Isolation of Bacterial Endophytes from *Phalaris arundinacea* and their Potential in Diclofenac and Sulfamethoxazole Degradation

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Abstract

Diclofenac (DCF), a non-steroidal anti-inflammatory drug (NSAID) and sulfamethoxazole (SMX), an antimicrobial agent, are in common use and can be often detected in the environment. The constructed wetland systems (CWs) are one of the technologies to remove them from the aquatic environment. The final effect of the treatment processes depends on many factors, including the interaction between plants and the plant-associated microorganisms present in the system. Bacteria living inside the plant as endophytes are exposed to secondary metabolites in the tissues. Therefore, they can possess the potential to degrade aromatic structures, including residues of pharmaceuticals. The endophytic strain MG7 identified as *Microbacterium* sp., obtained from root tissues of *Phalaris arundinacea* exposed to DCF and SMX was tested for the ability to remove 2 mg/l of SMX and DCF in monosubstrate cultures and in the presence of phenol as an additional carbon source. The MG7 strain was able to remove approximately 15% of DCF and 9% of SMX after 20 days of monosubstrate culture. However, a decrease in the optical density of the MG7 strain cultures was observed, caused by an insufficient carbon source for bacterial growth and proliferation. The adsorption of pharmaceuticals onto autoclaved cells was negligible, which confirmed that the tested strain was directly involved in the removal of DCF and SMX. In the presence of phenol as the additional carbon source, the MG7 strain was able to remove approximately 35% of DCF and 61% of SMX, while an increase in the optical density of the cultures was noted. The higher removal efficiency can be explained by adaptive mechanisms in microorganisms exposed to phenol (i.e. changes in the composition of membrane lipids) and by a co-metabolic mechanism, where non-growth substrates can be transformed by non-specific enzymes. The presence of both DCF and SMX and the influence of the supply frequency of CWs with the contaminated wastewater on the diversity of whole endophytic bacterial communities were demonstrated. The results of this study suggest the capability of the MG7 strain to degrade DCF and SMX. This finding deserves further investigations to improve wastewater treatment in CWs with the possible use of pharmaceuticals-degrading endophytes.

K e y w o r d s: endophytic bacteria, constructed wetlands, diclofenac, sulfamethoxazole, biodegradation

Introduction

The presence of pharmaceutical products in the environment has become a subject of great interest due to their widespread use and disposal. Belonging to this group, diclofenac (DCF, [2-(2,6-dichloro anilino)phenyl]acetic acid) and sulfamethoxazole (SMX, 4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide) are in common use.

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) administered to reduce inflammation and for relieving pain in patients. Recent studies have estimated that, at present, on average of 1443 ± 58 tons of DCF are consumed globally (Acuña et al. 2015). DCF sold OTC (over-the-counter) may also hamper any efforts to quantify its use. Due to DCF's high resistance to biodegradation and harmful impact on some environmental species in low concentration ($\leq 1\,\mu g/l$) (Schwaiger et al. 2004), in 2015 the drug was included in the first Watch List of substances in the EU that require environmental monitoring in all Member States (Barbosa et al. 2016).

Sulfonamides, including sulfamethoxazole (SMX), belong to the most commonly used antibiotics in clinical (in combination with trimethoprim) and veterinary medicine (Sukul and Spiteller 2006), and are applied to treat bacterial and protozoal infections. The worldwide consumption of antibiotics ranges from 100 000 to 200 000 tons per year and more than 20 000 tons of sulfonamides could be introduced to

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the environment each year (Baran et al. 2011). Antibiotics affect the environmental microbiome and pose the problems of development and the propagation of antibiotic resistance (Gao et al. 2012).

Both DCF and SMX are known to be recalcitrant to degradation in conventional activated sludge treatment systems and have been detected in wastewater effluents (Vieno and Sillanpää 2014), surface water (Hirsch et al. 1999), ground water (Sui et al. 2015) and even drinking water (Schaider et al. 2013). Therefore, searching for an alternative, and at the same time, effective methods of their removal, is still a challenge for researchers. Verlicchi and Zambello (2014) suggested that constructed wetlands (CWs) can represent a promising approach for treating wastewater containing recalcitrant compounds. CWs are engineered systems that use plants and plant-associated microorganisms' ability to remove contaminants from wastewater (Shelef et al. 2013). The effectiveness of these systems in wastewater treatment depends, e.g., on the presence and activity of plant-associated microorganisms, especially endophytic bacteria. They live inside plant tissues without causing any apparent symptoms of disease and can play a crucial role in the biodegradation or biotransformation of organic pollutants (Weyens et al. 2009). Recent studies have described many species of endophytes that are able to remove or transform different xenobiotics (Afzal et al. 2014). However, only few of these studies have focused on the degradation of pharmaceuticals (Sauvêtre and Schröder 2015; Syranidou et al. 2016).

Despite the ubiquity of pharmaceuticals, the response of the whole endophytic bacterial communities to the pressure caused by the presence of DCF and SMX, as well as the abilities of bacterial endophytes to degrade or transform these pharmaceuticals, are still not completely understood. Thus, the objectives of this study were: (i) the isolation of endophytic bacteria from the root tissues of *Phalaris arundinacea* (reed canary grass), taken from lab-scale CWs in which the plants were exposed or non-exposed to DCF and SMX pollution; (ii) the determination of the degradation potential of the selected endophytic isolate using DCF and SMX as the sole carbon source and in the presence of phenol as an additional carbon source in batch liquid cultures; (iii) the evaluation of diversity of endophytic bacterial communities in root tissues using PCR-DGGE.

Experimental

Materials and Methods

Plant source. The plant samples were obtained from a lab-scale constructed wetland system, consisting of 12 columns simulating the operation of intermittent
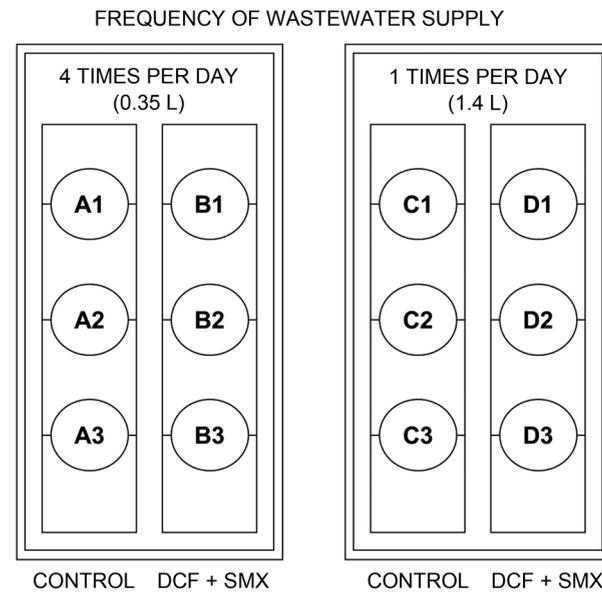
Fig. 1. The scheme of a lab-scale constructed wetlands system, from which the plant samples were collected.

downflow beds (Fig. 1). The columns were planted with reed canary grass. The CW system was fed with synthetic municipal wastewater (Nopens et al. 2001), which consisted of (mg/l): 208.76 Urea, 264 Yeast Extract, 118 Skim Milk Powder, 510.4 CH_3COONa, 40 Peptone, 41.37 KH_2PO_4, 0.62 NH_4Cl, 0.96 $KCr(SO_4)_2 \times 12H_2O$, 0.781 $CuSO_4 \times 5H_2O$, 0.108 $MnSO_4 \times H_2O$, 0.359 $NiSO_4 \times 7H_2O$, 0.1 $PbCl_2$, 0.208 $ZnCl_2$, 4.408 $MgSO_4 \times 7H_2O$, 11.6 $FeSO_4 \times 7H_2O$. Six columns (A1-3 and B1-3) were fed 4 times per day with a 0.35 l of wastewater, while the rest of columns (C1-3 and D1-3) were fed once a day with 1.4 l of wastewater.

The experimental system was operated using a high-pressure sodium lighting system with light/dark conditions that changed according to the season: for fall, 9 h of light and 15 h of darkness (9/15) and, for winter, 8/16. The mean night and day temperature during the experiment was 23.1°C and 29.1°C, and the mean humidity was 59.3%. DCF and SMX were added to the synthetic wastewater to a final concentration of 2 mg/l and were dosed to columns B1-3 and D1-3. A detailed description of the technical part of the experiment is provided in previous publication (Nowrotek et al. 2016).

Isolation of endophytic bacteria. The roots obtained from the columns not exposed and exposed to DCF and SMX mixed pollution, were separately sliced into 1–2 cm pieces, weighed and their surface was sterilized with 2% NaClO solution for 10 min. Afterwards, the samples were rinsed three times in sterile water for 1 min and dried on sterilized filter paper. Then, the samples were homogenized in a sterile ceramic mortar in 5 ml of phosphate-buffered saline (PBS). The crushed root tissues were used to obtain tenfold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) and 100 μl of each dilution was spread
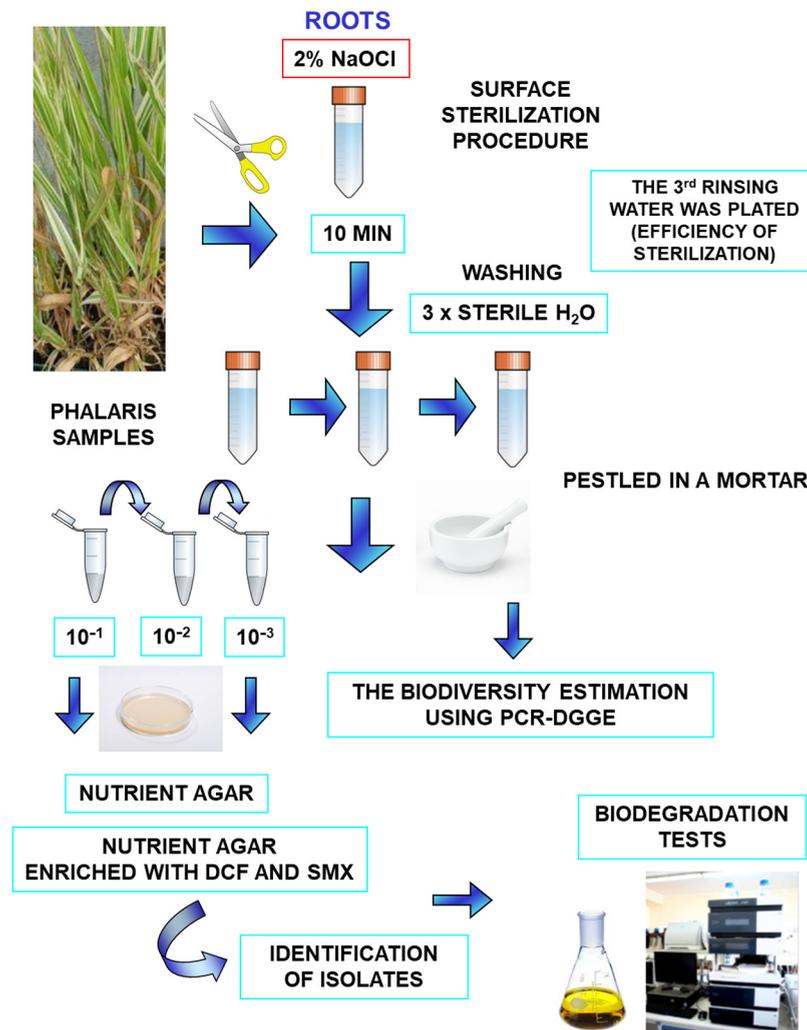
Fig. 2. Workflow for isolation of cultivable endophytic bacteria from *P. arundinacea* plants non-exposed and exposed to DCF and SMX mixed pollution (three plants per condition from each column were harvested).

onto nutrient agar plates enriched with DCF and SMX (2 mg/l, both). All plates were prepared in triplicate and incubated at 25°C for 7 days. A 100 μl sample of the third rinsing water was plated to confirm the efficiency of sterilization. The colonies on the plates were counted and the number of colony forming units per gram of fresh weight (CFU/g FW) was determined. The averages and standard errors were calculated from six replicates from a mixed sample of plants from three columns. The average CFU/g FW obtained after counting of the colonies grown on nutrient agar and nutrient agar enriched with DCF and SMX were compared using an unpaired two-sided t-test. The workflow for isolation of cultivable endophytic bacteria from reed canary grass is shown in Fig. 2.

Identification of selected endophytic bacteria. The isolates, representing the most abundant colony types grown on medium enriched with pharmaceuticals, were selected and identified. Genomic DNA was extracted from pure culture using the FastDNA™ SPIN kit for soil (MP Biomedicals) according to the recommendations of the manufacturer. The primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1401R (5'-CGGTGTGTACAAGACCC-3') were used to partially amplify the 16S rRNA gene. The reaction mixtures contained: $1 \times$ buffer, 2 mM MgCl$_2$, 5 pM/μl of 27F and 1492R primers, 20 pM/μl dNTPs and 1.5 U GoTAQ Flexi (Promega) in total reaction volume of 30 μl. Extracted DNA at a concentration of 0.15–0.2 μg/μl was added to the PCR mixture. After initial denaturation at 94°C for 5 min, each thermal cycling involved: 30 s denaturation at 95°C, 45 s annealing at 57°C and 1.5 min elongation at 72°C. After 30 cycles, a final elongation was performed at 72°C for 7 min. The presence of amplicons was confirmed by gel electrophoresis on 1% agarose (w/v) according to standard procedure. Using Clean Up Kit (A&A Biotechnology), PCR products were purified. Then, they were reamplified and sequenced with the BigDye®Terminator v3.1 kit (Applied Biosystems). The sequence of DNA was compared with GenBank NCBI (National Center for Biotechnology Information).

Biodegradation of DCF and SMX. For the biodegradation study, the isolate (MG7) representing the most abundant colony types grown on the medium enriched with DCF and SMX, was selected. The strain was cultivated in a nutrient broth at 30°C with agitation at 130 rpm for 24 h. Bacterial cells were harvested by centrifugation ($5000 \times g$ at 4°C for 15 min), washed with a sterile mineral salt medium, and used as an inoculum in experiments on the degradation of DCF and SMX.

The biological degradation of DCF and SMX in monosubstrate and in the presence of phenol as the additional carbon source was carried in 250-ml Erlenmeyer flasks containing 100 ml of a sterile mineral salt medium, which consisted of (g/l): $3.78\ Na_2HPO_4 \times \times 12H_2O$, $0.5\ KH_2PO_4$, $5.0\ NH_4Cl$, $0.2\ MgSO_4 \times 7H_2O$ and 0.01 yeast extract. The inoculum of bacterial cells prepared as described above was added to the medium to an initial optical density of about 0.2 to 0.4 at $\lambda = 600$ nm. Additionally, two control cultures (100 ml) were prepared: an uninoculated control involved the mineral salt medium and pharmaceuticals (abiotic control), and a heat-killed control consisted of bacterial cells destroyed by autoclaving (adsorption control). The optical density of the heat-killed control was the same as for the tested cultures.

DCF and SMX were added to each flask to a final concentration of 2 mg/l. In the monosubstrate culture, DCF or SMX was the sole carbon and energy source. In the flasks enriched with phenol as the additional carbon source, 20 mg/l of phenol was added every 5 days of the culture, to give a final concentration of 100 mg/l. All cultures were grown in triplicate and incubated with shaking at 130 rpm at 25°C for 20 days.

Determination of DCF and SMX concentration. High-performance liquid chromatography, coupled with variable wavelength detector (UltiMate 3000 system; Dionex Corporation, Sunnyvale, CA, USA), was used for quantification of SMX and DCF concentrations in all cultures. The chromatographic separation was performed using C18 Hypersil TM Gold column (250 mm × 4.6 mm; pore size: 5 μm) (Thermo Scientific, Polygen, Poland). The mobile phase consisted of a mixture of acetonitrile and acetate buffer (pH = 5.7), in a volumetric ratio 40:60 (v/v). During the analysis, an isocratic flow rate was used, and it was set at 1.0 ml/min. In the above-described conditions, the retention times (RT) of the compounds investigated were equal to 8.4–0.3 min and 6.4–0.2 min for DCF and SMX, respectively. The limit of quantification of DCF and SMX was equal to 0.2 mg/l. It was established as the first lowest calibration point of their calibration curves (linear regression, $R2 > 0.999$). The calculated value of "signal to noise" ratio in the case of both compounds investigated was greater than 10. The method was validated according to ISO/IEC 17025 (2005), which means that all method parameters as: accuracy, precision and recovery meet the criteria of this ISO standard. The analyses were performed and confirmed at four different wavelengths, i.e., 220 nm; 240 nm; 268 nm and 280 nm. The data was evaluated by means of Dionex Chromeleon™ 6.8 software.

The efficiency of removal was calculated using the formula:

$$\%R = (Cas - Cs)/Cac \times 100\%$$

where Cs is concentration of DCF or SMX in the sample, Cas is the concentration of DCF or SMX in the appropriate abiotic control.

Biodiversity of endophytic bacteria. All genomic DNA from tissues homogenates, obtained from surface sterilized roots of *P. arundinacea*, was extracted using FastDNA® SPIN KIT FOR SOIL (MP Biomedicals, USA), according to manufacturer's instructions and stored at –20°C until PCR amplification. Primers (Muyzer et al. 1993): 338f (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3') and 518r (5' ATT ACC GCG GCT GCT GG 3') were used for partial 16S rRNA bacterial gene PCR amplification. PCR was carried out in a 30 μl (total volume) reaction mixture containing: 19 μl sterile MiliQ water, 6 μl PCR buffer (GoFlexi TAQ, Promega), 2.4 μl $MgCl_2$ (2 mM), 0.25 μl of both primers (5 pmol/μl), 1.3 μl dNTPs (20 pmol/μl), 0.5 μl of genomic DNA (0,15–0,2 μg/μl) and 0.3 μl Taq DNA polymerase (1.5 U).

PCR amplification was performed using an Eppendorf thermal cycler and the following steps: (i) the initial denaturation step (10 min at 95°C); (ii) 30 cycles, each single cycle consisting of denaturation (10 min at 95°C), annealing (1 min at 53°C), and elongation (2 min at 72°C); and (iii) the final extension step (12 min at 72°C). Products were analyzed in agarose gel (0.8% w/vol agarose, 1 × TBE buffer), stained with ethidium bromide (1% w/vol) in MiliQ water and photographed under UV light. The DGGE of PCR products obtained in reaction with 338f and 518r primers were performed using the Dcode Universal Mutation Detection System (BioRad). The polyacrylamide gel (8% (v/v) with a gradient of 30–60% denaturant) was run for 9 h at 55 V in a 1 × TAE buffer at a constant temperature of 60°C. The gel was stained with SYBR GOLD (1:10 000, Invitrogen) in MiliQ water for 20 min and washed in MiliQ water twice for 15 min, then visualized under UV light and photographed. The DGGE banding patterns with 16S rDNA PCR products were analyzed using Quantity One 1D Software (BioRad). The structural diversity of the bacterial communities was calculated based on the Shannon-Weaver diversity index, H' (Shannon and Weaver 1963), estimated from the relative band intensities obtained from the DGGE fingerprints.
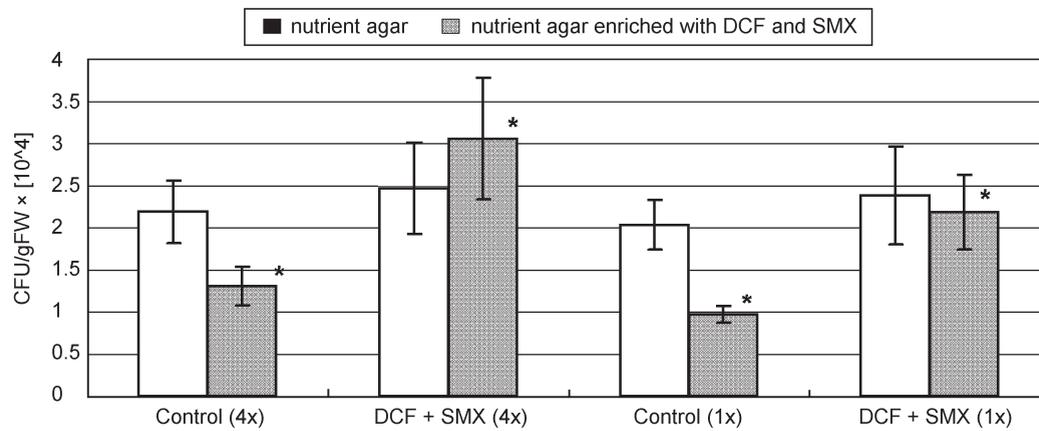
Fig. 3. The number of bacteria (CFU/g FW) obtained from the surface of the sterilized reed canary grass root samples.
Light bars – the number of bacterial endophytes grown on nutrient agar. Grey bars – the number of bacterial endophytes grown on nutrient medium enriched with DCF and SMX. N = 6. Significant differences have been marked using * ($p < 0.05$; t-test). Time regime of wastewater supply in brackets.

Results

Characterization of endophytic bacteria. Endophytic bacteria were found in *P. arundinacea* root tissues not exposed and exposed to DCF and SMX. After seven days of incubation, no significant differences in CFU/g fresh weight (FW) were observed, when nutrient agar was used as the growth medium (Fig. 3). The number of endophytic bacteria was comparable for the samples not exposed and exposed to DCF and SMX mixed pollution. When nutrient agar was enriched with DCF and SMX and used as the growth medium, significant differences in CFU/g FW were noticed. The number of endophytic bacteria was higher in the samples exposed to DCF and SMX mixture. However, the diversity of colony morphologies was lower than in the samples non-exposed to pharmaceuticals. The time regime of wastewater supply to CWs did not affect the CFU/g FW values.

Twelve isolates showing different colony morphologies were selected from the medium enriched with pharmaceuticals and transferred to a new plate. Cultures were considered to be pure after two successive passages on streak plates. Four isolates, representing the most abundant colony types grown on the medium enriched with pharmaceuticals, were identified by using partial 16S rRNA gene sequencing. The results are shown in Table I.
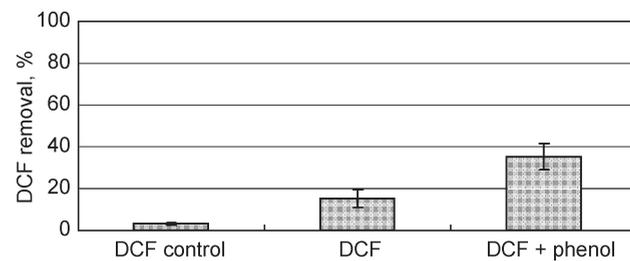
Fig. 4. The removal of 2 mg/l DCF by MG7 strain in the adsorption control, without additional carbon source and with phenol at a concentration of 100 mg/l as the additional carbon source.

Biodegradation of DCF and SMX. For the biodegradation test, the isolate (MG7) representing the most abundant colony types grown on the medium enriched with DCF and SMX was selected. In the first set of experiments, DCF and SMX were used as a single substrate at a concentration of 2 mg/l, respectively. After 20 days of incubation, strain MG7 removed approximately $15.23 \pm 4.21\%$ of DCF (Fig. 4) and $9.12 \pm 3.56\%$ of SMX (Fig. 5). A decrease in the optical density of the cultures was observed, from 0.350 to 0.200 (Fig. 6) and from 0.387 to 0.198 (Fig. 7), respectively. The removal of DCF and SMX in the adsorption cultures was low, and was equal to $3.04 \pm 0.56\%$ and $2.41 \pm 1.01\%$, respectively.

In the cultures enriched with phenol, as an additional carbon source, an improvement of SMX removal efficiency was observed, and $61.24 \pm 5.09\%$ of this

Table I
Identification of bacterial endophytes, based on the 16S rRNA gene sequence.

Isolate	Clostest match	Similarity, %	Sequence ID
MG2	*Variovorax boronicumulans*	96	NR_114214.1
MG5	*Bacillus wiedmannii*	97	NR_152692.1
MG7	*Microbacterium flavescens*	98	NR_029350.1
MG11	*Agrobacterium tumefaciens*	97	NR_041396.1
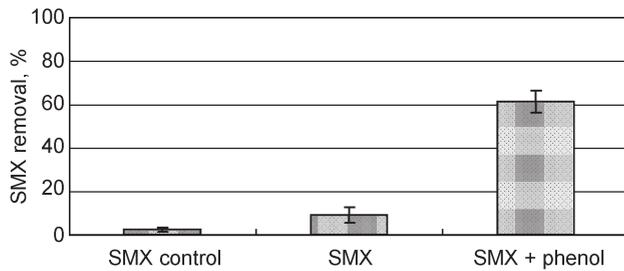
Fig. 5. The removal of 2 mg/l SMX by MG7 strain in the adsorption control, without additional carbon source and with phenol at a concentration of 100 mg/l as the additional carbon source.

substrate was removed after 20 days of incubation (Fig. 5). Moreover, an increase in the optical density of MG7 strain was observed (from 0.256 to 0.721) (Fig. 7). The efficiency of DCF removal was lower ($35.23 \pm 6.17\%$) (Fig. 4) in comparison to the cultures with SMX, as well as an increase in the optical density was not as efficient as it was observed for the SMX-containing cultures (from 0.321 to 0.509) (Fig. 6).

Biodiversity of endophytic bacteria. Total DNA extracts, obtained from the surface-sterilized root tissues of *P. arundinacea* non-exposed and exposed to DCF and SMX, were subjected to PCR amplification, followed by analysis *via* DGGE. The fingerprints obtained from DGGE separation of 16S rRNA gene fragments are shown in Fig. 8. All the samples yielded similar DGGE profiles. Some bands (signed as 3, 7 and 8) appeared in all samples. Bands signed as 6 were visible only in samples obtained from columns B1-3 and D1-3, in which plants were exposed to DCF and SMX. Bands signed as 5 appeared in all samples, except the samples obtained from columns B1-3. Bands signed as 4 were visible only in samples obtained from columns A1-3 and B1-3, i.e. columns exposed to wastewater four times a day. Bands signed as 1 and 9 appeared in samples obtained from columns D1-3 and C1-3, respectively. In profiles obtained from columns D1-3, there was a lack of the band signed as 2, visible in the other samples.
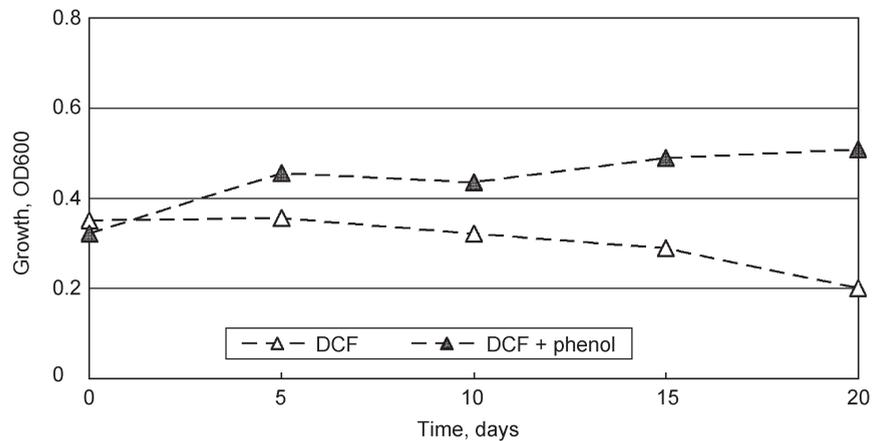
Fig. 6. Changes in biomass of MG7 strain in the cultures that contained DCF, monitored as optical density at 600 nm, without additional carbon source and with phenol at a concentration of 100 mg/l as additional carbon source.
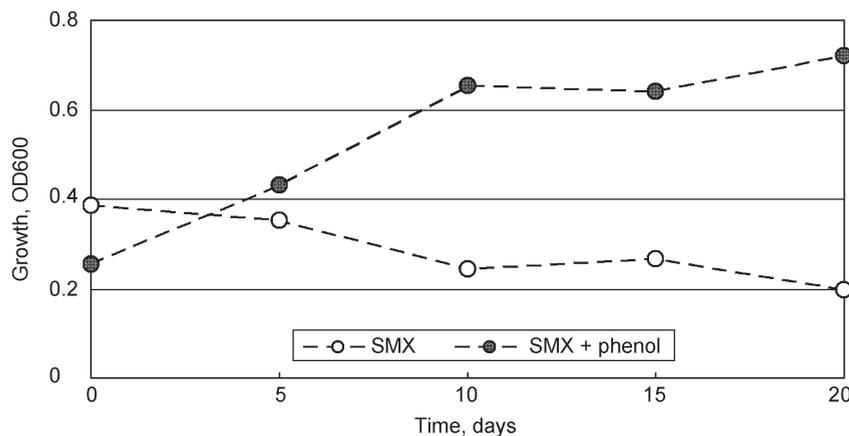
Fig. 7. Changes in biomass of MG7 strain in the cultures that contained SMX, monitored as optical density at 600 nm, without additional carbon source and with phenol at a concentration of 100 mg/l as additional carbon source.
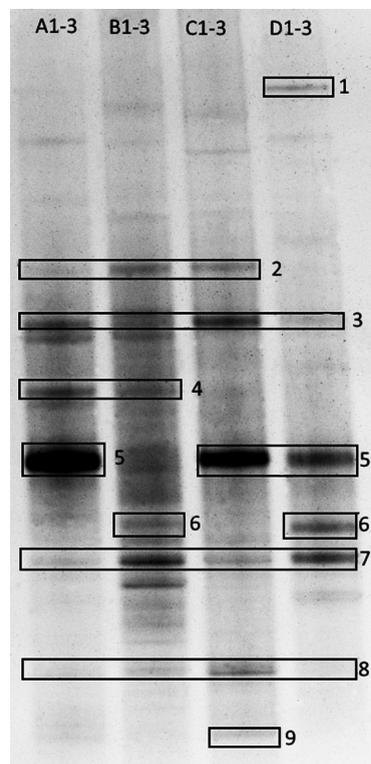
Fig. 8. DGGE pattern of 16S rRNA gene fragments of 180 bp amplified from DNA obtained from the surface-sterilized root tissues of *P. arundinacea*.

A1-3, C1-3 – the root samples non-exposed to DCF and SMX, B1-3, D1-3 – the root samples exposed to DCF and SMX. The frequency of wastewater supply: A1-3 and B1-3 – 0.35 l four times a day; C1-3 and D1-3 1.4 l – once a day. Description of the bands in the manuscript text.

The Shannon biodiversity index was determined based on densitometry analysis. The results are presented in Fig. 9. The highest value of Shannon biodiversity index was calculated for the sample obtained from columns B1-3, in which plants were exposed to DCF and SMX and the lowest for the sample obtained from the columns C1-3, which were non-exposed to DCF and SMX. The values of Shannon biodiversity index were comparable for all samples.
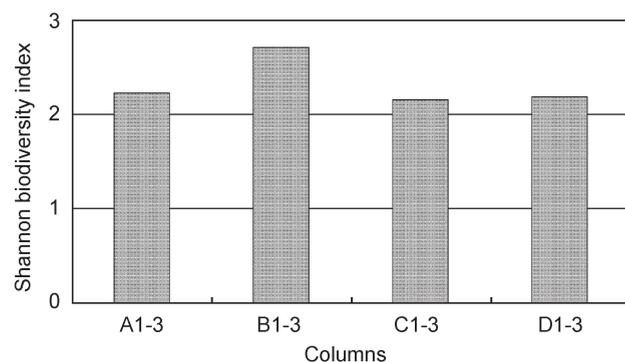
Fig. 9. Shannon biodiversity index based on DGGE profiles.

A1-3, C1-3 – the root samples non-exposed on DCF and SMX; B1-3, D1-3 – the root samples exposed on DCF and SMX. The frequency of wastewater supply: A1-3 and B1-3 – 0.35 l four times a day; C1-3 and D1-3 – 1.4 l once a day.

Discussion

Biological methods have been increasingly used for the removal of various xenobiotics, including pharmaceuticals from the environment. Constructed wetlands could be a sustainable solution for wastewater treatment in small villages and developing countries (Zhang et al. 2014), for a final treatment of troublesome effluents (Hijosa-Valsero et al. 2016) or the wastewater containing selected anthropogenic (micro)pollutants (Felis et al. 2016). Microbial endophytes reside within plant tissues and are therefore exposed to many plant metabolites (including phenolic compounds) and could possess efficient enzymatic systems for their transformation. Siciliano et al. (2001) showed that plants grown in soil contaminated with xenobiotics naturally recruited endophytes with the necessary contaminant-degrading capabilities. Inside the plant, the first step of different xenobiotic removal could be supported by the endophytic communities living in roots and rhizomes. Therefore, biodegradation of such compounds using plant-associated endophytic bacteria, has recently gained considerable attention.

In our investigations, the number of endophytic bacteria from roots non-exposed and exposed to DCF and SMX was determined. It was expected that pharmaceuticals, in particular SMX, would exert a selective pressure on endobacterial communities. The number of endophytic bacteria grown on nutrient medium were comparable and the decrease of this value in the samples exposed to DCF and SMX was not observed. However, the diversity of the colony morphologies was lower. When the nutrient agar enriched with DCF and SMX was used as the growth medium, significant differences in CFU/g fresh weight (FW) were observed. A higher number of endophytic bacteria was noticed in the samples exposed to DCF and SMX, what suggests that isolates can be better adapted to the presence of pharmaceuticals and be able to use these compounds as a carbon source or they can be resistant to SMX. A higher number of endophytic bacteria was observed in the samples obtained from *C. pepo* exposed to DDE (degradation product of DDT) (Evers et al. 2016) and the authors concluded that the xenobiotic can be used as a growth substrate by endophytes.

For biodegradation tests, the isolate (MG7) representing the most abundant colony types grown on medium enriched with DCF and SMX, identified by partial 16S rRNA gene sequencing as *Microbacterium flavescens,* was selected. The general potential of different *Microbacterium* species for the biodegradation of pharmaceuticals has already been highlighted (Kim et al. 2011; Ricken et al. 2013; Ricken et al. 2017).

In our investigations, the MG7 strain removed approximately 9% of SMX and 15% of DCF (the initial

concentration was 2 mg/l, each) from the liquid medium in monosubstrate culture. However, a decrease in the optical density of the cultures was also observed. One reason could be that the applied carbon and energy source didn't provide enough energy for bacterial growth and proliferation. When the carbon source was used up, the dying cells could lyse and provide a source of nutrients (Maier 2000). However, this hypothesis should be confirmed in follow-up studies focusing on endogenous metabolism. The literature data shows that the decrease in the optical density can be used for determination of bacterial cell lysis (Yin et al. 2013; Danevčič et al. 2016). A decrease in the optical density can be observed because of cellular damage and the leakage of cytoplasmic contents. The refractive index of bacterial suspension decreases and therefore, a decline in optical density can be noted because of lower light scattering.

The experiments on the autoclaved bacterial cells (heat-killed) showed that the removal of DCF and SMX was low and equal to $3.04 \pm 0.56\%$ of DCF and $2.41 \pm 1.01\%$ of SMX, respectively. Autoclaving leads to the destruction of the bacteria (Berns et al. 2008); therefore, the adsorption of the pharmaceuticals to damaged cell structures might not fully reflect the process that is characteristic of non-autoclaved cultures. The conclusion that adsorption of SMX and DCF onto biomass is negligible, should be confirmed in further investigations by using a different strain, similar to the tested one, but not able to degrade or transform pharmaceuticals. In many research studies, the adsorption of different pharmaceuticals was tested by using autoclaved biomass and our results agreed with those obtained by Yu et al. (2011), who found that SMX is weakly adsorbed to the microorganisms. Herzog et al. (2013) observed that adsorption of SMX onto autoclaved biomass of strains isolated from sulfamethoxazole-acclimated activated sludge was negligible. Moreira et al. (2018) didn't find any adsorption of DCF onto the heat-killed bacterial strain *Labrys portucalensis* F11.

The adsorption process may influence the biodegradation of pharmaceuticals. The affinity of SMX and DCF to solids was usually determined for activated sludge. Several approaches were used, based on e.g. the solid-water distribution coefficient (K_d) of the respective compound. Carballa et al. (2008) determined the log K_d values ranging from 1.3 to 2.2 for DCF and 0.8 to 1.8 for SMX. These values were significantly lower than those obtained for the musk fragrances or estrogens, indicating that these compounds do not adsorb onto sludge to an appreciable extent.

Considering the above information, it can be assumed that in our investigations the MG7 strain is involved in SMX and DCF removal. Some studies have reported on the microbial removal of SMX and DCF. Jiang et al. (2014) described the strain *Pseudomonas psychrophila* HA-4, which was able to remove 34.30% of 100 mg/l of SMX at 10°C after 192 h of incubation. Aissaoui et al. (2017) demonstrated that strains identified as *Pseudomonas* sp. and *Arthrobacter nicotianae* were able to remove 34.27% and 32.95% of 3 mg/l of SMX, respectively after 48 h of incubation. Biodegradation of DCF was mainly observed in white-rot fungi. Marco-Urrea et al. (2010) investigated the transformation of DCF by *Trametes versicolor*, and after 4 h, the efficiency of removal was 94%, when the initial concentration of DCF was 10 mg/l, but the adsorption on the surface of the fungus cells was significant. The efficiency of DCF removal was also investigated in activated sludge communities and microbial transformation did not lead to complete degradation (Kosjek et al. 2009). Langenhoff et al. (2013) demonstrated the ability of cultures enriched from activated sludge to biodegrade DCF (from 50 to 300 mg/l) over three weeks. However, the responsible strain(s) were not identified. Bessa et al. (2017) demonstrated that strain *Brevibacterium* sp. D4 was able to biodegrade 35% of 10 mg/l of DCF as the sole carbon source.

In our investigation, only SMX and DCF removal was measured. However, further research should focus on the determination of intermediates and/or stable metabolites and the description of the possible degradation pathway. Only a few studies reported information on the enzymes and different reaction mechanisms involved in SMX breakdown. Ricken et al. (2013) described the molecular mechanism in *Microbacterium* sp. strain BR1 for the breakdown of SMX. In this bacterium, degradation was initiated by ipso-hydroxylation and SMX fragmentation was observed. The NADH-dependent hydroxylation of the carbon atom attached to the sulfonyl group resulted in the release of sulfite, 3-amino-5-methylisoxazole, and benzoquinone-imine. The last compound was transformed to 4-aminophenol. Mulla et al. (2018) reported that *Chrobactrum* sp., *Labrys* sp. and *Gordonia* sp. were able to degrade 45.2%, 62.2% and 51.4% of SMX, respectively (the initial concentration was 5 mg/l). In all three bacterial cultures, SMX was sequentially converted into 3-amino-5-methylisoxazole, 4-aminophenol, and hydroquinone. Larcher and Yargeau (2011) reported that the arylamine N-acetyltransferase, which shows high specificity for aromatic amines, is involved in the degradation of SMX in *P. aeruginosa* and *Rhodococcus* species. Hu et al. (2017) proposed the pathways of SMX degradation by *Enterobacter cloacae* strain T2. The initial metabolites of SMX biodegradation follow four pathways: methylation, substitution by hydroxyl and double bond oxidation, desulphurization and acetylation. N-(4-Hydroxyphenyl)acetamide might be attributed to hydroxylation of N-phenylacetamide; N-phenylacetamide was also converted to benzoic and phenylacetic acid through

other pathways. The final process was the degradation of the benzene ring. Compounds containing a benzene ring are hydroxylated to form catechol and its structural isomers under catalysis of dioxygenases.

Despite the existing reports on the microbial degradation of DCF, there are still knowledge gaps related to the degradation process. Gröning et al. (2007) observed the removal of DCF in a concentration of 3–35 µM in a river sediment, with transient accumulation of a major metabolite, the pbenzoquinone imine derivative of 5-hydroxy-DCF, which is further abiotically adsorbed on the biofilm and this reveals a lack of biodegradation potential for this intermediate compound. Moreira et al. (2018) reported that the bacterial strain *L. portucalensis* F11 degraded 70% of 34 µM of DCF, supplied as the sole carbon source after 30 days of the culture; while its complete degradation was achieved *via* co-metabolism with acetate after 25 days. DCF degradation was mainly followed by hydroxylation reactions. Hydroxylation of DCF resulted in the formation of four isomers, from which it was possible to elucidate the structure of 4- and 5-hydroxy-DCF. Further degradation can occur through different pathways involved, e.g., methylation or decarboxylation. The formation of benzoquinone imine species seems to be a central step in the degradation pathway.

In our investigations, the additional carbon source used in the biodegradation tests of DCF and SMX was phenol. In plants, microbial endophytes can be exposed to secondary metabolites as phenolic compounds (Balasundram et al. 2006). Phenolic compounds are usually produced as a defense compounds against an attack on the plant tissue or in a stressful environment (e.g. unfavorable temperature, light, pH conditions or presence of xenobiotics in the soil). The basic structure of a phenolic compound is a benzene ring with hydroxyl substituent(s) (Cohen and Kennedy, 2010). Plant-microbial interaction requires sustained and prolonged reactions of the endophytes against the host defense mechanisms. Therefore, the selective pressure could be exerted on endobacterial communities and the growth of microorganisms that possess the specific enzymatic systems could be promoted.

In our investigations, in the presence of phenol as the additional carbon source, the efficient removal of pharmaceuticals, particularly SMX, was determined. Phenol was added to cultures gradually, to give a final concentration of 100 mg/l. An increase in optical density of the suspension was observed. However, the growth of biomass of the MG7 strain was more extensive in cultures containing SMX. The higher degradation of pharmaceuticals could be explained by adaptive mechanisms in microorganisms exposed to phenol. There is a correlation between an increase in membrane fatty acids saturation and the increased tolerance towards the toxic compounds in phenol-degrading strains (Mrozik et al. 2010). Among the adaptive mechanisms, changes in the fatty acid composition of membrane lipids are the most important reactions of bacteria to membrane-active substances (Neumann et al. 2004). Owing to this, the bacterial membranes become more resistant to the fluidizing action of aromatic compounds, which allows the cells to survive in the contaminated sites (Salar et al. 2014). The other adaptive mechanisms might involve changes in the following: membrane protein pattern, energy metabolism, cell morphology, the cell wall as well as the vesicle formation and transformation or degradation of xenobiotics (Heipieper et al. 2007; Schrewe et al. 2013). The higher degradation of SMX and DCF in the presence of phenol could also be explained by co-metabolic interaction. Non-growth substrates (pharmaceuticals), which have a similar structure to growth substrates (phenol) can bind to enzymes and be transformed due to their non-specific activity (Fatta-Kassinos et al. 2010). Most pathways on the degradation of aromatics go through oxygenation reactions catalyzed by mono- or dioxygenases, to convert these compounds to dihydroxy aromatic intermediates. As was mentioned before, the final step in SMX breakdown is the degradation of the benzene ring and compounds containing a benzene ring are hydroxylated to form catechol, which is also produced by the oxidation of phenol. The dihydroxyl compounds produced subsequently undergo a ring fission by dioxygenases, to produce intermediates of the Kreb's cycle (Cao et al. 2009)

The bacterial endophytes that can be grown in the laboratory consist only a small fraction of the total diversity that exists in the plant tissues. The literature generally suggests that only 0.001% to 1% of the endophytes present in plant tissues are cultivable (Torsvik and Øvreås, 2002; Alain and Querellou 2009). Therefore, the response of all endophytic bacterial communities to the pressure caused by the presence of DCF and SMX in wastewater should also be taken into consideration. In this study, the biodiversity of endophytic bacterial communities of reed canary grass, non-exposed and exposed to DCF and SMX was assessed using PCR-DGGE based on DNA extracted from root tissues. Analyzing the results, the values of Shannon biodiversity index were comparable for all samples. However, the highest value was calculated for sample obtained from columns B1-3, in which plants were exposed to DCF and SMX. This result is surprising because it was expected that pharmaceuticals, in particular SMX, would have exerted a selective pressure on endophytic bacterial communities and a loss of biodiversity should be observed. Taking into consideration the fingerprint obtained from DGGE separation of 16S rRNA gene fragments, different band patterns among the samples were observed. This situation may be associated with the adaptation of the

endobacterial communities to the presence of pharmaceuticals. It should also be mentioned that a similar difference in bands' composition was not clearly visible in the samples obtained from columns D1-3, in which plants also were exposed to DCF and SMX but in a different time regime. The fingerprints obtained for samples from columns in which plants were exposed to pharmaceuticals showed some similarities. However, it seems that the diversity of endophytic bacteria communities depends on both the presence of DCF and SMX and frequency of supply of the constructed wetland systems with the wastewater. Therefore, further experiments should be conducted to confirm this hypothesis.

Conclusions

Constructed wetland systems are a promising technology for treating wastewater containing micropollutants, including residues of pharmaceuticals. The bacterial endophytes can be exposed to secondary metabolites in plant tissues. Therefore, they can possess the potential to transform or degrade aromatic structures, including pharmaceuticals. When the initial concentration was 2 mg/l, the strain MG7 identified as *Microbacterium flavescens* was able to remove approximately 15% of DCF and 9% of SMX after 20 days of incubation in monosubstrate culture. A decrease in the optical density of the cultures was observed, which indicated that the applied carbon source didn't provide enough energy for bacterial growth and proliferation. The adsorption of DCF and SMX onto autoclaved bacteria was negligible. However, this observation should be confirmed with other methods. In the presence of phenol as the additional carbon source, the strain MG7 removed approximately 35% of DCF and 61% of SMX, when the initial concentration was also 2 mg/l. The adaptive mechanisms in microorganisms exposed to phenol (i.e. changes in the composition of membrane lipids) or a co-metabolic mechanism, when non-growth substrates can be transformed by non-specific enzymes could participate in this phenomenon. Both the presence of pharmaceuticals and frequency of supply of CWs with the contaminated wastewater affected the diversity of the whole endophytic bacterial communities. However, further experiments should be conducted to confirm this hypothesis. The results obtained suggest the capability of the strain MG7 for degradation of DCF and SMX. However, further research should focus on the determination of intermediates and/or stable metabolites and assessment of the strains' potential for bioaugmentation in CWs.

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