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Aerobic biodegradation of norfloxacin and ofloxacin by a microbial consortium

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Abstract: Since fluoroquinolone (FQ) antibiotics are extensively used both in human and veterinary medicine their accumulation in the environment is causing increasing concern. The aim of the study was to isolate a microbial consortium resistant to ofloxacin and norfloxacin and able to biodegrade both antibiotics. Green compost was used as a source of microorganisms. The biodegradation efficiency was monitored by changes of antibiotics concentrations and toxicity. The microbial consortium was composed of two bacterial isolates: *Klebsiella pneumoniae* (K2) and *Achromobacter* sp. (K3) and two fungi *Candida manassasensis* (K1) and *Trichosporon asahii* (K4). All the isolates were characterized as highly resistant to both antibiotics – ofloxacin and norfloxacin. FQs were supplied individually into the culture medium in the presence of an easily degradable carbon source – glucose. Biodegradation of norfloxacin was much faster than ofloxacin biodegradation. During 20 days of the experiment, the norfloxacin level decreased by more than 80%. Ofloxacin was generally biodegraded thereafter at relatively slow biodegradation rate. After 28 days the ofloxacin level decreased by 60%. Similarly, the toxicity of biodegraded antibiotics decreased 4-fold and 3.5-fold for norfloxacin and ofloxacin, respectively. The ability of the bacterial-fungal consortium to degrade antibiotics and reduce toxicity could help to reduce environmental pollution with these pharmaceutical.

Introduction

Emerging pollutants are a group of compounds which have no specific legal regulations and whose toxic effects to the environment and human health coupled with high occurrence make them subject to future regulations (Miralles-Cuevas et al. 2015). This group includes various types of globally widespread organic compounds, such as pesticides, dyes, pharmaceuticals, personal care products, polymers and plastics.

Antibiotics are natural or synthetic pharmaceuticals that can eliminate or prevent the multiplication of bacteria (Doruk Aracagök et al. 2018, Nzila et al. 2018). They have been used for therapy of infectious diseases in humans and animals for almost one hundred years. However, wide and prevalent application of antibiotics has recently created great public concern (Peng et al. 2012, Wang and Wang 2016). Antibiotics in field-applied manure can be leached during rain events and thus contaminate surface water and groundwater. In addition, current water treatment technologies are not able to remove antibiotics effectively and thus, antibiotics are discharged into the environment (Marcelino et al. 2016). In addition, the excessive use of antibiotics and their disposal after expiration increase their presence in the environment. Bacteria have been observed to develop drug resistance genes during long-term contact with antibiotics or through gene transfer (Bouki et al. 2013). Therefore, the health risk of antibiotic efficacy originated from their environmental occurrence is of the major research concern.

Among the antibiotics, fluoroquinolones are ubiquitously detected in wastewater, surface, soil and sediments (Vazquez-Roig et al. 2010). Thus, their environmental fate deserves special attention. Fluoroquinolones (FQs) are synthetic broad--spectrum chemotherapeutic antibacterial substances due to their enhanced pharmacokinetic properties, mechanism of action as well as extensive and potent activity. FQ is one of the most commonly used antibiotics, not only in the hospital sector, but also in everyday life, to reduce the wide range of infections. The four quinolone antibacterial agents include acrosoxacin, ciprofloxacin, ofloxacin, and norfloxacin. Ciprofloxacin (CIP), norfloxacin (NOR) and ofloxacin (OFL) belong to the 2nd generation FQs (Jia et al. 2012). CIP and NOR have almost similar structural characteristics, the substituent at the nitrogen atom from the pyridin-carboxilic ring (cyclopropyl and ethyl side chain, respectively) being the only difference between the two, while OFL is a tricyclic derivative and has a methyl substituent on the piperazine ring (Jia et al. 2012).

Recently, several studies have been published describing the removal techniques of different antibiotics occurring in nature using biotic and abiotic methods (Wang and Wang 2016). The conventional methods used to degrade antibiotics are sorption, hydrolysis, photolysis, oxidation and reduction. However, there are some limitations connected with these methods, e.g. they are expensive and unsustainable. Hence, there is a need for an inexpensive biotic degradation method for remediating the antibiotics from the environment (biodegradation). It is important that this technology is sustainable so that it is not only inexpensive, but also ensures complete removal/degradation of the contaminant without having any adverse effects on the environment.

In this context, the aim of the study was to isolate the microbiological consortium resistant to ofloxacin and norfloxacin and evaluate its ability to biodegrade these pharmaceuticals.

Materials and methods

Green compost characterization

The mixed green compost used for this study was collected from compost plant in Katowice (50°16'11.02"N 18°57'34.19"E Poland), where the MUTDANO system is applied. This system is based on a technological line equipped with two biostabilizers with a capacity of 120 t per day. The physico--chemical parameters of the compost were obtained from the compost plant. The values of the selected compost parameters are presented in Table 1.

Table 1. Microbiological and physico-chemical parameters of the compost used

Characteristics	Units	Values
pH	–	6.81
Conductivity	[uS/cm]	770.24
Humidity	%	52.00
Total organic carbon (TOC)	%	30.45
Total organic matter (TOM)	%	43.45
Total nitrogen	%	0.59
Total phosphorus	%	1.11
C/N ratio	%	19.26
Aerobic bacteria	CFU/g	$1.1 \cdot 10^8$
Fungi	CFU/g	$1.3 \cdot 10^5$
Actinomycetes	CFU/g	$4.4 \cdot 10^4$

Microbiological characterization of green compost

Culturable bacterial and fungal numbers were evaluated by a series of 10-fold dilutions of green compost suspensions. One gram of the compost sample was dispersed in 9 mL of sterilized physiological water (0.9%, NaCl) by shaking for 2 min. After dilutions (10^{-1}–10^{-6}) 1 mL of aliquots of different dilutions was pipetted onto plates. Pour-plate method was used for quantification of microorganisms. Aerobic bacteria were incubated on SMA medium (Standard Methods Agar, BioMérieux) containing 100 mg cycloheximide per liter. Bacteria were incubated at 30°C for 24 h. Fungi were incubated on MEA medium (Malt Extract Agar, BioMérieux) with 100 $mg \cdot L^{-1}$ chloramphenicol at 25°C for 7 days.

Isolation of norfloxacin and ofloxacin resistant microorganisms

At the beginning of the experiment, 10 g of green compost was added to 90 mL of saline solution (0.85%, NaCl) and incubated at 30°C for 48 hours. Then, 0.1 ml of compost solution was spread on the solid Luria-Bertani medium (LB), pH 7.0, containing in $g \cdot L^{-1}$ casein peptone (10.0); yeast extract (5.0), NaCl (5.0) and agar (20) supplemented with norfloxacin and ofloxacin with a final concentrationof 10 $mg \cdot L^{-1}$. The plates were incubated at 30°C for 24–48 h. The isolates were preserved at -70°C in Luria-Bertani (LB) medium supplemented with 20% (v/v) glycerol.

Identification of isolates involved in antibiotic biodegradation

Genomic DNA was extracted according to the manufacturer's protocol of Roche PCR Master Kit (Ref. 11 636 103 001), and it was stored at -20°C until further use. The 16S rRNA gene (size of amplicon: 1400 bp) or 18S rRNA gene (size of amplicon: 650 bp) was amplified and sequenced at the Blirt company (Poland). The 16S rRNA gene sequences and 18S rRNAs were compared to the NCBI GenBank database, using BLAST software to determine the genetic similarities of the isolates. Sequences of the 16S rRNA and 18S rRNA were aligned using the Clustal W algorithm in MEGA 7 and edited manually. Phylogenetic tree was constructed with the MEGA 7 software using the Maximum Likelihood Estimation method and the Tamura-Nei model on default settings. Bootstrapping was carried out using 100 replicates and values were indicated on the nodes (Kumar et al. 2016).

Moreover, the identification of selected bacteria (K2 and K3) was performed by the GEN III MicroPlate™ test panel of the Biolog system as described in the manufacturer's instruction of Biolog, Inc. (Hayward, CA, USA). The phenotypic fingerprint of purple wells was compared with the Biolog extensive species library. If a match was found, a species-level identification of the isolates could be made.

Evaluation of MIC values

To determine the MIC values of norfloxacin and ofloxacin for the isolates, axenic cultures of microbes were grown in the Mueller-Hinton base (BioMerieux). The assay was performed on 96-well plates. Both norfloxacin and ofloxacin concentrations were tested in the range from 0 to 15.0 $mg \cdot L^{-1}$, with dilution ratio of 2. The initial optical density of each culture was 0.1 (OD_{600}). After 24–48 h of incubation with shaking at 30°C, the optical density of the cultures was measured. The MIC value was estimated using Statistica 12 software.

Biodegradation assay

To create the co-culture consortium, the strains were grown in separate flasks containing 100 mL of LB liquid medium. Flasks were incubated at 30°C on a shaker rotating at 120 rpm for 48 h. After the incubation period, 50 mL of each culture slurry was split into two separate 50 mL sterile Corning vials and centrifuged at 10.000 rpm for ten minutes at 4°C.

After the initial centrifugation the biomass was washed twice in PBS solution (Phosphate-buffered saline) at the same speed. After the final washing the appropriate volume of PBS solution was added to each vial and vortexed to create working culture stocks of the density of about 10^7–10^8 CFU·L^{-1} each. The optical density (OD) of the samples was measured at 600 nm (OD_{600} nm) using UV-Vis spectrophotometer (Eppendorf).

Short-term batch biodegradation tests were conducted for two antibiotics separately: norfloxacin and ofloxacin. Norfloxacin (NOR, P98%) and ofloxacin (OFL, P98%) were obtained from Sigma-Aldrich (USA). All glassware and media were autoclaved, and solutions of the antibiotics were filter sterilized. Sterilization of different materials and chemicals was performed separately. In addition, the antibiotics batch study was carried out in different individual bottles.

Antibiotic degradation studies were performed in 300 ml flask containing 150 ml of M9 medium which consisted of (g·L^{-1}): $Na_2HPO_4 \cdot 12H_2O$ 3.78, KH_2PO_4 0.5, NH_4Cl 5.0, $MgSO_4 \cdot 7H_2O$ 0.2, and 0.01 yeast extract. The microbial consortium prepared as described above was added to the medium to the initial optical density of 0.1 at λ=600 nm. Filter-sterilized antibiotics (Sigma-Aldrich, USA) were added to the flasks by filter syringes to obtain the final concentrations of 10 mg·L^{-1}. The cultures were supplemented with filter-sterilized glucose solution at the final concentration of 0.1%.

Two different treatments were performed: (I) MM with the consortium composed of 4 strains (K1–K4) and (II) MM as an abiotic control. All of these treatments were prepared in triplicate and incubated at 100 rpm, 30±1°C. Periodically, five milliliters of biotic and abiotic samples were taken to determine the concentration of antibiotics. Each sample was drawn at the beginning of the trial (T0) and every second day to determine the antibiotics concentration as well as to estimate the toxicity. A decrease in the antibiotics concentration relative to the sterile control concentrations was the evidence for biodegradation. The decrease in the concentration of the antibiotics over time provides an indication of the overall biodegradation rate. Each experiment was conducted in triplicate.

Analytical procedure

During the experiments, 2 mL of each sample was withdrawn at the specified time points. The degradation rate of tested antibiotics was determined with the HPLC technique using Merck Hitachi HPLC reversed-phase chromatograph equipped with an Ascentis Express® C18 column, Opti-Solw® EXP pre-column, and UV/VIS DAD detector. The mobile phase consisted of acetonitrile and 1% acetic acid (10:90 v/v; flow-rate 1 ml/min). The detection wavelength was set at 270 nm.

Evaluation of the toxicity during the experiment period

Acute ecotoxicity test – Microtox® – 15-min bacterial luminescence inhibition assay – was conducted using luminescent marine bacteria *Vibrio fischeri* to evaluate the toxicity of the samples. The assay was performed according to the standard procedure described in the manufacturer's instruction. A two-step determination of the acute toxicity was performed. In the first step – screening assay the toxicity was determined in non-diluted samples. Percent Effect (PE) was calculated for this test. In the second step the toxicity tests were performed on a 2-fold dilution series of the samples which resulted in a more than 50% effect in the screening assay. The endpoint specific for the test (EC_{50} or LC_{50}) was expressed as a percentage for all the samples. The obtained effect results were transformed into toxic units according to the following formula:

$$TU = \frac{1}{EC_{50}} \cdot 100$$

The toxicity of the samples was ranked into one of 5 classes on the basis of the TU, as described by Persoone et al. (2003).

Results and discussion

Based on the preliminary experiments, 4 out of a total of 47 isolates named K1, K2, K3 and K4 were chosen based on colonial morphology and macroscopic characteristics. The selected isolates occurred most frequently and were the most characteristic in the initial selection of strains. Based on the results obtained from gene sequencing of 16S and 18S rRNA (see section: Identification of isolates involved in the biodegradation of antibiotics), K1 and K4 isolates were estimated as fungi: *Candida manassasensis* (K1) and *Trichosporon asahii* (K4), two others isolates: K2 and K3 were classified as bacteria – *Klebsiella pneumoniae* (K2) and *Achromobacter* sp. (K3).

These microbial consortiums were used in biodegradation of norfloxacin (NOR) and ofloxacin (OFL). As it was mentioned, the isolates were identified by the 16S rDNA and 18S rDNA sequencing analysis and additionally confirmed with GEN III Biolog system (Table 2). Strains named K1, K2, K3 and K4 were shown to be in bacteria genus *Klebsiella pneumoniae* (K2) and *Achromobacter* sp. (K3) and fungi genus *Candida manassasensis* (K1) and *Trichosporon asahii* (K4). Phylogenetic trees of K1, K2, K3 and K4 strains were rooted with outgroup strains and *Pleurotus cornucopiae* P-38, respectively (Fig. 1 A–D).

Before the biodegradation experiment, the MIC values for the strains were evaluated (Table 3). According to the obtained results the strains were resistant to both antibiotics, however, the fungi were much more resistant than bacteria. The MIC values were estimated above 15 mg·L^{-1} for both fungi: *Candida manassasensis* (K1) and *Trichosporon asahii* (K4).

Table 2. Identification of isolates by 16S or 18 rRNA gene sequencing and GEN III Biolog system

Strain	Biolog System Similarity (%)	16S or 18S rDNA Similarity (%)
Candida manassasensis (K1)	nd	98
Klebsiella pneumoniae (K2)	78	99
Achromobacter sp. (K3)	69	99
Trichosporon asahii (K4)	nd	99

nd – no detected

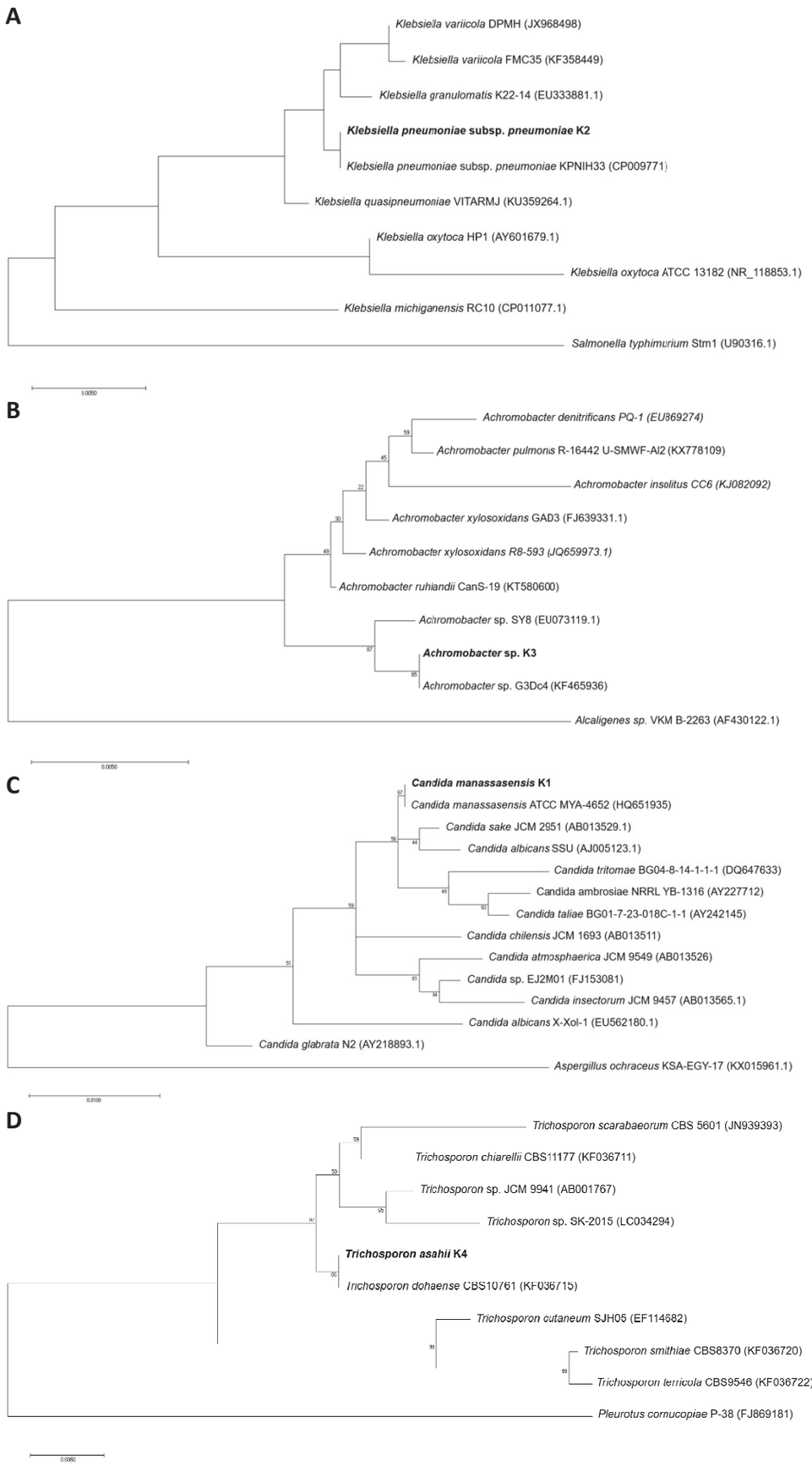

Fig. 1. Phylogenetic tree of bacterial (A–B) and fungal (C–D) strains based on 16S rRNA/18S rRNA gene sequences constructed using neighbor-joining method

The results obtained in the biodegradation experiment of norfloxacin and ofloxacin are presented in Figure 2 A and B. The results revealed that both antibiotics were degraded by the mixed bacterial-fungal consortium. As observed, biodegradation of norfloxacin was much faster than biodegradation of ofloxacin. In 20 days of the experiment, the norfloxacin level decreased by more than 80% compared to the control level. Ofloxacin was generally biodegraded thereafter at a relatively slow biodegradation rate. In 28 days, the ofloxacin level decreased by 60%.

Toxicity was evaluated in terms of percentage inhibition of bacterial luminescence and toxicity unit values (TU). After the biodegradation process all samples were non-toxic. The results are presented in Table 4. During the experiment the process became more efficient and the TU values decreased to 0.4 and 0.6 for norfloxacin and ofloxacin biodegradation, respectively.

The review by Petrie et al. (2015) identifies understudied areas of emerging contaminant (EC) research in wastewater and the environment, and recommends a direction for future monitoring. Also, dissemination of antibiotic resistant bacteria in the environment caused by the presence of antibacterial drugs (antibiotics) is presented in this review. This emphasizes the level of concern posed by the family of environmental pollutants. Large numbers of non-regulated ECs have been observed mainly in the ng to mg per liter range in surface water. To date, more than 200 different pharmaceuticals alone have been reported in river water, with concentrations up to a maximum of 6.5 mg·L^{-1} for ciprofloxacin.

Table 3. Target Minimum Inhibition Concentrations (mg·L^{-1}) for isolated strains

Strains	Minimal inhibitory concentrations (MICs)	
	Norfloxacin (NOR)	Ofloxacin (OFL)
Candida manassasensis (K1)	>15	>15
Klebsiella pneumoniae (K2)	12.5	10
Achromobacter sp. (K3)	10	10
Trichosporon asahii (K4)	>15	>15

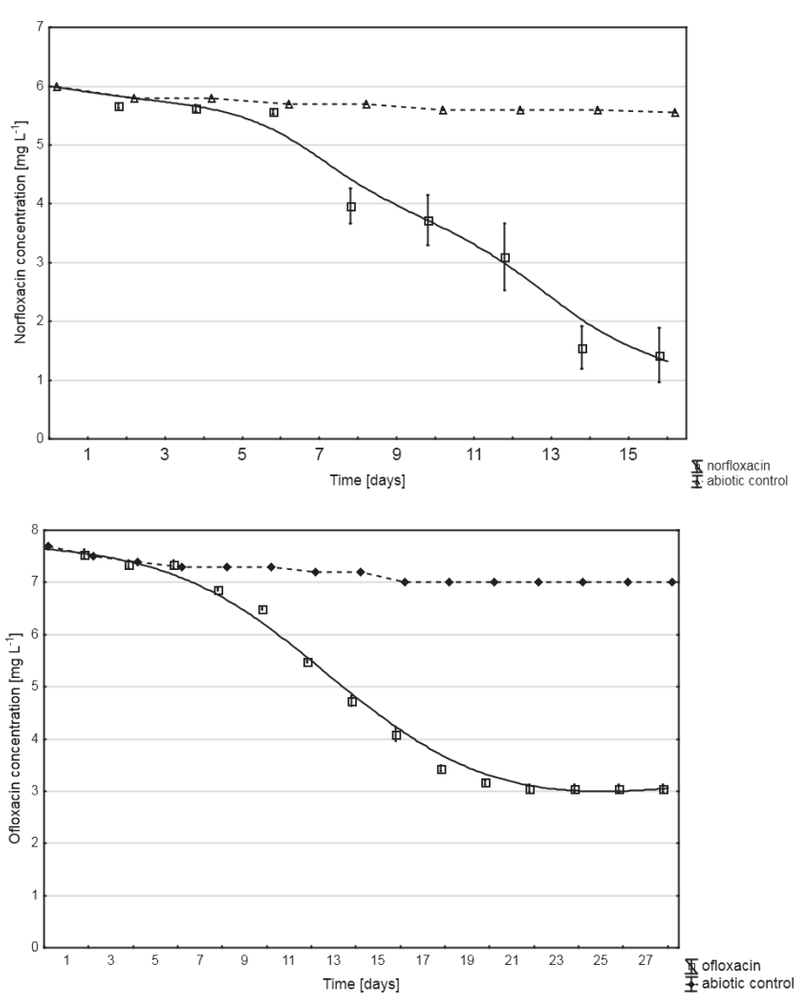

Fig. 2. Biodegradation of norfloxacin (A) and ofloxacin (B) by the bacterial-fungal consortium

Table 4. Evaluation of toxicity samples before and after biodegradation process

Antibiotics	Time (days)	Toxicity units (TU_{50})	Toxicity class
Norfloxacin biodegradation	T0	1.8	Acute toxicity
	T16	0.4	Low acute toxicity
Ofloxacin biodegradation	T0	1.6	Acute toxicity
	T27	0.6	Low acute toxicity

The knowledge about the presence of antibiotics in different parts of environment and the consequences of this presence (e.g. increased resistance to antibiotics in bacteria) is still being widened. However, the presence of these compounds in the environment implies the need to search for natural methods to remove these compounds from the environment without any negative impact on the microorganisms living there. Hence, the main aim of researchers is to find the way to eliminate pharmaceutical compounds by using microorganisms which are present in the environment. There is still lack of knowledge in this area, so there is a need for further research in this topic.

Biodegradation is usually accepted as the major way to dissipate environmental pollutants (Dai et al. 2015, Guo et al. 2014, Zhang et al. 2012). Some studies examined the biodegradability of antibiotics in controlled batch tests (Halling-Sorensen et al. 2000, Kümmerer et al. 2000, Rodríguez et al. 2017), and some data are available for full-scale wastewater treatment plants regarding the overall removal of these antibiotics from the aqueous phase (Jelic et al. 2011, Jia et al. 2012, Verlicchi et al. 2012). However, the significance of microbial degradation to quinolones dissipation in the environment remains scarce.

A number of previous studies suggested that quinolones were not readily biodegradable (Baginska et al. 2015, Girardi et al. 2011, Kummerer et al. 2000, Li and Zhang 2010, Thuy and Loan 2014, Wu et al. 2009), while a few studies reported the biotransformation of quinolones in a mixed culture or wastewater treatment bioreactor (Dorival-Garcia et al. 2013, Liao et al. 2016, Liu et al. 2013a, b). Dorival-Garcia et al. (2013) developed laboratory-scale batch experiments to investigate the main removal routes for 6 commonly found quinolones, e.g. ciprofloxacin, moxifloxacin, norfloxacin, ofloxacin, pipemidic acid, and piromidic acid in wastewater from a wastewater treatment plant. In the experiments, an aerobic sludge system from a membrane bioreactor (MBR) pilot plant was used. It was demonstrated that sorption and biotransformation were the main removal routes for the target antibiotics over other possible pathways as volatilization or hydrolysis, under the experimental conditions. Liao et al. (2016) investigated the biodegradation potential of ciprofloxacin by a mixed culture and the influential factors and depicted the structure of ciprofloxacin-degrading microbial community. The original microbiota were obtained from a biological activated carbon filter system treating antibiotic-polluted lake water. The authors identified novel biotransformation products, and four different metabolic pathways for ciprofloxacin were proposed. Bacterial community structure illustrated a profound shift with ciprofloxacin biodegradation. The ciprofloxacin-degrading bacterial community was mainly composed of classes *Gammaproteobacteria*, *Bacteroidia*, and *Betaproteobacteria*. Microorganisms from genera *Pseudoxanthomonas*, *Stenotrophomonas*, *Phenylobacterium*, and *Leucobacter* might have links with the dissipation of ciprofloxacin.

To date, the pathway for quinolone biodegradation and the influencing factor regulating the biodegradation of this antibiotic substance remain essentially unknown. Only one bacterial strain *Labrys portucalensis* able to degrade ciprofloxacin has been documented (Amorim et al. 2014). According to our survey there is no literature on norfloxacin and ofloxacin biodegradation by the individual strains or mixed consortia. Our report provides one of the first descriptions of biodegradation of ofloxacin and norfloxacin by a mixed bacterial-fungal consortium.

Conclusions

The biodegradation tests showed that the biodegradation of antibiotics was achieved. In the current study, NOR was degraded more rapidly than OFL by mixed bacterial-fungal consortium. Further studies on the biodegradation of antibiotics should be focused on the evaluation of microbes' biodegradability, by-products and metabolic pathways of biodegradation, and other factors that have been identified as necessary for biodegradation, as well as on the optimization of factors already identified as necessary for the biodegradation of antibiotics.

Due to the identification of a new bacterial-fungal consortium the present study can provide new insight into biodegradation of norfloxacin and ofloxacin.

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Kompost jako źródło mikroorganizmów biorących udział w biodegradacji norfloksacyny i ofloksacyny

Streszczenie: Antybiotyki to zróżnicowana grupa związków, która nie ma konkretnych uregulowań prawnych, dotyczących ich występowania w środowisku, zarówno wodnym jak i glebowym. Farmaceutyki przedostają się do środowiska m.in. wraz ze ściekami oczyszczonymi z oczyszczalni ścieków i jako substancje czynne biologicznie stanowią poważne zagrożenie dla organizmów żywych. Ich akumulacja w środowisku prowadzi do nieodwracalnych zmian w ekosystemach oraz szerzenia się zjawiska oporności wśród mikroorganizmów. Fluorochinolony (FQ) to syntetyczne substancje antybakteryjne o zwiększonym potencjale farmakokinetycznym i szerokim spektrum działania. FQ to jedna z najszybciej rozwijających się klas antybiotyków coraz częściej stosowanych zarówno w szpitalach, jak i w społecznościach lokalnych w leczeniu różnego typu zakażeń. Norfloksacyna i ofloksacyna to FQ II generacji o podobnej budowie strukturalnej wykazujące aktywność głównie wobec bakterii Gram-ujemnych. Ze względu na swoja budowę antybiotyki te w niewielkim stopniu są rozkładane w środowisku, przez długi czas kumulują się w wodzie i w glebie, oddziałując w na organizmy żywe. Celem pracy była ocena toksyczności ofloksacyny i norfloksacyny po biodegradacji przez zespół mikroorganizmów wyizolowany z kompostu. Proces biodegradacji przeprowadzono w bioreaktorach New Brunswick™ BioFlo® 415 o pojemności 5,5 l. Stopień degradacji określono za pomocą chromatografii cieczowej w odwróconym układzie faz. Do oceny toksyczności wykorzystano test Microtox® oparty na pomiarach aktywności bakterii luminescencyjnych *Vibrio fischeri*.