The influence of antibiotics on wastewater treatment processes and the development of antibiotic-resistant bacteria

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ABSTRACT

The influence of antibiotics, namely doxycycline, gentamicin, penicillin, nitrofurantoin, and rifampicin, on wastewater treatment was assessed. The presence of 100–300 μg/L of antibiotics (63.52–134.41 mg/g.d.w·d) marginally influenced organic matter degradation, without impacting nitrogen or phosphorus concentrations. However, a significant increase in the number of antibiotic-resistant bacteria was observed, which varied with different antibiotics. The largest number of bacteria became resistant to nitrofurantoin and penicillin. After the process, some multi-resistant strains were isolated from the sludge. Two of them revealed the activity of carbapenamase, the enzyme directly related to resistance against β-lactam antibiotics.

Key words | antibiotics, β-lactamase, microbial resistance, wastewater treatment

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INTRODUCTION

Over the last five years a systematic growth of the pharmaceutical industry has been observed. Pharmaceutical and biotechnology companies are market leaders in terms of innovation world-wide. The industry's global revenue in 2010 totaled 875 billion USD in annual sales (Vazquez-Roig *et al.* 2013). Antibiotics are one of the most popular pharmaceuticals used in veterinary care, medicine, and farming (Baquero *et al.* 2008; Bergeron *et al.* 2015). Therefore, they can appear as contaminants in wastewater, soil, surface and ground water, municipal sewage, and in the influents and effluents of wastewater treatment plants (Kümmerer 2009). These pharmaceuticals are biologically active substances that can adversely affect ecosystems. Antibiotics have been formulated to kill or inhibit the growth of target microbial organisms and persist in the host organism (Fent *et al.* 2006). They are excreted in feces or urine in the form of metabolites or as the parent compounds (Zupanc *et al.* 2013). Bacterial resistance towards antibiotics is a protective mechanism, which allows bacteria to gain an advantage and survive in the environment (Russell *et al.* 1999). Microorganisms can acquire resistance against antibiotics through mutation or gene transfer (Łebkowska 2009). The available data show that conventional wastewater treatment does not completely eliminate antibiotics or their metabolites. For example, the concentrations of tetracycline and sulfonamide in raw wastewater in China were 1,129.2 ng/L and 1,535.9 ng/L, respectively, and the decrease in their concentration during the wastewater treatment was 42.2% for tetracycline and 83% for sulfonamide (Gao *et al.* 2012). β-lactam antibiotics are one of the most common bactericidal pharmaceuticals. They include several types based on similar chemical structure: carbapenems, penicillins, monobactams, cephalosporins and β-lactamase inhibitors. The antibiotic resistance of some bacteria can be related to the presence and activity of β-lactamases. It has been shown that gram-negative bacteria capable of producing extended-spectrum β-lactamases (ESBL) and metallo-β-lactamases (MBL) are responsible for numerous infections (Rzewuska 2009).

Most of the published studies in the literature focus on the fate and elimination of antibiotics from wastewater at sewage treatment plants (Ghosh *et al.* 2009; Le-Minh *et al.* 2010; Jelić *et al.* 2012; Michael *et al.* 2013). However, there are still scarce data concerning the behavior, change in numbers, and occurrence of multi-resistant bacteria in relation to the effectiveness of wastewater treatment in the presence of antibiotics.

This research is the first laboratory-scale simulation of wastewater treatment with increasing concentrations of the antibiotics doxycycline, nitrofurantoin, gentamicin,

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rifampicin, and penicillin, allowing the ability to trace the changes in microbial resistance during the wastewater treatment process. Furthermore, the multi-resistance profile and presence of carbapenamases were examined for the predominating strains.

MATERIALS AND METHODS

The sample of activated sludge was collected in October 2015 from a municipal wastewater treatment plant receiving wastewater from hospitals, urban households, industries, and other facilities. The sample was stored in a 5 L sterilized bottle and transported to the laboratory for processing and microbiological analysis.

The wastewater treatment process was carried out in a laboratory-scale activated sludge bioreactor (4.8 L). It operated in four cyclic phases: filling, aeration, sedimentation, and decantation. In the experiment, synthetic wastewater (imitating domestic sewage) was used for feeding the activated sludge. The composition of the synthetic wastewater was as follows:

- casein-peptone 226 mg/L
- nutrient agar 152 mg/L
- ammonium chloride 20 mg/L
- sodium chloride 7 mg/L
- calcium chloride 7.5 mg/L
- magnesium sulfate 2 mg/L
- potassium phosphate monobasic 16.9 mg/L
- potassium phosphate dibasic 40 mg/L

Treatment with antibiotics started after two weeks of adaptation of the sludge, accompanied by monitoring of physico-chemical parameters. Five commonly used antibiotics (doxycycline, gentamicin, penicillin, nitrofurantoin, and rifampicin) were added to the sludge. A mixture of antibiotics in equal amounts was added to achieve the total concentration of 100 μg/L, 200 μg/L, 300 μg/L at the beginning of the third, fifth, and seventh weeks, respectively. The total antibiotic load was 63.52 mg/gdm·d, 84.00 mg/gdm·d and 134.41 mg/gdm·d. The experiment was carried out for 7 weeks.

The control parameters of the process covered chemical oxygen demand (COD) (HACH cell test), total nitrogen content (HACH cell test), phosphorus content (molybdate method, HACH 8114), sludge dry matter concentration (according to PN-PN-78/C-04541), microscopic observations of the sludge biocenosis, and microbiological assays.

The chemical analyses and microscopic observations were performed once a week.

Microbiological analyses were performed after four, six, and seven weeks in duplicate. They covered the determination of the total number of bacteria in activated sludge, as well as the number of bacteria resistant to individual antibiotics. Bacteria were cultivated on nutrient agar medium, with the addition of individual antibiotics (if applicable), for 24 hours at 37 °C. The results were given as the average number of colony forming units per milliliter (CFU/mL).

The predominating strains of antibiotic-resistant bacteria were isolated from the activated sludge at the end of the experiment. They were purified and tested for resistance to a broad spectrum of commonly used antibiotics. Antibiograms were performed using 12 pharmaceuticals: amikacin, minocycline, tigecycline, polymyxin, cefadroxil, azithromycin, ampicillin, gentamicin, rifampicin, doxycycline, nitrofurantoin, and vancomycin. Five parallel measurements of the zones of inhibition were carried out, and the average zone diameters and standard deviations were calculated for active antibiotics. The bacterial resistance/sensitivity was evaluated according to data from The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2014).

The enzymes involved in the antibiotic resistance: carbapenamase, metallo-β-lactamase, and extended-spectrum β-lactamase were assessed using the double-disc method (Rosco). Selected strains of penicillin-resistant bacteria (potentially containing the enzymes responsible for the resistance to β-lactam antibiotics) were applied in the test. The presence of the enzymes was evaluated based on the zone diameters around the discs with meropenem, meropenem + phenylboronic acid (carbapenamase inhibitor), meropenem + dipicolinic acid (metallo-β-lactamase inhibitor), and meropenem + cloxacillin (AmpC inhibitor). Average values of five determinations and standard deviations were calculated. The results were interpreted according to the test producer's instructions.

RESULTS AND DISCUSSION

The concentration of antibiotics in wastewater can reach several milligrams per liter (Hirsh *et al.* 1999). It usually decreases during the wastewater treatment process, although a certain amount of antibiotics and their metabolites can still remain in surface waters (Kümmerer 2009).

In this research, the concentration of antibiotics was not extremely high (100–300 μg/L), but they were introduced into the activated sludge reactor in a comparatively short period of time achieving antibiotic loads of 63.52 mg/g.d.w·d to 134.41 mg/g.d.w·d.

There are some data in the literature suggesting that antibiotics can have an effect on biological wastewater treatment. The analysis of basic parameters in this study revealed that the organic matter content in the activated sludge bioreactor was not affected by the presence of antibiotics. After the initial drop at the beginning of the process (without antibiotics), it remained at a similar level (2.5–3.5 g/L) throughout the experiment (Figure 1). The proper functioning of the sludge was confirmed by microscopic observation. No impact of antibiotics on the structure of activated sludge biocenosis was revealed. It was characterized by the typical biological diversity with regular sludge flocs. Increases in the number of filamentous bacteria were not observed.

There is some evidence that antibiotics can influence nitrogen metabolism, although these results are inconclusive. Ghosh *et al.* (2009) observed no effect of individual antibiotics (50 μg/L) on ammonia oxidation, although antibiotics in combination revealed a stronger impact on the wastewater treatment process. Campos *et al.* (2001) described a 50% inhibition of the nitrification process with the stepwise increase of the oxytetracycline concentration from 100 to 250 mg/L; the effect was not observed in concentrations below 100 mg/L. No inhibition of the nitrification process was noticed for chloramphenicol 10–250 mg/L. Ampicillin and benzylpenicillin did not inhibit the nitrification process, up to 250 mg/L (Gomez *et al.* 1996). No effect of oxytetracycline and sulfathiazole on anaerobic ammonium oxidation was observed (Lotti *et al.* 2012). Chen *et al.* (2015) stated that 2–5 mg/L of tetracycline caused a 20% decrease in nitrogen removal, due to the negative impact on denitrifiers. Schmidt *et al.* (2012) noticed the inhibition of nitrate formation with 30 mg/L of antibiotics.

In our research, the nitrogen concentration in the effluent varied during the experiment (Figure 2). A decrease in nitrogen concentration was noticed at the beginning of the dosage of antibiotics and after 6 weeks of the process. The phenomenon is difficult to interpret, because, according to the literature data quoted above (Campos *et al.* 2001; Schmidt *et al.* 2012), the most common effect of several antibiotics is the inhibition of the nitrification process, resulting in increased nitrogen concentration in the effluent. This negative effect was not observed in our study and the nitrogen concentration for the highest antibiotic load was even lower than in the effluent at the beginning of the experiment (without antibiotics).

The concentration of phosphorus in the effluent was at a similar level throughout the experiment (Figure 3). According to published data, antibiotics do not influence the elimination of phosphorus from wastewater. Chen *et al.* (2015) observed no effect with 2–5 mg/L of tetracycline on the effectiveness of phosphorus removal.

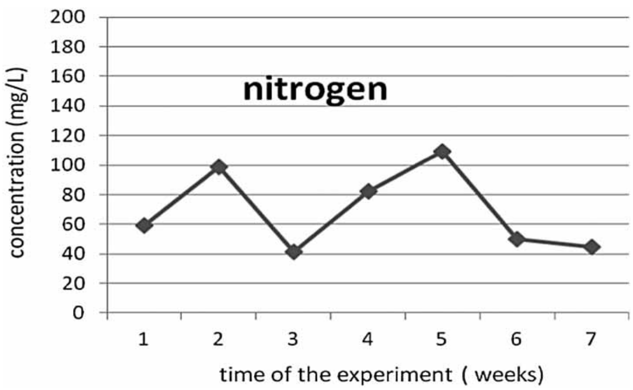

Figure 2 | Changes in nitrogen concentration during the process.

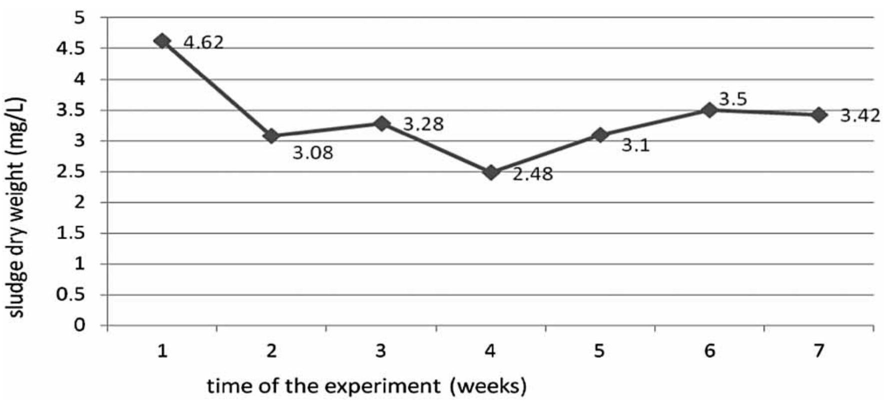

Figure 1 | The sludge dry weight during the experiment.

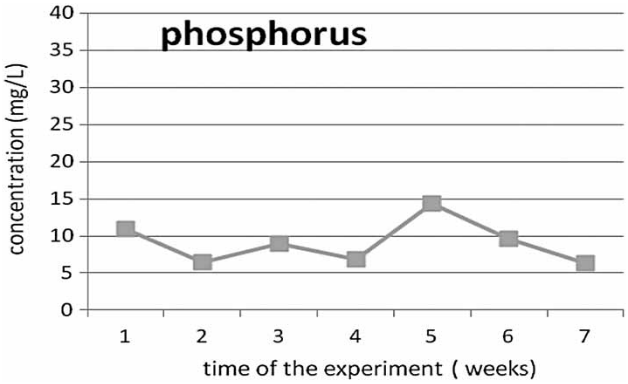

Figure 3 | Changes in phosphorus concentration during the process.

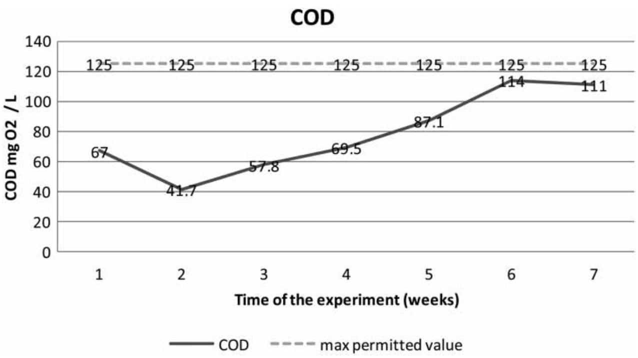

Figure 4 | The changes of chemical oxygen demand (COD) in the effluent from the reactor. The dashed line indicates a maximum value of COD that can be introduced into water and soil.

The COD removal during our experiment was slightly affected, but remained below the permitted level (Figure 4). A similar effect was observed by Schmidt *et al.* (2012) for the mixture of ciprofloxacin, gentamicin, sulfamethoxazole, vancomycin, and trimethoprim in concentrations up to 20 mg/L. On the other hand, Chen *et al.* (2015) quoted several examples of the influence of antibiotics on COD removal and the mineralization process, with the inhibition of COD removal and sludge yield observed for tetracycline.

Our quantitative study showed that the resistant bacteria were already present in the sludge from the wastewater treatment plant, with a higher number of bacteria resistant to gentamicin, rifampicin, and penicillin (Figure 5). There is evidence that during the wastewater treatment process, resistance can spread between microorganisms, resulting in increased numbers of antibiotic-resistant bacteria (Łebkowska 2009). In this research the number of antibiotic-resistant bacteria increased significantly with increasing concentrations of antibiotics. This finding confirms the impact of antibiotics on the selection of antibiotic-resistant strains. The highest numbers of bacterial colonies were observed on the culture media with penicillin and nitrofurantoin, with the minimum number in the presence of doxycycline. After 7 weeks, the number of nitrofurantoin-resistant and penicillin-resistant bacteria increased to more than 10^5 CFU/mL. This effect was not observed for gentamicin, rifampicin, and doxycycline.

The number of antibiotic-resistant bacteria in the bioreactor was similar to those obtained in other studies. Huang *et al.* (2012) detected 1.5×10^4–1.9×10^5 CFU/mL of penicillin-resistant bacteria and 3.1×10^2–6.1×10^4 CFU/mL of rifampicin-resistant bacteria in the effluent of a wastewater treatment plant in China. Munir *et al.* (2011) reported the occurrence of 6.1×10^3 CFU/mL of antibiotic-resistant bacteria in the final effluents from wastewater utilities in the USA. The amount of bacteria in the treated biosolids reached 10^9 CFU/g. According to Gao *et al.* (2012), the

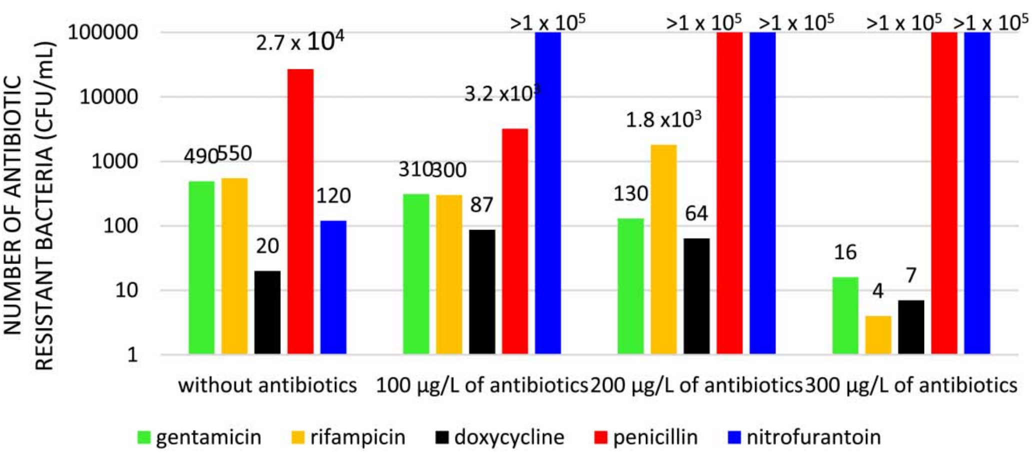

Figure 5 | Numbers of antibiotic-resistant bacteria [CFU/mL].

number of tetracycline- and sulfonamide-resistant bacteria in the final effluent from the wastewater treatment plant reached 10^3 CFU/mL, while in treated sludge it was 10^4–10^6 CFU/mL. The increase in percentages of antibiotic-resistant bacteria during the wastewater treatment process has also been observed (Novo *et al.* 2013).

Subsequent analyses revealed that predominating strains of resistant bacteria (labeled as strain 1, strain 2, and strain 3, respectively) were multi-resistant. The resistance patterns of the strains are presented in Table 1. It was observed that two of them were resistant to 90% of tested antibiotics. Such extended resistance can be related to several mechanisms: prevention of its transport into the cell, active removal from the cell, creating alternative metabolic pathways, or enzymatic deactivation (Markiewicz 2001).

The results of double disc tests confirmed that two of the most resistant strains isolated from the experimental activated sludge bioreactor had carbapenamase activity, the enzyme directly related to the resistance against *β*-lactam antibiotics. The results of the test are presented in Table 2 and Figure 6. The lack of an inhibition zone around the meropenem disc and the inhibition zones around the discs with appropriate enzyme inhibitors allowed the identification of the enzyme activity. The examples of the occurrence of *β*-lactamases in bacteria after the wastewater treatment were previously published by Yi *et al.* (2015).

Table 1 | Antibiotic resistance in bacteria isolated from the activated sludge in the bioreactor (R-resistant, S-sensitive)

	Strain 1		Strain 2		Strain 3	
Antibiotic	Inhibition zone diameter (mm) (average/standard deviation)	Resistance	Inhibition zone diameter (mm) (average/standard deviation)	Resistance	Inhibition zone diameter (mm) (average/standard deviation)	Resistance
Amikacin	0	R	0	R	0	R
Minocycline	0	R	26.28/0.71	S	0	R
Tigecycline	0	R	18.89/0.59	S	0	R
Polymyxin	15.28/0.97	S	0	R	15.37/1.20	S
Cefadroxil	0	R	0	R	0	R
Azithromycin	0	R	0	R	0	R
Ampicillin	0	R	0	R	0	R
Gentamicin	0	R	0	R	0	R
Rifampicin	0	R	14.87/1.26	R[a]	0	R
Doxycycline	0	R	14.24/1.33	S	0	R
Nitrofurantoin	0	R	15.39/0.62	S	0	R
Vancomycin	0	R	17.89/0.65	S	0	R

[a]Too low diameter of the inhibition zone.

Table 2 | The inhibition zones diameters in enzymatic tests

	Strain 1		Strain 2		Strain 3	
Antibiotic	Inhibition zone diameter (mm) (average/standard deviation)	Enzymes	Inhibition zone diameter (mm) (average/standard deviation)	Enzymes	Inhibition zone diameter (mm) (average/standard deviation)	Enzymes
Meropenem	0		19.76/0.53		0	
Meropenem + phenylboronic acid	13.37/0.38	+[a]	23.13/0.57	–	14.52/0.35	+[a]
Meropenem + dipicolinic acid	9.29/0.47	–	21.81/0.82	–	7.42/0.41	–
Meropenem + cloxacillin	0	–	22.23/0.84	–	0	–

[a]Carbapenamase activity

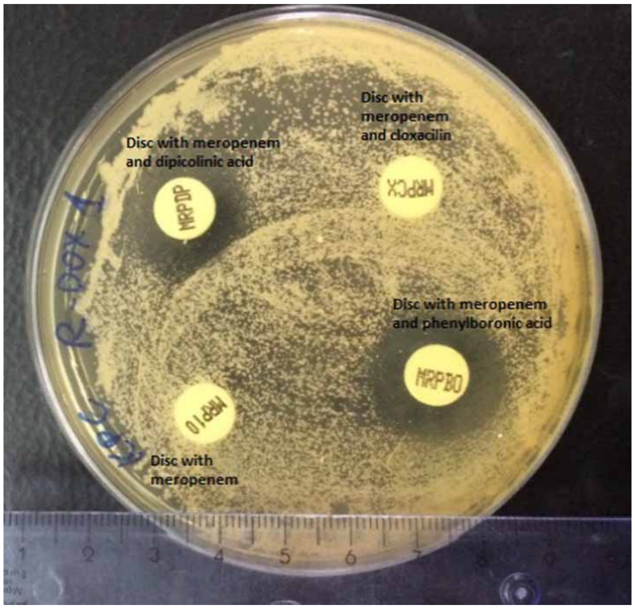

Figure 6 | The results for the carbapenamase producing strain 1.

CONCLUSIONS

The novel laboratory simulation of wastewater treatment performed in this study allowed the evaluation of aspects of the wastewater treatment process otherwise unavailable in standard, full-scale processes in a wastewater treatment plant (i.e. the influence of the rapidly increasing antibiotic concentration on the sewage treatment parameters and the microflora of activated sludge). Moreover, this study determined that the rate of development of antibiotic-resistant microorganisms can be extremely high, even in the absence of any significant disturbances in organic matter degradation, changes in nitrogen and phosphorus concentrations, and the structure of the sludge biocenosis. Based on these findings, it is possible that increasing concentrations of commonly used antibiotics can force the appearance of some extremely multi-resistant strains, equipped with special enzymes, which can enter the environment together with the treated wastewater, thus posing a serious health problem.

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