Research Article

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Treatment of hospital wastewater: emphasis on ecotoxicity and antibiotic resistance genes

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Abstract

BACKGROUND: Hospital wastewater (HWW) charges wastewater treatment plants (WWTPs) with a mixture of contaminants such as pharmaceutically active compounds (PhACs) and pathogenic bacteria. This matrix is considered highly toxic to the ecosystem and organisms, and it may induce the development of antibiotic resistant bacteria (ARB) and the transfer of antibiotic resistance genes (ARGs) within microbial communities. Conventional WWTPs cannot treat HWW effectively, because they have not been designed to confront this challenge. Therefore, this study investigated the applicability of photocatalysis to purify HWW, regarding its ecotoxicity and the removal rates of targeted substances, selected pathogenic bacteria and specific ARGs.

RESULTS: The HWW samples showed high toxicity towards the bioindicator *Daphnia magna* population, while they also contained significant levels of ARB and ARGs. Upon application of the photocatalytic treatment, the pharmaceutical concentrations decreased at a rate of >80% and the removal rates of the examined bacteria (*Escherichia coli*, Enterococci, *Klebsiella* sp. and *Staphylococcus* sp.) were >80%. Importantly, the bacteria remaining after photocatalysis were sensitive to the tested antibiotics. Conversely, the examined ARGs were present in high concentrations before and after photocatalytic treatment. For example, the concentrations of the selected genes, namely ampC, sul2, tetA and qnrA, in the effluents were from 10^4 to 10^6 gene copies L^{-1}.

CONCLUSIONS: Photocatalysis may be a promising treatment technique for the elimination of PhACs and pathogenic bacteria from HWW. Moreover, it proved capable of altering the antibiotic resistance profile of the bacteria surviving after treatment, making them sensitive to certain antibiotic compounds. However, the main concern regarding public health protection remains, as the presence of ARGs in effluents in considerable concentrations may induce antibiotic resistance in bacterial communities of aquatic environments.

Supporting information may be found in the online version of this article.

Keywords: hospital wastewater; wastewater treatment; antibiotics; pharmaceuticals; antibiotic resistant bacteria; antibiotic resistance genes

NOMENCLATURE

HWW	Hospital wastewater
POCW	Pathology & Oncology clinic wastewater
TWW	Total wastewater
PSE	Primary sedimentation effluent
MBRE	Effluent from MBR

INTRODUCTION

Healthcare services generate wastewater, whose concentration and composition are quite different from domestic wastewater. In particular, hospital wastewater (HWW) is characterized by higher values of biochemical oxygen demand (BOD), chemical

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oxygen demand (COD), ammonia and nitrogen than domestic wastewater, making it more difficult to treat.[1]

Depending on hospitals premises, HWW contains a wide spectrum of contaminants; among others, pharmaceutically active compounds (PhACs) such as antibiotics, analgesics, β-blockers, hormones, stimulants, antidepressants and antiepileptics have been detected.[2,3] These substances are discharged into sewer systems either in their original form or extracted as metabolites from human bodies inasmuch as the latter are capable of absorbing only a small percentage of them.[4] As a result, hospitals charge wastewater treatment plants (WWTPs) with high loads of PhACs.[5]

Besides the aforementioned contaminants, pathogenic microorganisms also are contained therein, as plenty of them might be classified under the antibiotic resistant bacteria (ARB) category. ARB are of great concern, as they are responsible for many nosocomial infections, mortality and raising treatment costs.[6] For this reason, bacteria such as the methicillin-resistant *Staphylococcus aureus* and the extended-spectrum beta-lactamase *Klebsiella pneumoniae* have been characterized by WHO as being of high priority for the development of new antibiotics.[7-9] The evolution of resistant bacteria stems mainly from one of the mechanisms of horizontal gene transfer, by way of which bacteria exchange antibiotic resistance genes (ARGs).[10] Quantities of ARB and ARGs might have originated from patients' bodies, where the coexistence of antibiotics and human microbiota facilitate their dissemination.[11] What is more, the ubiquity of residual antibiotics in this kind of wastewater could subject bacteria to selective pressure, enhancing ARG exchange and therefore the creation of new ARB.[12] Many studies have alluded to the abundance of ARB and ARGs in hospital effluents.[1,5]

In this respect, conventional WWTPs cannot treat HWWs effectively because they have not been designed to confront the challenge of their complex composition.[3,13] Thus, residual concentrations of such substances have been reported in the effluents of conventional WWTPs.[14,15] Removal efficiencies are different depending on the substances, for instance, > 90% has been reported for compounds such as the analgesic acetaminophen and the nonsteroidal analgesic drugs (NSAIDs), < 10% for carbamazepine and metoprolol, and no removal for β-blockers.[3,5] Recent studies suggest the combination of biological processes with other treatment technologies such as advanced oxidation processes (AOPs), in order to treat real HWW in a more efficient and economically feasible way.[16]

Concerning ARGs, concentrations in the order of 10^{10} copies L^{-1} have been reported in WWTPs' effluents.[6] In addition, WWTPs have been characterized as hotspots for the creation of ARB, because the presence of antibiotics and bacteria facilitates their gene transfer. Additionally, the toxicity of PhACs prevents microbial growth and hampers the degradation process in WWTPs.

The aforesaid pollutants have beenproven to cause anomalies in the reproductive system, locomotion and metabolism and, therefore, could be problematic for aquatic organisms.[3] For example, diclofenac and trimethoprim may decrease metabolic activity, whereas ciprofloxacin (CPF) and sulfamethoxazole (SMX) may be responsible for reduced growth.[13,17]

In this context, the present study investigated the characteristics of real HWW in terms of its ecotoxicity, the content of ARB and ARGs and the applicability of photocalysis to treat HWW. More specifically, the aims were (i) to assess the removal efficiency of the targeted substances, the selected pathogenic bacteria and the selected ARGs, (ii) to examine isolated strains of the bacteria for their resistance to antibiotics, and (iii) to assess the potential ecotoxicological risks using the aquatic bioindicator *Daphnia magna*.

MATERIALS AND METHODS

Real hospital wastewater samples

Real HWW samples were collected from the Venizelion General Hospital of Heraklion (Crete, Greece), which accommodates 440 beds and approximately 1000 employees. Samples were collected from two different spots, one from the Pathology and Oncology clinics effluent, and the other from the tank where waste streams from all clinics and departments converge (referred to as total wastewater for the rest of the text).

Simulated hospital wastewater samples

Certain pharmaceutical compounds were spiked to different matrices taken from the Heraklion WWTP (194 000 inhabitants equivalent) to simulate HWW, which then was subject to photocatalysis. These are (i) the effluent of the primary sedimentation tank and (ii) the effluent of a membrane bioreactor (MBR). Physicochemical parameters of the wastewaters used are presented in Supporting Information, Table S1.

Chemicals and reagents

The following pharmaceuticals were used to spike the wastewater: Acyclovir (ACV), Amoxicillin (AMX), Diclofenac sodium salt (DFNa) and with purity ≥98.5% from Sigma Aldrich (St Louis, MO, USA); analytical standards of Metronidazole (MTZ) and Valsartan (VAL) from Supelco Inc. (Bellefonte, PA, USA); Trimethoprim (TMP) and SMX with purity >98% from Acros Organics (Thermo Fisher Scientific, Shanghai, China) and TCI chemicals (Tokyo, Japan), respectively; Carbamazepine (CBZ) and Cefadroxil (CDX) with purity >95% from Alfa Aesar (Thermo Fisher, Kandel, Germany) and Carbosynth (Berkshire, UK), respectively. The initial spiked concentrations are presented in Table S2.

Two kinds of photocatalysts were used: Degussa AEROXIDE® TiO_2 P25 (Japan) and ZnO with purity >99% from Sigma Aldrich. Titanium dioxide is a widely used semiconductor in photocatalytic applications as a result of its outstanding optical and electronic properties, physicochemical characteristics, high stability against photochemical corrosion, nontoxicity and commercial availability. Moreover Degussa TiO_2 P25 presents better surface properties and higher photocatalytic activity than other TiO_2 forms, because it contains >70% of anatase crystallites, which is the most photoactive polymorph of TiO_2.[18] Zinc oxide has been proposed as an alternative catalyst to TiO_2 owing to similar properties including its excellent electrical, mechanical, optical and antibacterial performance.[19]

For the evaluation of the ARB, the antibiotics AMX, CPF (Sigma Aldrich, St Louis, MO, USA), TMP - SMX (1:19), Vancomycin (VAN) (Sigma Aldrich, St Louis, MO USA) and Tetracycline (TCL) (Sigma Aldrich, St Louis, MO, USA) were used.

Ecotoxicity

For ecotoxicity assessment, the crustacean *D. magna* was selected as the bio-indicator. Bioassays were performed using the Daphtoxkit F Kit (Microbiotests, Gent, Belgium) following the manufacturer's protocol and exposing the population of *D. magna* to the wastewaters for 24 and 48 h.

Microbiological quality of wastewater

The isolation of selected bacteria was performed by filtration through nitrocellulose membranes (0.45-μm pore size, 47 mm diameter; Whatman GmbH, Dassell, Germany) followed by plating on selective media and incubation at 37 °C. The media used were Hi Crome Agar (HiMedia Laboratories GmbH, Einhausen,

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Germany), Slanetz & Bartley Medium (HiMedia), Klebsiella selective medium and Manitol Agar for *Escherichia coli*, Enterococci, *Klebsiella* sp. and *Staphylococcus* sp. isolation, respectively. Viable counts were performed after 24 h for *E. coli*, *Klebsiella* sp. and *Staphylococcus* sp., and after 48 h for the Enterococci. The latter also were confirmed by transferring the membranes onto Bile Aesculin Agar (HiMedia) followed by incubation at 44 °C for 2 h. Typical colonies of *Klebsiella* sp. and *Staphylococcus* sp. grown on the media were chosen for further identification and confirmatory tests. Biochemical identification was performed using the API® 20 E and Staph test (Biomérieux, Marcy-l'Étoile, France) for the identification of *Klebsiella* sp. and *Staphylococcus* sp., respectively.

Assessment of antibiotic resistance

Selected colonies of *E. coli*, Enterococci, *Klebsiella* sp. and *Staphylococcus* sp. were tested for antibiotic resistance using the broth microdilution method and the MIC_{60} (i.e. the minimum concentration needed for 60% reduction in bacterial population) was estimated. Sterile 96-well micro titre plates were labelled with the appropriate concentrations of each antibiotic. Each well was inoculated with the bacterial strain, with a final concentration of 10^5 CFU mL^{-1}. Microtitre plates were incubated at 37 °C for 18–24 h, followed by optical density measurement at 630 nm, using a microplate reader (EZ Read 400; Biochrom Ltd, Cambridge, UK) and GALAPAGOS software. Susceptibility/resistance breakpoints were determined in compliance with EUCAST (European Committee on Antimicrobial Susceptibility Standards) and CLSI (Clinical and Laboratory Standards Institute criteria).[20,21]

The concentration of each antibiotic that the bacteria were deemed resistant to is presented in Table 1.

Nucleic acid extraction and quantitative PCR-detection of target ARGs

Total DNA was collected from hospital samples by centrifuging 40 mL wastewater at 4000 × *g* for 20 min. In simulated wastewater, samples were divided into cell-associated DNA and cell-free eDNA. The entire DNA extraction process was performed using the NucleoSpin® Soil (Macherey-Nagel GmbH & Co. KG, Dueren, Germany) kit. For cell-associated DNA, 10 mL sample were filtered through 0.22-μm nylon membranes (Branchia) and the filter was washed with phorphate-buffered saline (PBS) to limit the possibilities of non-cell or phage-related DNA presence thereon. The filter was sliced into small pieces for cell-associated DNA extraction from the filters.[22]

The filtrate (10 mL) was used for the extraction of cell-free DNA (eDNA), applying a standard precipitation method. Specifically, a mixed solution was prepared containing the filtrate, 1 mL of 3 mol L^{-1} sodium acetate (pH 5.5) and 20–30 mL ethanol 95–100% (v/v), and stored at −20 °C overnight. The precipitates were obtained by centrifugation at 10 000×*g* for 10 min at 4 °C and afterwards the supernatant was discarded.[23] The DNA extraction was performed using the abovementioned kit. The quantity and purity of the extracted DNA were determined by absorbance measurement at 260 and 280 nm (BioPhotometer® D30; Eppendorf, Hamburg, Germany). All samples were stored at −20 °C before analysis.

Real-time quantitative PCR assays were used to quantify the target resistance genes (Table 2). The concentrations of the ARGs were estimated by the SYBR green method using the StepOne Plus System (Applied Biosystems/Therm Fisher Scientific). Real-time PCR reactions were carried out in a 20-μL reaction mixture consisting of 1.0 × SYBR Green Master Mix (Kapa Biosystems, Potters Bar, UK), 2 μL DNA template and 200 nmol L^{-1} primers for ampC, sul2 or tetA quantification, and 400 nmol L^{-1} of primers for qnrA detection. All PCR reactions were run in triplicate. Standard curves were generated using bacterial reference strains, namely *E. coli* DSM 498 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) and *K. pneumoniae* NCTC 5056 (Public Health England Culture Collections). Cycling conditions and sequences of the primers are shown in Table 2.

Table 1. Minimum inhibitory concentrations (MIC_{60}; mg L^{-1}) of the antibiotics to which bacteria in the wastewaters were considered resistant

Antibiotic	*E. coli*	Enterococci	*Klebsiella* sp.	*Staphylococcus* sp.
Trimethoprim-Sulfamethoxazole (1:19)	>4	>1	>8	>4
Ciprofloxacin	>0.5	>4	>0.5	>1
Amoxicillin	>8	>8	>8	≥0.25
Tetracycline	>4	≥16	>4	>2
Vancomycin				>2

Photocatalysis

The interior of the in-house constructed photocatalytic reactor is made of stainless steel, resulting in a mirror-like appearance that promotes the reflectance of the incident irradiation. A magnetic stirrer is fitted in the centre of the reactor, while aeration is supplied by fans that operate only when the temperature reaches a specific value defined by the user, thus maintaining a constant temperature in the interior of the photocatalytic reactor. Four UV-A black light lamps (TL 4W BLB 1FM/10X25CC; Philips, Eindhoven, the Netherlands) with a main emission line at 365 nm served as the source of irradiation.

Two matrices, according to the section of 'simulated wastewater samples' (the effluent of the primary sedimentation tank of WWTP and the effluent of a MBR) were filtered with 0.7μm glass fibre filters (Merck Serono Ltd, Dublin, Ireland) before the addition of the mixture that contained all nine pharmaceutical compounds in order to avoid the interference of solid materials with the photocatalytic process. The initial concentration of each pharmaceutical to the final working volume is presented in Table S2. A volume of 400 mL matrix was used for each experiment and placed into a beaker, suitable for UV irradiation, in which 200 mg L^{-1} catalyst were added. Samples were collected before and after 3 h of irradiation from the reaction mixture and then they were filtered through 0.7μm glass fibre filters (Merck Serono Ltd, Dublin, Ireland). All the experiments were conducted in triplicates.

Analytical methods

Pharmaceuticals were analyzed using a LC20-AD liquid chromatographer (Shimadzu, Kyoto, Japan) associated with a SPD-M20A prominence diode array detector (LC-DAD) and a SIL-20AC auto sampler. A Zorbax SB-C18 4.6 mm × 15 cm (5 μm) connected with a Zorbax SB–C18 pre-column (Hewlett Packard, Palo Alto, CA, USA) was used to achieve separation. Column and pre-column were heated at 35 °C in a CTO-20AC column oven (Shimadzu). The mobile phase consisted of H_2O with 0.01% formic acid (solvent A) and methanol (MeOH, solvent B). Flow-rate was 0.5 mL min^{-1}. Gradient elution was performed as follows: from 15% MeOH to 90% in 10 min, hold at 90% for 8 min and then decrease to 15%

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Table 2. Real-time PCR primers and cycling conditions

Target gene	Primer sequence (5′→3′) Forward/Reverse	Product size (bp)	Cycling conditions	Reference
ampC	TTCTATCAAMACTGGCARCC/CCYTTTTATGTACCCAYGA	550	94 °C for 5 min; 35 cycles: 94 °C for 30 s, **49** °C for 30 s and 72 °C for 1 min	24
sul2	GCGCTCAAGGCAGATGGCATT/GCGTTTGATACCGGCACCCGT	293	94 °C for 5 min; 35 cycles: 94 °C for 15 s, **69** °C for 30 s and 72 °C for 1 min	25
tetA	CGATATCACTGATGGCGATG/GTCCGACAAGTTGCATGAT	318	94 °C for 5 min; 35 cycles: 94 °C for 1 min, **55** °C for 1 s and 72 °C for 5 min	26
qnrA	GATAAAGTTTTTCAGCAAGAG/ATCCAGATCGGCAAAGGTTA	543	95 °C for 5 min; 35 cycles: 94 °C for 1 min, **64** °C for 30 s and 72 °C for 1 min	27,28

Note: Temperatures in bold are the annealing temperature of the primers.

in 1 min and hold there for 14 min. The diode array detector (DAD) was set at 210 nm.

The pH value and temperature of samples were measured using a C932 (Consort BVBA, Turnhout, Belgium) portable electrochemical analyzer. Chemical oxygen demand (COD), total phosphorus (TP), phosphate and ammonium nitrogen (NH_4-N) were measured according to Standard Methods.[29]

RESULTS AND DISCUSSION

Real hospital wastewater

Ecotoxicity

Figure 1 shows the ecotoxicity of real HWW in relation to its effect towards a *D. magna* population. Generally, the wastewater from the Pathology and Oncology clinics showed higher toxicity (100% immobilization) than the total HWW (60–70% immobilization). This may be attributed to the fact that the wastewater from the Pathology and Oncology clinics contained higher concentrations of contaminants than the total wastewater. The extent of toxicity decreased when the samples were diluted at various ratios (i.e. from 6.25 to 50%) but still remained at considerable values.

This has been discussed in a previous study,[30] where 122 compounds were analyzed in terms of detection frequency and concentration, whereas the gross physicochemical properties of the two wastewaters also were given. The concentrations and frequency of detection of selected contaminants are presented in Figs S1 and S2. In brief, although the two wastewaters exhibited similar COD values, the concentrations of the individual compounds predominant in the two streams were substantially different; in fact, the concentration in the Pathology and Oncology clinic wastewater was 2–3-fold greater than in the total wastewater.

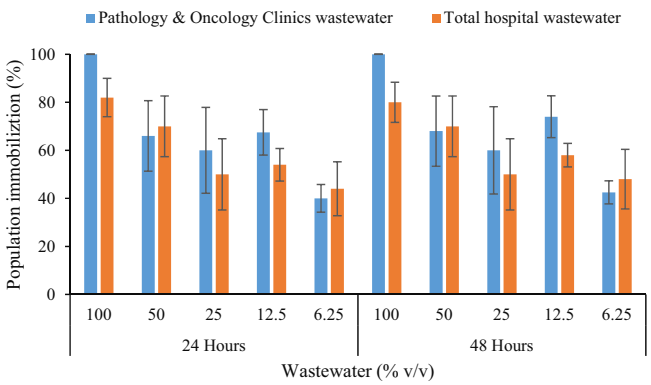

Figure 1. *Daphnia magna* immobilization after exposure to HWW for 24 and 48 h at various wastewater dilution ratios.

The above results confirm that substances that are present in HWW could be toxic even at low concentrations. Many studies have alluded to the adverse impacts of these substances on the aquatic bioindicators, such as *D. magna* and *Vibrio fischeri*.[31,32]

Microbiological quality of wastewater and detection of antibiotic-resistant bacteria

The examined bacteria were expectedly found at high concentrations in HWW. The concentrations of *E. coli*, Enterococci, *Staphylococcus* sp. and *Klebsiella* sp. in Pathology and Oncology clinic wastewater were 7×10^4, 5×10^6, 2×10^2 and 8×10^4 CFU 100 mL^{-1}, respectively, and 7×10^5, 9×10^6, 10^3 and 3×10^5 CFU 100 mL^{-1} in total wastewater. These results are in accordance with previous studies reporting similar concentrations from hospitals in Europe.[1,33]

The MIC analysis demonstrated that among the bacteria counted, high numbers of ARBs were present. According to Fig. 2, *E. coli* showed the highest percentage of resistant isolates followed by enterococci, *Klebsiella* sp and *Staphylococcus* sp. In particular, most of the strains showed resistance to AMX, and then to TCL, CPF and SMX. Strains of *Staphylococcus* sp. showed sensitivity to CPF and SMX, whereas no obvious differences occurred between the sources of wastewater.

Moreover, a high percentage of the examined isolates seemed resistant to two or more of the selected antibiotics. Thus, these strains could be characterized as multidrug-resistant (MDR) bacteria. According to Fig. 3, most of the MDR strains belong to *E. coli* followed by *Staphylococci*, Enterococci and *Klebsiella* sp. Also, the biochemical tests showed that among the MDR *Staphylococci* strains, 25% corresponded to *S. aureus*.

It is clear that not only did the HWW contain high concentrations of pathogenic bacteria, but also a high percentage thereof seemed to be resistant to more than one of the examined antibiotics. The MDR bacteria are of great concern for the public health as they could enforce nosocomial infections, which have been proven to require hard and costly treatment. *Staphylococcus aureus* is the most virulent species of the genus, and changes in its drug susceptibility profile have been reported;[7] it also has been

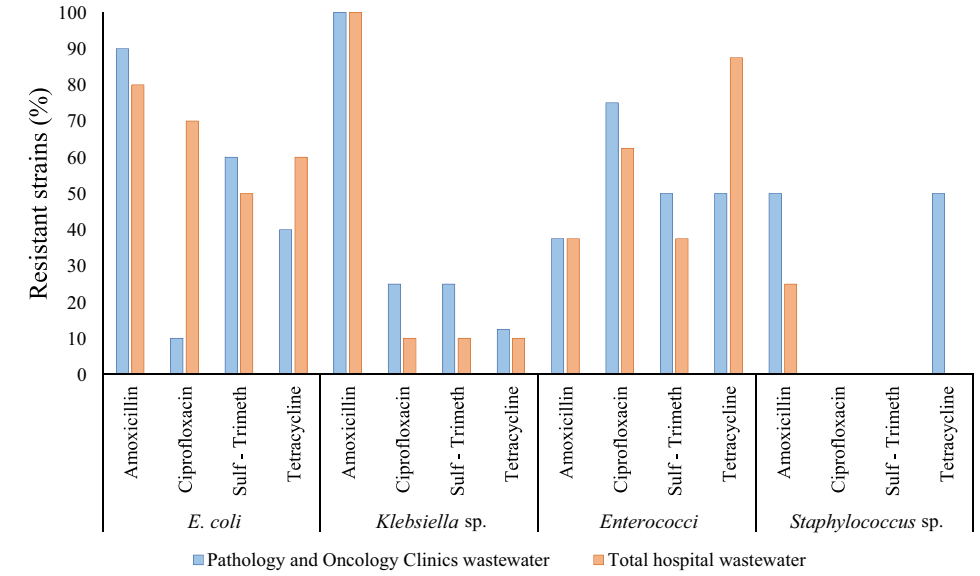

Figure 2. Percentage of resistant strains of the isolated bacteria to the examined antibiotics.

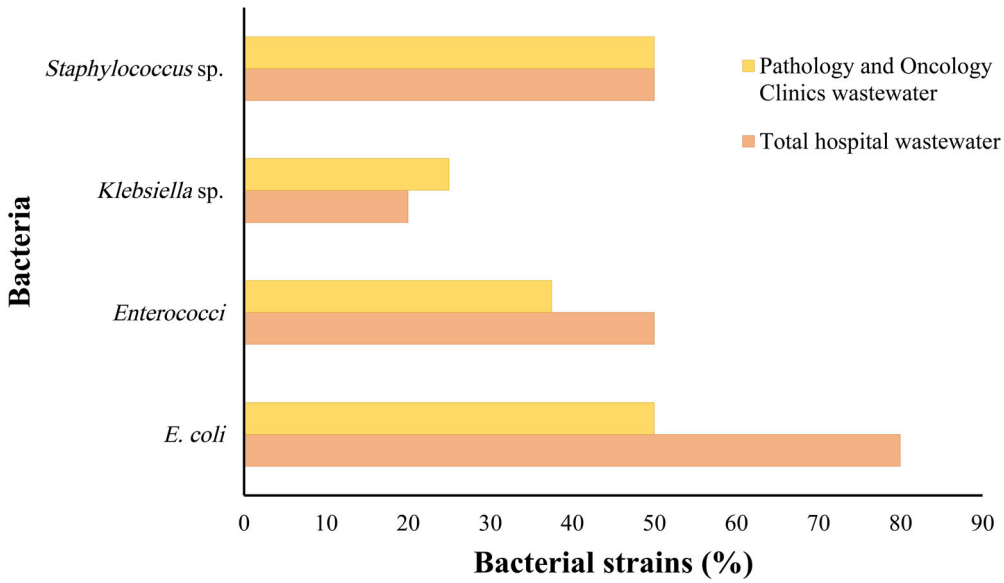

Figure 3. Percentage of bacteria strains showing resistance to two or more antibiotics.

detected in treated HWW and its competency to escape treatments also has been observed.[34] Moreover, antibiotic resistance genes are located on the mobile genetic elements in *S. aureus*, so it can be assumed that these genes could be transferred to other pathogenic bacteria in the wastewater environment.[34]

ARG occurrence and quantification

Quantification of ARGs (Fig. 4) showed that the resistance genes appear in abundance in HWW. The *sul2* gene was quantified at the highest concentrations followed by *tetA*, *ampC* and *qnrA*, while the examined genes were detected in all samples except for the *qnrA*. No important differences were observed between the concentrations of ARGs in the Pathology and Oncology clinic wastewater in comparison with the total wastewater.

It is notable that the two sampling spots gave similar concentrations of ARGs; therefore, it can be hypothesized that the Pathology and Oncology clinic wastewater (which is a fraction of the total wastewater) is the main contributor of the ARGs in the total wastewater.

High concentrations of ARGs in HWW also have been reported in other studies.[35,36] ARGs eventually reach WWTPs, thus contributing to the creation of suitable conditions for the proliferation of ARB.

Simulated hospital wastewater

Contaminant removal

Figures 5 and 6 show the extent of photocatalytic removal of the various pharmaceuticals spiked in primary sedimentation and MBR effluents, respectively. In general, degradation in the MBR

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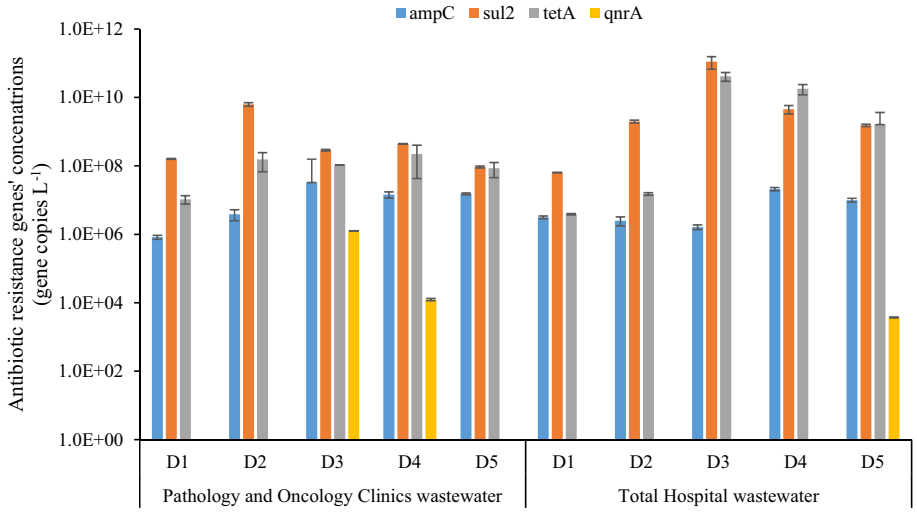

Figure 4. Concentrations of the examined ARGs in the hospital wastewaters on five different sampling days (D1 to D5).

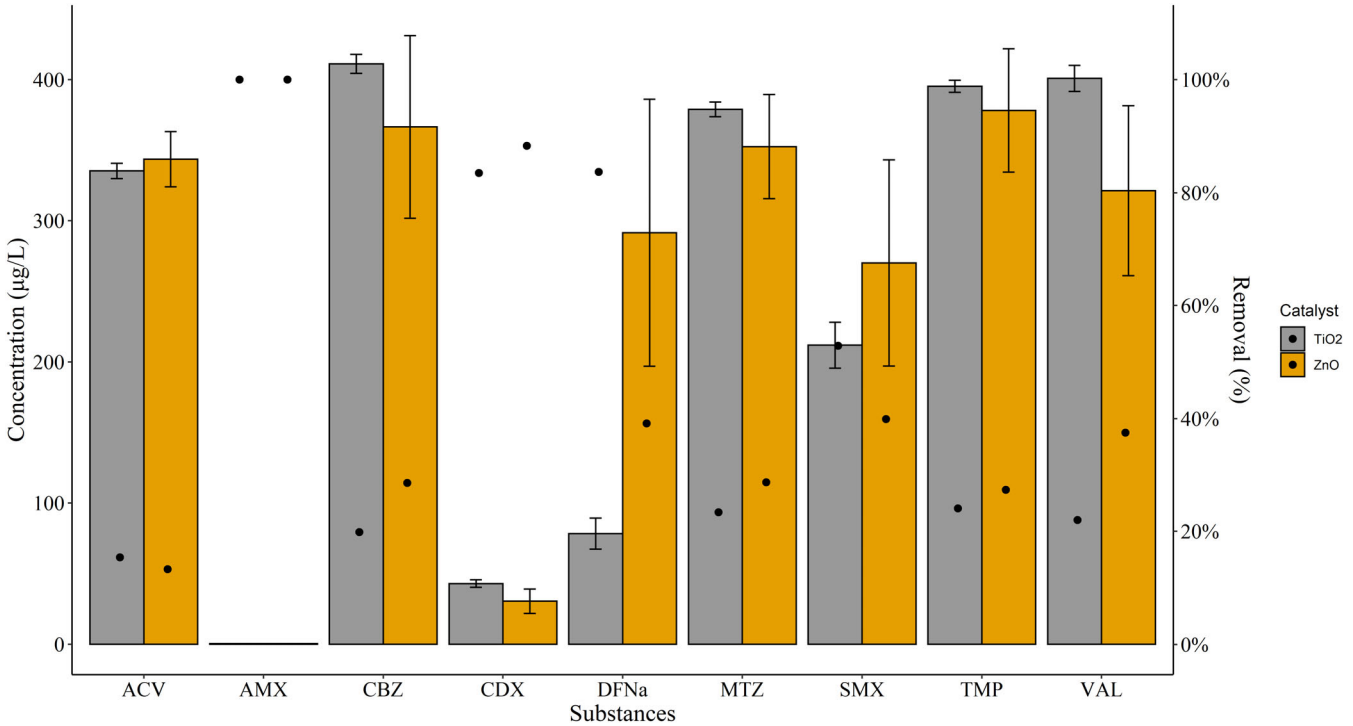

Figure 5. Photocatalytic removal for each pharmaceutical spiked in the primary sedimentation tank effluent after 3 h irradiation with P25 TiO_2 and ZnO. Bars indicate the final concentrations of substances and dots indicate the substance removal.

effluent was more effective than in the primary sedimentation tank, although both photocatalysts exhibited similar activities. Regarding the pharmaceuticals tested in this work, CDX and AMX were the more susceptible in photocatalysis in either matrix and photocatalyst used, yet ACV exhibited substantial resistance to photocatalytic degradation.

The use of TiO_2 resulted in high decrease percentages (>80%) for SMX in both matrices and DFNa for the second matrix. Concerning VAL and CMZ, the decrease reached up to 60% for TiO_2 and 50% for ZnO. Similar results were observed for TMP and MDZ, whereas ACV presented the lowest efficiency of all tested compounds for both matrices.

Based on the above results, it can be assumed that photocatalytic treatment is capable of considerably decreasing the concentration of pharmaceuticals. This is in accordance with other studies, reporting degradation as high as 99%, 100%, 90%, 88%, 100%, 95%, 100%, 95% and 90%, for CPF, erythromycin, trimethoprim, TCL, SMX, paracetamol, naproxen, atenolol and metoprolol, respectively.[1] Notably, various pharmaceuticals exhibit different reactivities to photocatalytic treatment because their molecular structures may induce different degradation mechanisms and pathways.[37]

Moreover, results support the fact that the quality of the water matrix is a crucial factor for photocatalysis because various interferences are likely to occur between photocatalysts, oxidants,

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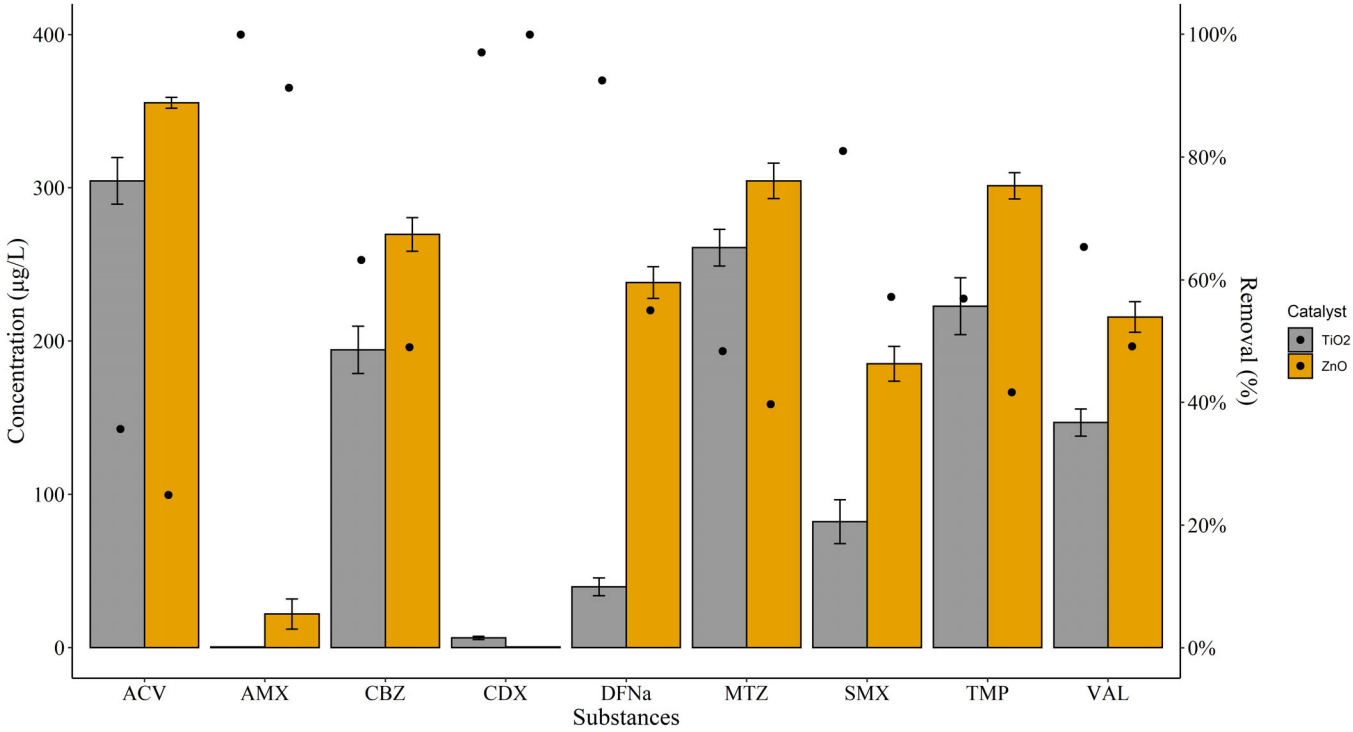

Figure 6. Photocatalytic removal for each pharmaceutical spiked in the MBR effluent after 3 h of irradiation with P25 TiO_2 and ZnO. Bars indicate the final concentrations of substances and dots indicate the substances removal.

the inherently present nontarget species and the target pharmaceuticals; such complex interactions usually negatively affect degradation.[38]

Ecotoxicity

The ecotoxicity of either matrix (i.e. primary sedimentation or MBR effluent) to *D. magna* was quite low because only 20% population immobilization was recorded. Noticeably, this did not change after the addition of the pharmaceutical cocktail. Photocatalytic treatment with either photocatalyst did not affect the sample's ecotoxocity although most of the spiked pharmaceutucals were effectively removed; this implies that the residual, yet quite low, ecotoxicity is associated with other substances in the matrix that either were originally present and were unaffected by the photocatalytic treatment and/or formed as degradation by-products. In real matrices, the existence of several different substances even at minute concentrations may lead to enhanced ecotoxicity as a result of synergistic effects that, to a great extent, remain unknown because the contributing substances are difficult to identify. For example, a mixture of CBZ and clofibric acid was reported to be more toxic to *D. magna* than the individual compounds at the same concentration.[39]

Microbiological quality of wastewater and detection of antibiotic resistant bacteria

The effective photocatalytic degradation of pharmaceuticals was accompanied by a substantial removal of the selected bacterial indicators, in terms of *E. coli*, Enterococci, *Klebsiella* sp. and Staphylococci. Treatment in the primary sedimentation effluent through photocatalysis resulted in >90%, 80% and 77% removal of *E. coli*, Staphylococci and *Klebsiella* sp. (respectively) with TiO_2, whereas the corresponding values were 96%, 80% and 80% for ZnO. Enterococci were not detected after treatment with either photocatalyst. Regarding experiments in the MBR effluent, complete elimination of all bacteria except Staphylococci occurred; a few remaining colonies of the latter were detected in the effluents. No ARB were detected at any stage of the photocatalysis experiments. This is an important finding because ARB are a growing global problem creating potential risks for public health.[40] Not only do ARB cause risks arising from their direct contact with humans, but also from the coexistence of ARB with environmental bacterial communities that could facilitate their dissemination in ecosystems and possibly induce their transfer into the food chain. Although photocatalytic treatment does not completely eliminate the tested antibiotics, it is a suitable technology to restrict the dissemination of ARB in ecosystems. However, it should be pointed out that even low concentrations of antibiotics in the environment could cause selective pressure and activate the SOS mechanism of bacteria. Thus, it is crucial to take into consideration not only the removal rates upon treatment, but also the final concentrations of contaminants dispersed into the environment.[41]

ARG occurrence and quantification

Figure 7 shows the concentration of ARGs before and after photocatalytic treatment in the primary sedimentation effluent. The examined ARGs were present at high quantities in samples from bacterial DNA and remained unchanged after treatment with either photocatalyst. The concentration of genes in the effluents were 10^6 gene copies L^{-1} for *ampC* and *tetA*, and 10^4 gene copies L^{-1} for *sul2* and *qnrA*.

Results from eDNA showed that ARGs were detected at higher concentrations, almost by two orders of magnitude, in comparison with the bacterial DNA. No differences in the quantities were observed following photocatalytic treatment

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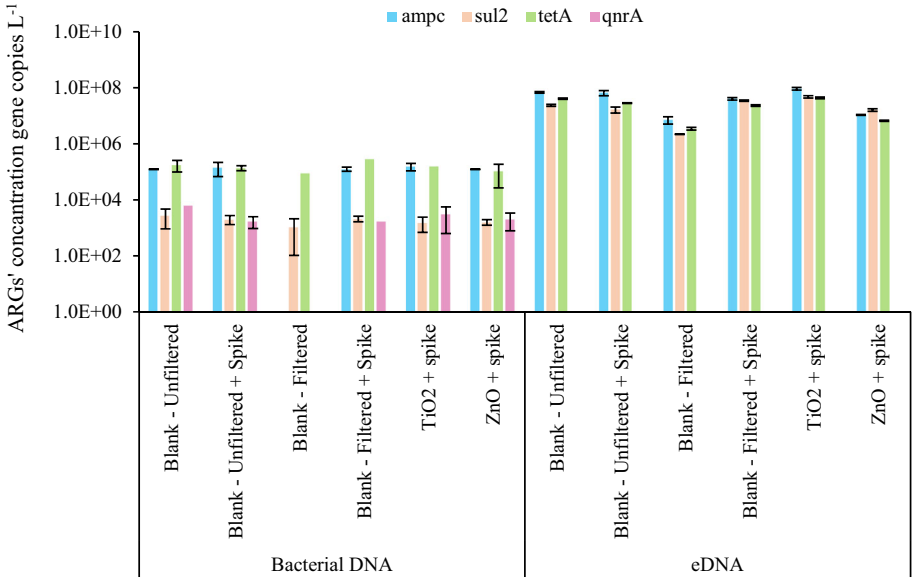

Figure 7. Concentrations of ARGs from bacterial DNA and eDNA following photocatalytic treatment in the primary sedimentation effluent.

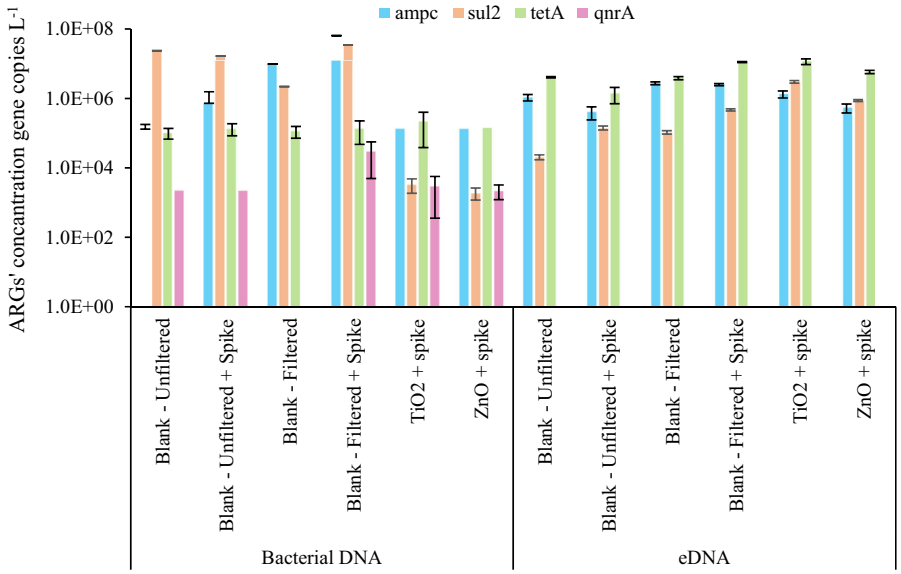

Figure 8. Concentrations of ARGs from bacterial DNA and eDNA following photocatalytic treatment in the MBR effluent.

with TiO_2. In fact, the *ampC* and *qnrA* genes were quantified at higher values post-treatment. This did not happen post-treatment with ZnO, where all ARGs showed a slight decrease.

Figure 8 shows the concentration of ARGs before and after photocatalytic treatment in the MBR effluent. Photocatalytic treatment seemed to have an influence on bacterial DNA, namely a decrease in the concentration of all examined ARGs. This could be due to the fact that MBR effluent contains higher concentration of humic acids and other dissolved inorganic matter that increase turbidity and inhibit the permeation of UV radiation. Conversely, the concentration of ARGs from eDNA samples were not affected by the photocatalytic treatment. Generally, contradictory results have been reported in the literature regarding photocatalytic treatment. For example, the study by Karaolia *et al*. showed that the concentration of *sul1* and *ampC* genes remained unaffected after photocatalytic treatment with simulated solar light and TiO_2, whereas the *ermb* gene was eliminated.[42]

Regarding the presence of ARGs in samples from eDNA, there were no differences before and after the treatment with TiO_2; treatment with ZnO led to a slight decrease in *ampC* and *tetA*, whereas *qnrA* was not detected. The final concentrations were 10^6 gene copies L^{-1} for *ampC* and *sul2*, and 10^8 gene copies L^{-1} for *tetA*. In the case of eDNA, the ARG concentrations in the MBR effluent were lower than those in the primary sedimentation tank.

CONCLUSIONS

Healthcare services constitute the main source of antibiotics entering the WWTPs, but their treatment in conventional units seems to be problematic. This is of great interest because WWTPs

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are the last barrier before their discharge into the environment, so the quality of their final effluent is critical to the ecosystems and consequently to public health.[4]

Results of the present study confirm that wastewaters containing the abovementioned groups of contaminants could have a hazardous impact on aquatic ecosystems and increase risks to public health and safety. It is mandatory that HWW be treated so as to eliminate their toxic effects[43] and prevent the dissemination of potential ABR strains and their related genes.[34,36]

Different methods, such as ozonation, chlorination and UV disinfection, have been examined in the literature for the efficient treatment of HWW. In this work, heterogeneous photocatalysis induced by UVA irradiation was applied in simulated effluents spiked with several pharmaceuticals and the effectiveness was promising in reducing the concentration of antibiotics and bacteria substantially. Conversely, ARGs remained at high concentrations in the effluents post-photocatalytic treatment, despite the elimination of the examined bacteria.

In a nutshell, coupling conventional treatment in WWTPs to a final advanced photocatalysis treatment stage is probably a step to the right direction.[44] This approach is capable of decreasing ecotoxicity and the concentrations of emerging contaminants and ARB,[45,46] preventing the fostering of new ARB strains.[5,47,48] What is more, photocatalysis with the reported catalysts may be applied under simulated or natural solar light, exploiting the UVA part of the irradiation and making this method even more sustainable and environmentally friendly.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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