it is the most ecotoxic compound within the group of analgesics and antiinflammatories with a PNEC of 0.02 μg/L. Although the concentration of paracetamol in the wastewater of MFH2 was almost sixfold that of diclofenac in the wastewater of MFH1, paracetamol had an HQ_{HWW} of 31.86 because it is the less ecotoxic compound with a PNEC of 2.04 μg/L. In this group, naproxen had moderate risk, and ketoprofen, phenazone, and mefanaminic acid with $0.1 < HQ_{HWW} < 1$ had low risk in the analyzed hospital wastewaters.

Some compounds such as cilastatin in the group of antibiotics and oxcarbazepine in the group of psychotropic drugs had no available PNEC values, so their ecotoxicological hazard in the selected hospital wastewaters could not be described.

Oxazepam and citalopram in the group of psychotropic drugs treatments had HQ_{HWW} of 21.05 and 14.17 in MFH1 wastewater, respectively. Carbamazepine, which had the highest concentration in the group of psychotropic drug treatments for all hospital wastewaters, presented a moderate risk for MFH1 and low risk for MFH2 and TRH. Metoprolol, the only detected compound in the group of β-blockers, was determined to be hazardous compound with an HQ_{HWW} of 50 for MFH2 wastewater.

According to HQ_{HWW} values, 14 pharmaceuticals were classified as "high risk" with $HQ_{HWW} > 10$, 8 of which were antibiotics. HQ_{HWW} values higher than 100 were calculated for five antibiotics and one analgesic. HQ is directly proportional to concentration and indirectly proportional to PNEC. Thus, high concentrations and/or low PNEC values lead to high HQ. As shown in Table 3, throughout the calculated antibiotics, clarithromycin had one of the high concentrations (15 μg/L) and low PNEC (0.02 μg/L) values. Therefore, clarithromycin has a high-risk potential with the calculated HQ_{HWW} of 750 in the MFH2 wastewater. In addition, the antibiotic ofloxacin had the highest HQ_{HWW} value because of the high concentration (200 μg/L) in TRH wastewater with almost the same PNEC value (0.022 μg/L). Ciprofloxacin with an HQ_{HWW} value of 666.67 for the MFH2 wastewater was classified with high risk. Similarly, the research in Germany (Sui et al. 2012) reported that ciprofloxacin had the highest HQ values for different hospital wastewaters with an extremely low PNEC value of 0.036 μg/L. Diclofenac was also remarkable with an HQ_{HWW} value higher than 100 for the MFH1 wastewater as an analgesic compound.

As previously mentioned, the wastewaters from MFH1 and MFH2 are discharged to the Sea of Marmara after pre-treatment while the wastewaters from TRH are discharged to the same sea passing through a biological wastewater treatment plant. Escher et al. (2011) reported that high-risk pharmaceuticals are excreted mainly with feces. In our study, the feces in the hospital wastewater and the contribution of pharmaceuticals in domestic wastewater, however, were not taken into account in the calculation of the $PEC_{WWTP\ influent/effluent}$ and $HQ_{WWTPinfluent/effluent}$. The $HQ_{WWTPinfluent}$ and $HQ_{WWTPeffluent}$ for the wastewater of MFH1 and MFH2 should be the same, because the pharmaceuticals were analyzed in the soluble phase and also the wastewaters from both hospitals were discharged to the sea after pre-treatment. The moderate-risk pharmaceuticals ($1 < HQ < 10$) in the hospital wastewaters diminished to mainly the insignificant risk category in the effluent of the WWTP due to dilution in the sewer, while some of the high-risk pharmaceuticals ($HQ > 10$) failed to the low-risk category. A few high-risk pharmaceuticals in the hospital wastewaters of MFH1 and MFH2, namely, diclofenac, trimethoprim, ciprofloxacin, and claritromycin, were in the moderate-risk category after dilution in sewer (Table 3). The wastewater of TRH was firstly diluted in the sewer and then removed in the biological WWTP. Ciprofloxacin, the high-risk pharmaceutical in the wastewater of TRH, diminished the insignificant risk category in the influent of the WWTP. The most ecotoxic pharmaceutical with HQ_{HWW} of 9090 in TRH, ofloxacin dropped to the moderate-risk category ($HQ_{WWTPinfluent}$ of 5.75) in the influent of WWTP, and then was degraded to the low-risk category ($HQ_{WWTPeffluent}$ of 0.13) in the effluent of WWTP.

The order of toxic unit for hospital wastewater, TU_{HWW} values calculated in Table 3, was TRH ≫ MFH2 > MFH1. The highest TU_{HWW} value of TRH was remarkable;

however, 99.3% of the TU_{HWW} value were sourced from ofloxacin. The contribution of each therapeutic group to the TU_{HWW} value for the hospital wastewaters is given in Table 4. The contributions of antibiotics to TU_{HWW} were found to be 49.7, 94.1, and 99.1% for MFH1, MFH2, and TRH, respectively. Moreover, the contributions of analgesics and antiinflammatories were found to be 46.5, 3.1, and 0.8% for MFH1, MFH2, and TRH, respectively. It is clearly observed that the wastewaters of three hospitals had different environmental risks owing to varying pharmaceutical types and concentrations. The largest percentage of environmental risk consisted of antibiotics for all hospitals. Moreover, the contributions of analgesic and antiinflammatories were found to be the second high environmental risk for MFH1. $TU_{WWTPinfluent}$ and $TU_{WWTPeffluent}$ are same for MFH1 and MFH2.The order of toxic unit for the influent of the WWTP, $TU_{WWTPinfluent}$, did not change with TU_{HWW} because only dilution in sewer was taken into account. Only in TRH, $TU_{WWTPeffluent}$ decreased from 9.7 to 5.7 because of degradation and sorption in WWTP (Table 3).

The contributions of each antibiotic to the TU_{HWW} value of the therapeutic group antibiotics are given in Fig. 1. In the TRH wastewater, ofloxacin had a TU_{HWW} of 99.3% in the group of antibiotics, whereas it was 0.6% for MFH1 and 3.4% for MFH2. The wastewater of MFH1 had a more diverse distribution of TU_{HWW} in the antibiotic group. In the wastewater of MFH1, trimethoprim, ceftazidime, sulfamethoxazole, and ciprofloxacin were predominantly found, and clarithromycin, ciprofloxacin, and sulfapyridine were in the MFH2 wastewater. The occurrence of antibiotic resistance bacteria was also investigated hereinafter. However, the contribution of each antibiotic to the $TU_{WWTPinfluent/effluent}$ for MFH1 and MFH2 did not again change with TU_{HWW} because dilution does not affect the contribution of each compound to TU_{HWW}. Actually, dilution is not an elimination and/or removal method for micropollutant. The total load of micropollutant in the environment should be considered, and biological and advanced biological treatment processes must be applied to remove these compounds.

Table 4 Percent contribution of the pharmaceuticals to the TU value of the wastewater of each hospital

Therapeutic group	TU (%)		
	MFH1	MFH2	TRH
Antibiotics	49.7	94.1	99.1
Analgesics and antiinflammatories	46.5	3.1	0.8
Psychotropic drugs	2.4	0.2	0.06
Antineoplastics and immunomodulant agents	0.006	0.02	–
Antihypertensive pharmaceuticals	0.7	2.5	0.02

Toxicological assessment

Genotoxic chemicals induce DNA damage and mutations, and chronic exposure to low doses of those chemicals might increase the risk of cancer development (Giulivo et al. 2016). Some epidemiological investigations have shown a link between pharmaceuticals in wastewaters/surface waters and genotoxic/cytotoxic effects (Gupta et al. 2009; Dizer et al. 2002; Besse et al. 2012).

In the present study, the cytotoxicity of the wastewater samples was evaluated based on mitochondrial, membrane, and lysosomal damage in NRK-52E kidney cells at 0–4% exposure concentrations of the non-filtered and filtered samples. Kidney cells were used because the kidney is the main route of excretion of most substances present in waters. Cell death was observed for more than 90% of the non-filtered and filtered samples (data were not shown in detail). In addition, the samples showed bactericidal activity in *S. typhimurium* at concentrations higher than 4%. Similar results were reported by various researchers (Bagatini et al. 2009; Sharma et al. 2014).

The Ames assay, a useful tool for quantifying the genotoxicity of complex matrix in the water, was used in the present study. However, it should be noted that a positive result does not necessarily indicate that the substance is a carcinogen. The Ames assay confirms that only whether the substance is mutagenic to the particular bacterial strain used and for the genetic endpoint tested. By the Ames assay, we observed that (i) the samples showed strongly mutagenic activity with a maximal increase of ≤10.4 times the negative controls, (ii) there was no difference in the mutagenic activity between the non-filtered and filtered samples, (iii) the TA98 strain was more sensitive to the samples than the TA100 strain in the sense that the incidence of base-pair substitutions of the samples was higher owing to their potential to form frameshift mutation, and (iv) the mutagenic potentials of the samples were generally concentration and metabolic activation dependent. However, the non-filtered samples show mutagenic activity

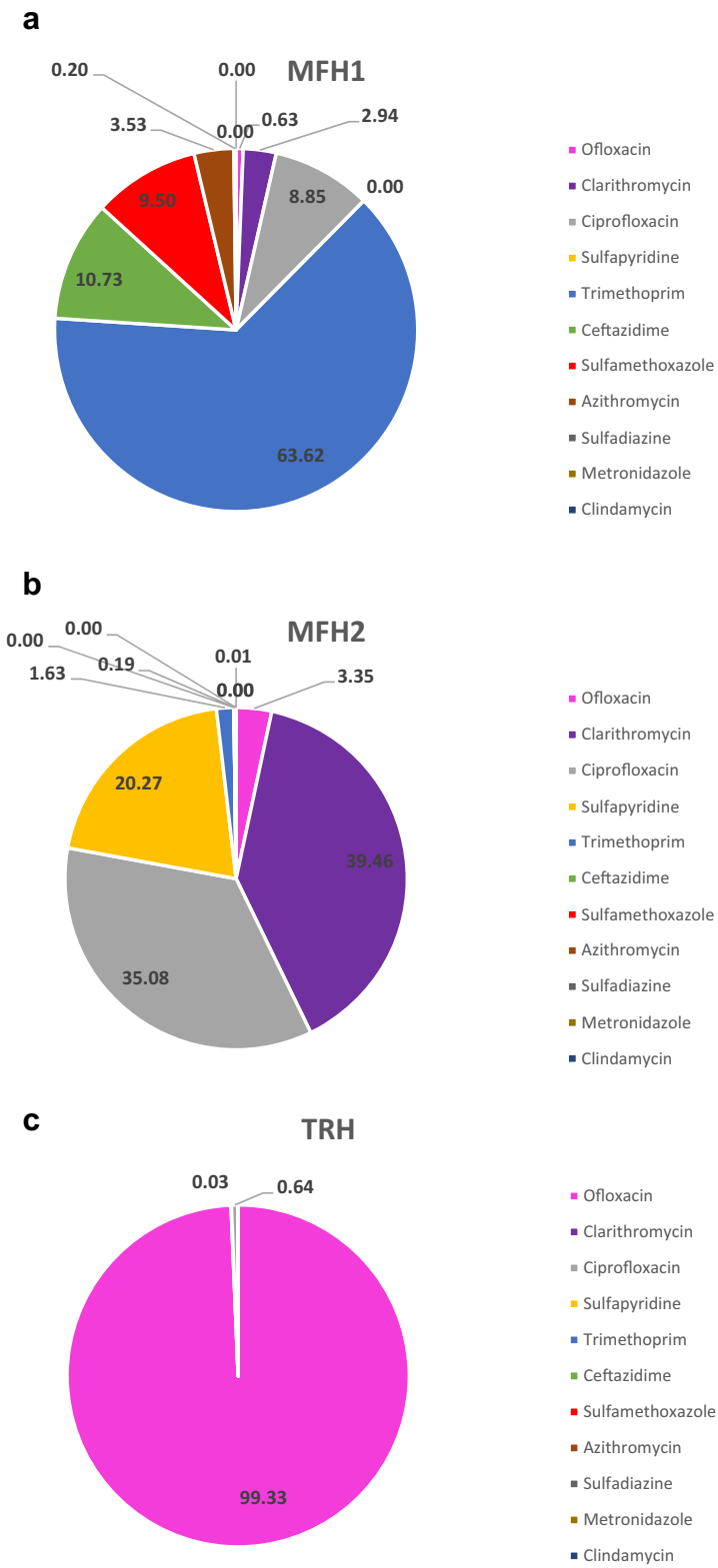

Fig. 1 Percent contribution of each antibiotic to the TU value of the therapeutic group antibiotics **a** MFH1, **b** MFH2, and **c** TRH

with the TA98 strains independent of metabolic activation at concentrations higher than 1% (Table 5).

A comparison based on the toxicological assessment among the various studies is difficult because the hospitals feature different wastewater compositions, sizes, and levels of activity. In addition, there is no standard protocol for sample collection, sample processing, or selection of tests. However, similar to the present study, many of the hospital wastewater samples were identified as cytotoxic and mutagenic (Giuliani et al. 1996; Hartmann et al. 1998; Hartmann et al. 1999; Jolibois et al. 2003; Jolibois and Guerbet 2005; Gupta et al. 2009; Mater et al. 2014; Sharma et al. 2015).

Determination of antibiotic resistance bacteria

The occurrence and spread of antibiotic resistance is an expected and important result of the wastewater discharge to the sea. Regarding the results of evaluation of the studied three hospitals with respect to the high contribution to TU and Danish guideline ranking system, antibiotics presented the most important group. Release of hospital wastewaters with antimicrobial/antibiotic residuals into the sea may cause to the development of resistant strains (Barraud et al. 2013; Harris et al. 2014). Antibiotics, therefore, should be monitored for the environmental risk assessment. The antibiotic susceptibility patterns of isolated and identified strains were investigated for the wastewater of MFH1. According to the results of microbiologic studies, 7.70×10^6 total aerobic bacteria and 1.5×10^3 total funguses were counted from 1 mL of wastewater obtained from the hospital collector system. Among them, ten clinically serious bacteria were detected by the additional identification studies. *Staphylococcus aureus* was one of them and the others belonged to the family of *Enterobacteriaceae*. The *Enterobacteriaceae* family has been linked to well-known antibiotic-resistant gene pools. These genes are transferred into the normal flora of humans and animals (Lin and Biyela 2005), where they exert a strong selective pressure for the emergence and spread of resistance in both pathogenic and commensal bacteria. Eventually, they find their way into the environment via wastewaters, manure, and sewage sludge (Dancer 2004). In the present study, based on the interpretive antibiotic resistance criteria of CLSI, the numbers of resistant strains of *Enterobacteriaceae* to ciprofloxacin, trimethoprim, and ceftazidime were 4, 6, and 6, respectively. In addition, the multiple antibiotic resistances were demonstrated with tested strains 1 and 2. Multiple antibiotic-resistant phenotypes were generated for isolates that showed resistance to three or more antibiotics (Rota et al. 1996). Moreover, *S. aureus* is a bacterial pathogen that colonizes multiple body sites, most commonly the nostrils, and causes a number of infections, including skin and soft tissue infections, pneumonia,

Table 5 Results of mutagenic activity with Ames MPF™ 98/100 assay by using bacterial strains TA98 and TA100 exposed to the wastewater samples with/without metabolic activation

Exposure concentration (%)	Mutagenicity ratio, MR (mean)*							
	Non-filtered water samples				Filtered water samples			
	TA98		TA100		TA98		TA100	
	S9−	S9+	S9−	S9+	S9−	S9+	S9−	S9+
0	0.92	1.03	1.25	1.08	0.92	1.03	1.25	1.08
0.125	1.21	*2.05*	0.96	0.91	0.95	*2.14*	0.82	0.63
0.25	0.34	*3.11**	1.14	0.86	0.85	*4.04**	0.85	0.41
0.5	0.34	*4.00**	1.28	1.13	1.03	*5.35**	0.80	0.72
1	*5.60*	*7.12**	1.21	1.31	1.62	*10.46**	0.92	*2.17*
2	*5.46*	*8.01**	1.42	1.27	1.39	*10.70**	1.09	*2.99**
4	*3.44*	*7.65**	1.67	*2.67**	1.44	*10.70**	1.24	*3.35**
Positive controls	16.28	15.90	18.04	20.52	16.28	15.90	18.04	20.52

Italic indicates the MR of ≥2

*Statistically significantly mutagenic ($p < 0.05$) as the evaluation of the Ames assay

and septicemia. Because these bacteria can be aerosolized from water and are capable of colonizing skin and soft tissues, exposure through inhalation is of concern, particularly among people who get in contact with the contaminated water. Our isolated *S. aureus* strain exhibited intermediate resistance to clindamycin and trimethoprim; however, it was susceptible to ciprofloxacin, azithromycin, and ceftazidime. The in vitro activities of the studied antibiotics against ten strains isolated from wastewaters are summarized in Table 6.

Conclusions

In this study, 38 compounds within the 55 compounds analyzed were detected in hospital wastewaters. The results of HQ_{HWW} studies conducted for each individual compound presented high-risk potential and ecotoxicological hazardousness, especially for the groups of antibiotics, analgesics, and antiinflammatories in hospital wastewaters. Cytotoxicity and mutagenicity tests were applied to both non-filtered and filtered hospital wastewater samples. Both non-filtered and filtered samples of hospital wastewater had strong cytotoxic and mutagenic effects. Multi-drug resistance to commonly used antibiotics (ciprofloxacin, trimethoprim, and ceftazidime) was also high. The water contamination by antibiotics and other compounds led to an increase in resistance owing to the selection pressure. The presence of antibiotic-resistant organisms should be taken into consideration since the release of these organisms to the sea leads to development of antibiotic resistance in marine organisms/fish and consequently in human via consuming them. The results show that the hospital wastewater in MFH1 presented the lowest HQ_{HWW} in the selected hospital wastewaters; however, MFH1 was classified as a point source for four antibiotics. This means that the characterization of hospital wastewater for each individual compound and calculation of HQ might be not enough for toxicity assessment. Therefore, in vitro toxicity screening assays with short, rapid, and effective results should be recommended instead of trying to identify one or several toxic especially cytotoxic and genotoxic compounds. This study will provide the basis for comprehensive research for hospital wastewaters in Turkey. Considering the results of the study, MFH1 was selected as the pilot hospital because of the occurrence of the various pharmaceuticals, considerable environmental risk, cytotoxic–genotoxic effects, and multi-antibiotic resistance. Since conventional wastewater treatment units are insufficient in the treatment of hospital wastewaters, membrane bioreactor (MBR) processes including advanced oxidation ($UV/O_3/H_2O_2$) and/or adsorption processes (powdered activated carbon (PAC)) should be applied to reduce the chemical load and toxicological effects of the hospital wastewaters. In our future work, complete characterization and treatment of MFH1 wastewater with MBR-PAC will be studied.

CLSI, clinical and laboratory standards; df, dilution factor; DMEM, Dulbecco's modified eagle medium; ECOSAR, ecological structure activity relationships;

Table 6 The in vitro activities of the studied antibiotics against ten strains isolated from wastewater

Microorganisms		Antibiotic inhibition zone (mm)				
Number	Strains	Ciprofloxacin	Clindamycin	Azithromycin	Trimethoprim	Ceftazidime
1.	*Klebsiella pneumoniae*	13.2 (R)	–	–	3.00 (R)	6.50 (R)
2.	*Proteus mirabilis*	11.7 (R)	–	–	3.00 (R)	20.0 (S)
3.	*Serratia marcescens*	12.2 (R)	–	–	16.4 (S)	16 (R)
4.	*Proteus mirabilis*	3.00 (R)	–	–	3.00 (R)	3.00 (R)
5.	*Escherichia coli*	23.5 (S)	–	–	16.1 (S)	19.0 (I)
6.	*Salmonella typhimurium*	31.2 (S)	–	–	9.70 (R)	16.0 (R)
7.	*Erwinia* spp.	21.7 (S)	–	–	3.00 (R)	15.0 (R)
8.	*Pantoea* spp.	25.0 (S)	–	–	21.5 (S)	22.0 (S)
9.	*Proteus mirabilis*	32.0 (S)	–	–	3.00 (R)	16.0 (R)
10.	*Staphylococcus aureus*	21.5 (S)	20.8 (I)	≥21.6 (S)	14.5 (I)	22.0 (S)

R resistant to, *S* susceptible to, *I* intermediate to tested antibiotics, – not studied

FBS, fetal bovine serum; HWW, hospital wastewater; HQ, hazard quotient; IC_{50}, half maximal inhibitory concentration; LDH, lactate dehydrogenase; MEC, measured environmental concentration; MFH, medical faculty hospital; MHA, Mueller Hinton agar; MR, mutagenicity ratio; NR, neutral red; OD, optical density; PAC, powdered activated carbon; PEC, predicted environmental concentration; PNEC, predictive no effect concentration; SDA, sabouraud dextrose agar medium; SD, standard deviation; TRH, training and research hospital; TSA, trypticase soy agar medium; TU, toxic unit; QSAR, quantitative structure activity relation; WWTP, wastewater treatment plant.

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