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Effect of erythromycin on biochemical activity of microorganisms of activated sludge

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ABSTRACT

The steadily increasing production of pharmaceutical substances, their widespread use in health care for people and animals and inadequate disposal methods have attracted much interest in risks involved in the presence of these substances and their metabolites in the environment. Drugs present in wastewater are often resistant to biodegradation and can disturb biological processes of removing contaminants from wastewater. It should be emphasized that conventional wastewater treatment plants are not adapted to disposal of such contaminants as pharmaceutical substances. The aim of this study was to evaluate the effect of erythromycin on changes in dehydrogenase activity in activated sludge and oxygen uptake rate of the whole population of microorganisms of active sludge, heterotrophic bacteria and nitrifying bacteria of the first and second phase. A decline in activity of dehydrogenases of activated sludge in the presence of erythromycin was observed. With the concentration of this antibiotic of 150 mg/L, the lowest value of dehydrogenase activity was 35.5 μmol TF/gSS, whereas the degree of inhibition for this concentration reached 33.7%. It was also found that oxygen uptake rate declined for the entire population of microorganisms of active sludge in the presence of the antibiotic. The highest value of the inhibition degree for this oxygen uptake rate was 28.8%. Furthermore, an insignificant decline in the degree of inhibition of oxygen uptake rate was observed for heterotrophic bacteria (13.7%). The most sensitive to erythromycin were nitrifying bacteria. Degree of inhibition of oxygen uptake rate in the presence of this antibiotic for nitrifying bacteria of the first phase reached 40–64.5%, whereas for the bacteria of second phase, this value was 28.2–62%.

Keywords: Activated sludge; Erythromycin; Dehydrogenase activity; Oxygen uptake rate

1. Introduction

With the increasing production of pharmaceutical substances, their widespread use in treating people and animals and inadequate utilization methods, much interest has been attracted to the risks involved in the presence of these substances and their metabolites in the environment. It should be emphasized that pharmaceutical substances are one of the major groups of microcontaminants present in natural environments because they represent biocidal substances with low concentrations while presence of antibiotics in environments is conducive to the growth of antibiotic-resistant microorganisms [1,2].

Annual consumption of individual groups of pharmaceuticals over the year varies depending on the country from several ten to several hundred tonnes [3], whereas annual consumption of antibiotics all over the world is 200,000 tonnes [4]. The Polish market of pharmaceuticals is sixth in Europe, whereas in terms of growth dynamics, it is second, following Spain [5].

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Major sources of contamination of water environment with antibiotics include hospital and pharmaceutical sewage (which is characterized by a substantial load of drugs), municipal wastewater, and sewage from agricultural farms and veterinary facilities [6–9]. Presence of antibiotics has been recorded with various concentrations in municipal sewage (0.01–1 μg/L), hospital sewage (0.1–100 μg/L), pharmaceutical sewage (0.1–100 mg/L) and surface waters (7–400 ng/L) [10]. The specimens of Taiwan rivers found very high concentrations of erythromycin, reaching 75 μg/L [11].

Pharmaceuticals present in wastewater, often resistant to biodegradation, can contribute to the disturbance of the processes of removing pollutants from waste [12], whereas conventional wastewater treatment plants are not adjusted to removal of such contaminants as drugs. Therefore, part of them is discharged to water receivers with treated wastewater and get to the soil with sewage sludge.

To date, Polish and European legislature and even world organizations have not prepared documents to standardize permissible concentrations of specific pharmaceuticals supplied to wastewater treatment. The only initiative is a substance observation list for monitoring purposes that concerns the entire European Union in terms of water policy.

The list contains only 10 groups of dangerous substances that concern pharmaceuticals, including macrolide antibiotics such as erythromycin. These substances have been monitored in treated sewage in selected sewage treatment plants located in the EU countries. The data collected allow for making a decision on classification of these compounds as priority substances [13,14].

Effects of biological treatment of sewage containing biochemical transition inhibitors or toxic compounds can be monitored by the measurement of activity of dehydrogenases in microorganisms of activated sludge. Effectiveness of sewage treatment depends on the condition of microorganisms present in sewage sludge, which, with enzymes present in cells, help decompose the contaminants that flow into the wastewater treatment plant. Dehydrogenases are enzymes from the group of oxidoreductases which catalyze oxidation of organic substances which are involved in cellular respiration.

Determination of dehydrogenases activity allows for evaluation of biochemical activity of microorganisms of active sludge, thus allowing for the control of biological sewage treatment. One of the methods to measure dehydrogenases is the method based on the use of triphenyltetrazolium chlorate (TTC) test.

The test is especially useful for examination of the correctness of treatment procedures in the case of sewage containing inhibitors of biochemical reactions and toxic substances [15]. The choice of the TTC test to characterize biochemical transitions in activated sludge is justified by the findings published by Rich et al. [16,33].

Measurement of dehydrogenase activity (DA) using the TTC test is used for the evaluation of the biological process of sewage treatment using the activated sludge method at varied composition of sewage, variable technical parameters in the presence of inhibitory or toxic substances [17–20].

The dynamics of the processes of biological sewage treatments that occur in the activated sludge chamber can be evaluated using the tests of oxygen uptake rate (OUR) of the microorganisms in the activated sludge [21,22]. Rate of oxygen uptake by the biomass of activated sludge can be evaluated by inhibition or toxicity of selected substances present in the sludge [23–25]. Measurements of OUR were used to evaluate the condition of activated sludge during treatment of municipal waste, industrial waste, effect of selected pollutants on biological activity of microorganisms of sewage sludge, and effect of industrial sludge on nitrification, denitrification, and transition of phosphorus compounds during sewage treatment [24,26–30].

Surmacz-Górska et al. [22,31] conducted the examinations concerning the process of nitrification using the oxygen consumption measurement test. The method consists in consecutive addition of selective inhibitors of *Nitrobacter*, followed by the inhibitor of *Nitrosomonas*. The researchers found the correlation between changes in oxygen consumption in both phases of the nitrification process with changes in individual forms of nitrogen, that is, decline in the concentration of the ammonium nitrogen and the increase in the concentration of nitrate nitrogen.

The aim of the study was to determine the effect of erythromycin on biochemical properties of activated sludge using the measurement of DA and OUR of heterotrophic and nitrifying bacteria.

2. Material and methods

The effect of erythromycin on biochemical properties of activated sludge was determined by comparison of DA and OUR of activated sludge after adding a specific amount of the antibiotic directly with DA and OUR of activated sludge without this addition. The experiments were conducted for erythromycin concentrations of 10–150 mg/L for DA and 100–1,000 mg/L for OUR.

The applied concentration of erythromycin in studies may seem high in relation to the levels administered in municipal wastewater or other works (from 0.1 to 20 mg/L). However, the concentrations used in the studies are in the same order of magnitude as the concentrations of antibiotics in pharmaceutical wastewater. Similar concentrations ranging from 1 to 300 mg/L have been used in the studies of Alighardashi et al. [39], by Louvet et al. [34] and in the range of 0.1 to 20 mg/L [35]. Higher erythromycin concentrations during determination of OUR and greater volumes of erythromycin solutions were used due to the specificity of determinations (i.e., different volumes of the samples of activated sludge) in order to obtain similar concentrations of antibiotic in the samples of activated sludge during determination of DA and OUR. Basic information concerning erythromycin is presented in Table 1.

Table 1
Characterization of erythromycin used in the experiments

Molecular formula	$C_{37}H_{67}NO_{13}$
Antibiotic group	Macrolides
Molar mass, g/mol	733.93
Density, g/ml	1.226
Solubility	
In water, g/L	$1.44 \cdot 10^{-3}$
In ethanol	Easy

The measurements of DA and OUR were based on the activated sludge sampled directly from the nitrification chamber of the Wastewater Treatment Plant "Warta" in Częstochowa. Sewage treatment in this plant is ensured by the use of the combined biological and chemical method using activated sludge in multifunction biological reactors. Activated sludge, with concentration of suspended solids of 5.4 g/L was sampled directly from the nitrification chamber. Total suspended solids were determined according to Hermanowicz et al. [32].

2.1. Determination of DA of the microorganisms in activated sludge

DA was determined based on the TTC test which uses TTC, reduced with dehydrogenase-catalyzed reactions to triphenylformazan (TF) [15,32]. TF has a red colour. Intensity of the red colour of the sample is directly proportional to the primary amount of dehydrogenases in activated sludge.

Before the determination of DA for activated sludge, we added erythromycin solutions with selected concentrations. Next, the mixture was thoroughly mixed and left for 30 min. The volumes of solutions added to the sample of activated sludge are contained in Table 2.

Tris-HCl buffer was added to all samples and quickly heated to the temperature of 37°C. Next, the TTC and glucose solution was added to all test tubes except for the blind sample, where distilled water was added. The deoxidation substance was sodium sulphite. All the samples were thoroughly mixed and again placed in the water bath. The next step was incubation in the darkness at temperature of 37°C. After 30 min, the reaction was stopped by adding the methyl alcohol to achieve the volume of 25 mL. The samples were filtered to the measurement flasks and methyl alcohol was added to achieve the volume of 50 mL.

Absorbencies of the coloured samples were determined spectrophotometrically at the wavelength of 490 nm by adopting the concentration of generated TF as a measure of DA. Three repetitions were performed for each sample. After determination of TF, the value of DA was determined using Eq. (1) (see Table 3).

2.2. Determination of OUR of the microorganisms in activated sludge

OUR of microorganisms of activated sludge was determined based on the test which consisted in determination of oxygen consumption after previous oxidation [22,29,31]. During the measurement, sewage sludge is mixed, whereas oxygen consumption by microorganisms is read after a specific measurement time.

The test can be used to determine activity of heterotrophic and nitrifying bacteria. The measurement method consists in adding, to activated sludge, of the selective inhibitors of nitrifying bacteria for *Nitrobacter* (nitrificants of the Phase II) – sodium chlorate ($NaClO_3$), followed by the inhibitor of *Nitrosomonas* (nitrificants of the Phase I) – allylthiourea ($C_4H_8N_2S$). Oxygen amount used by *Nitrosomonas* for oxidation of ammonia was evaluated based on the difference between oxygen consumption with respect to sodium chlorate and oxygen consumption with respect to sodium chlorate and allylthiourea. Furthermore, the amount of oxygen for oxidation of nitrites (III) was determined based on the difference between total oxygen consumption and oxygen consumption with respect to sodium chlorate. OUR of heterotrophic bacteria corresponded to oxygen consumption in the presence of sodium chlorate and allylthiourea.

Oxygen activity of microorganisms of activated sludge was measured in a tightly closed cylindrical-shaped glass container with capacity of 100 mL equipped in the oxygen probe with oxygen meter. Temperature during the measurement of OUR was 20°C. In the case of samples with antibiotic, erythromycin solution with selected concentration was added to the container with activated sludge, whereas distilled water was added to the samples without antibiotic. The volumes of solutions added to activated sludge are presented in Table 2. Next, activated sludge was saturated with oxygen by intensive mixing. The tightly closed container with the oxygen probe was placed on the magnetic mixer in order to ensure thorough mixing. After a specific time, oxygen consumption was read for the whole population of microorganisms of activated sludge. First, sodium chlorate was added and the oxygen consumption was read. Next, allylthiourea was added and oxygen consumption measurement was made again. All determinations were made in three repetitions. The results of bacteria OUR were given as unit

Table 2
Volumes of solutions added to the sample of activated sludge in mL

Determination of DA				Determination of OUR		
Sample	Blind	Without erythromycin	With erythromycin	Sample	Without erythromycin	With erythromycin
Activated sludge	5	5	5	Activated sludge	100	100
Erythromycin solution	–	–	2	Erythromycin solution	–	4
Distilled water	2	2	–	Distilled water	4	–
Tris-HCl buffer	5	5	5	Sodium chlorate	1	1
NA_2SO_3 solution	1	1	1	Allylthiourea	1	1
TTC solution	–	1	1			
Distilled water	1	–	–			

Table 3
Determination of DA and OUR and the degree of inhibition

Magnitude		Equation		Explanation
Dehydrogenase activity (DA), μmol TF/gSS		$AD = \frac{TF \cdot 1000}{V \cdot S}$	(1)	TF – triphenylformazan concentration in the sample of activated sludge, μmol TF V – volume of activated sludge used for determination, mL S – total suspended solids, g/L
Oxygen uptake rate (OUR), $mgO_2/gSS \cdot h$		$OUR = \frac{C_I - C_F}{\Delta T \cdot S}$	(2)	ΔT – time to change oxygen concentration from the initial to final value, h C_I – initial oxygen concentration in the sample in mgO_2/L C_F – final oxygen concentration in the sample in mgO_2/L S – total suspended solids, g/L
Heterotrophic bacteria		$OUR_{heter} = OUR_{chl+all}$	(3)	OUR of microorganisms: OUR_{wp} – the whole population without nitrifying inhibitors OUR_{chl} – in the presence of $NaClO_3$ $OUR_{chl+all}$ – in the presence of $NaClO_3$ and $C_4H_8N_2S$
Nitrifiers	Phase I	$OUR_{NI} = OUR_{chl} - OUR_{chl+all}$	(4)	
	Phase II	$OUR_{NII} = OUR_{wp} - OUR_{chl}$	(5)	
Degree of inhibition (I), %		$I = \frac{A_0 - A_E}{A_0} \cdot 100$	(6)	A_0 – activity of microorganisms without addition of erythromycin A_E – activity of microorganisms of activated sludge in the presence of erythromycin

oxygen consumption over 1 h with respect to total suspended solids in $mgO_2/gSS \cdot h$. The method to calculate OUR for heterotrophic and nitrifying bacteria of the phases I and II are presented in Table 3 (see Eqs. (2)–(5)).

Percentage decline of DA and OUR nitrificants of the first and second phases and heterotrophic bacteria caused by the antibiotic for all the measurements was determined based on the inhibition degree (see Eq. (6)).

3. Results

Changes in DA of activated sludge following the use of erythromycin were observed for concentrations of 10, 20, 50, 100 and 150 mg/L. Basal DA level without addition of erythromycin was 53.3 μmol TF/gSS.

Fig. 1 presents changes in DA of sewage sludge caused by selected concentrations of erythromycin and inhibition degree for the activity calculated according to Eq. (1). A declining tendency for DA was observed for the increase in erythromycin concentration. With the concentration of this antibiotic of 150 mg/L, DA decreased by 1.5 times compared with the basal value, reaching 35.5 μmol TF/gSS. Inhibition degree for this erythromycin concentration was 33.7%.

Examinations of the effect of erythromycin on OUR of microorganisms of activated sludge were performed for the concentrations of 100, 200, 500 and 1,000 mg/L. It was observed that OUR for the whole population of microorganisms in the presence of erythromycin decreased by 1.4 times from the

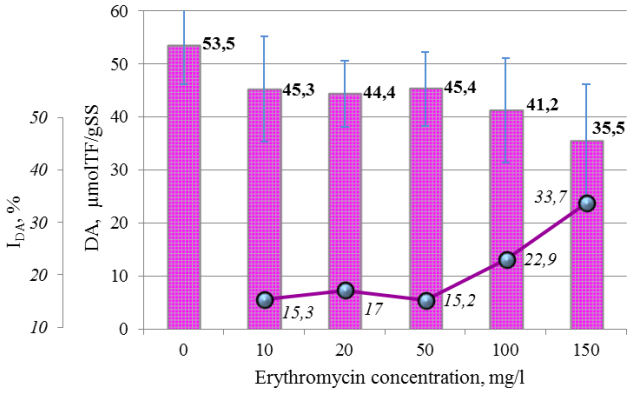

Fig. 1. Changes in dehydrogenase activity (DA) of microorganisms of activated sludge in the presence of erythromycin and inhibition degree (I_{DA}).

basal value of 10 mg $O_2/gSS \cdot h$ to 7.1 mg $O_2/gSS \cdot h$ with erythromycin concentration of 1,000 mg/L. Degree of OUR inhibition for all microorganisms present in activated sludge for this antibiotic concentration was 28.8%. Changes in OUR and degree of inhibition for the whole population of microorganisms in activated sludge in the presence of erythromycin are presented in Fig. 2.

The lowest OUR values were found for heterotrophic bacteria. Degree of OUR inhibition for these microorganisms in the presence of erythromycin did not exceed 13.7% (see Fig. 3).

Based on the results obtained for the sewage sludge, a substantial decline in OUR for both *Nitrosomonas* and *Nitrobacter* was observed after addition of erythromycin (see Fig. 4). It was observed that OUR of nitrifying bacteria of the phases I and II reduced with the increase in erythromycin concentration from 1.5 to 0.5 mg O_2/gSS·h for bacteria of the Phase I of nitrification and from 1.6 to 0.6 mg O_2/gSS·h. Degree of OUR inhibition for the bacteria of the Phase I of nitrification induced by erythromycin was 40%–64.5%, whereas the decline in OUR of the Phase II of nitrification changed in the range of 28.2%–62%.

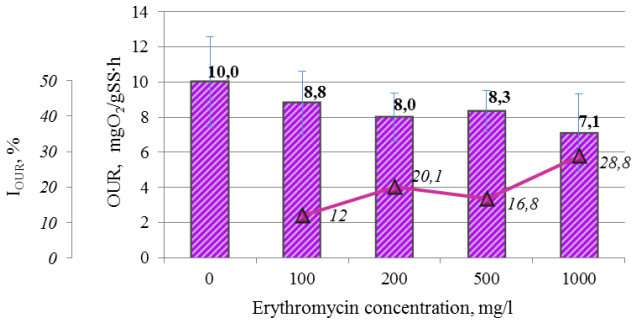

Fig. 2. Changes in oxygen uptake rate (OUR) for the entire population of microorganisms of activated sludge in the presence of erythromycin and its inhibition degree (I_{OUR}).

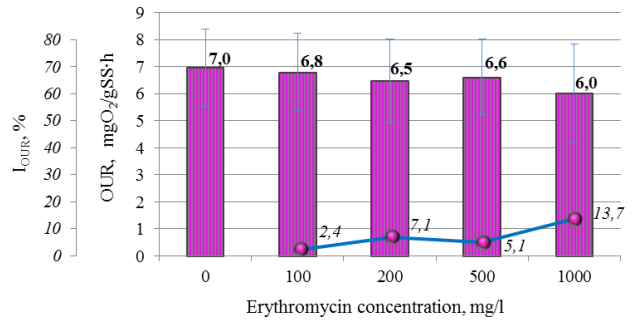

Fig. 3. Changes in oxygen uptake rate (OUR) of heterotrophic microorganisms in the presence of erythromycin and its inhibition degree (I_{OUR}).

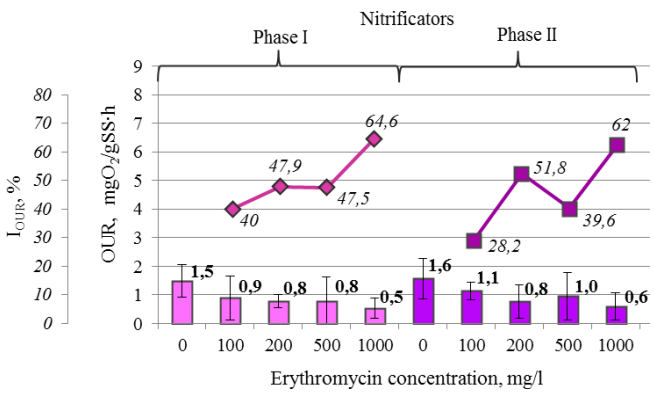

Fig. 4. Changes in oxygen uptake rate (OUR) for nitrifying bacteria of the phases I and II in the presence of erythromycin and its inhibition degree (I_{OUR}).

The results of our own studies confirm the inhibitory effect of erythromycin on both stages of nitrification. Louvet et al. [34] used lower doses of 10 and 20 mg/L erythromycin, giving average inhibition rates of 46% and 72%. Similar values (78% and 36%) were obtained for lincomycin at 11 mg/L [35]. Erythromycin is characterized by a stronger and much more drastic effect on the nitrification stages compared with other antibiotics, for example, tertacycline [36]. Louvet et al. [34] showed that the toxicity of erythromycin at a lower concentration increases with the exposure time. Noël Louveta et al. [37] showed that a 4 μg/L erythromycin concentration was toxic to heterotrophic bacteria on a 5-d time exposure, and a 5 mg/L concentration inhibited nitrification. Terzica et al.'s [38] studies showed the ability of activated sludge microorganisms of degrading high concentrations (10 mg/L) of three prominent macrolides, erythromycin, clarithromycin and azithromycin, which could be particularly important for the treatment of heavily polluted industrial wastewaters. It should be stressed that erythromycin and azithromycin have recently been proposed for inclusion into the EU Watch List as emerging contaminants of concern [38].

4. Conclusions

Biochemical transitions in sewage sludge induced by erythromycin were observed based on DA and OUR of the whole population of microorganisms in activated sludge of heterotrophic bacteria and nitrifying bacteria of the phases I and II.

A decline in DA in activated sludge in the presence of erythromycin was found. The lowest value of DA of 35.5 μmol TF/gSS was obtained for the concentration of this antibiotic of 150 mg/L. For this sample, the inhibition degree was almost 34%.

A decline in OUR for the whole population was also observed for the entire population of microorganisms present in activated sludge. The highest inhibition degree for this activity was 29%. An insignificant degree of inhibition of OUR was found for heterotrophic bacteria (below 14%).

Based on the obtained results, it was found that erythromycin inhibited the respiratory activity of activated sludge microorganisms only at high concentrations, whereas at the concentrations typical for wastewater it did not have any effect on activated sludge microorganisms.

The most sensitive to erythromycin were nitrifying bacteria. Degree of inhibition of OUR caused by erythromycin for nitrifying bacteria of Phase I reached 40%–65%, whereas for the bacteria of Phase 2, this value was 28%–62%. This is not surprising because nitrifying bacteria belong to one of the most sensitive to inhibitory and toxic substances of the groups of microorganisms that take part in the processes of sludge treatment, whereas the amount of the factors that would cause partial or total inhibition of nitrification is defined as substantial [39].

Similarly, Louvet et al. [34] observed inhibition of the nitrification rate caused by erythromycin in part of the sewage sludge they studied. These researchers also found that erythromycin led to the increase in nitrification rate. However, it should be stressed that the study used erythromycin concentration of up to 20 mg/L.

Abbreviations

DA	—	dehydrogenase activity
OUR	—	oxygen uptake rate
TF	—	triphenylformazan
TTC	—	triphenyltetrazolium chlorate
I_{DA}	—	degree of DA inhibition
I_{OUR}	—	degree of OUR inhibition

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