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Veterinary antibiotic oxytetracycline's effect on the soil microbial community

Natalia Danilova*, Polina Galitskaya and Svetlana Selivanovskaya

Abstract

Background: Antibiotics are widely used to treat animals from infections. After fertilizing, antibacterials can remain in the soil while adversely affecting the soil microorganisms. The concentration of oxytetracycline (OTC) in the soil and its effect on the soil microbial community was assessed. To assess the impact of OTC on the soil microbial community, it was added to the soil at concentrations of 50, 150, and 300 mg kg^{-1} and incubated for 35 days.

Results: The concentration of OTC added to the soil decreased from 150 to 7.6 mg kg^{-1} during 30 days of incubation, as revealed by LC-MS. The deviations from the control values in the level of substrate-induced respiration on the 5th day of the experiment were, on average, 26, 68, and 90%, with OTC concentrations at 50, 150, and 300 mg kg^{-1}, respectively. In samples with 150 and 300 mg kg^{-1} of OTC, the number of bacteria from the 3rd to 14th day was 2–3 orders of magnitude lower than in the control. The addition of OTC did not affect the fungal counts in samples except on the 7th and 14th days for the 150 and 300 mg kg^{-1} contaminated samples. Genes *tet*(M) and *tet*(X) were found in samples containing 50, 150, and 300 mg kg^{-1} OTC, with no significant differences in the number of copies of *tet*(M) and *tet*(X) genes from the OTC concentration.

Conclusions: Our results showed that even after a decrease in antibiotic availability, its influence on the soil microbial community remains.

Keywords: Antibiotics, Tetracyclines, Antibiotic-resistant genes, Soil microbial biomass, Soil microbial community, Oxytetracycline

Background

Antibiotics are medicines that are widely used to treat and prevent bacterial infections not only in human medicine but also in veterinary treatments (Sarmah et al. 2006; Thiele-Bruhn 2003). Additionally, in animal husbandry, antibiotics are often used for the growth stimulation of animals (Halling-Sorensen et al. 1998). Many antibiotics used for veterinary purposes are poorly absorbed in the animal's gut, so up to 90% of the administered dose is excreted in manure or urine (Sarmah et al. 2006). The use of manure as a component of organic fertilizer leads to the spread of antibiotics and antibiotic-resistant genes in the environment (Ramaswamy et al. 2010).

Antibiotics have a selective effect on various microbial groups; therefore, the relative abundance and diversity of soil microbial species may be damaged (Grenni et al. 2018). It has been reported that some antibiotics inhibit microorganisms, while others have stimulating effects on microbial growth and activity (Sarmah et al. 2006; Thiele-Bruhn 2003; Halling-Sorensen et al. 1998). As a result of the action of the antibiotic as an antimicrobial agent, the biomass of sensitive microorganisms can be reduced. However, there is evidence that some microorganisms are able to use the antibiotic as a carbon source for nutrition, which is expressed in increasing microbial biomass (Thiele-Bruhn and Beck 2005).

Antibiotics have a selective effect on different microbial populations, which leads to the formation of resistance, genetic and phenotypic differences and changes in the relative abundance of microbial species and the

* Correspondence: natasha-danilova91@mail.ru
Institute of Environmental Sciences, Kazan Federal University, Kazan 420008, Russia

disruption of the interactions between the various species. As a result, the presence of antibiotics can lead to the disruption of natural environmental processes such as methanogenesis, nitrogen transformation, destruction of organic matter, and nutrient cycling (Sarmah et al. 2006).

The effects of the antibiotic depend on the characteristics of the environment that determine its availability. For example, the persistence, transformation, and sorption of antibiotics are highly dependent on soil type, amount of organic matter, pH, humidity, and temperature. It is known that tetracyclines strongly bind to clay soil particles, humic substances, and montmorillonite. However, at certain pH values, they can be released and have a long-term effect on microorganisms. Additionally, the impact of the antibiotic on microorganisms depends on its concentration. At high concentrations, antibiotics have bactericidal (fatal) and bacteriostatic (growth-inhibiting) effects on bacteria (Mojica and Aga 2011).

Antibiotics can affect the enzyme activity and biomass production of bacterial communities. It has been reported that some antibiotics inhibit microorganisms, while others have stimulating effects on microbial growth and activity (Thiele-Bruhn and Beck 2005).

Antibiotics of the tetracycline group are among the most common and effective drugs for treating beef cattle, poultry, pigs, sheep, and goats from pneumonia, mastitis, salmonellosis, sepsis, genitourinary infections, and diseases of the gastrointestinal tract. The mode of action of tetracyclines is the prevention of the association of aminoacyl-tRNA and ribosomes in bacterial cells and inhibition of the synthesis of bacterial proteins (Chopra and Roberts 2001; Sarmah et al. 2006). Currently, one of the most commonly used antibiotics of the tetracycline group is oxytetracycline (OTC), discovered in 1949 (Qingxiang et al. 2009), and is actively used in agriculture in Russia.

When introduced into the soil, tetracycline differentially influences various microbial groups and functions. As a whole, the presence of tetracyclines in the soil increases the amount of ammonia-oxidizing bacteria (Cao et al. 2016). Gram-positive bacteria are more sensitive to tetracyclines than gram-negative bacteria because tetracycline-resistant bacteria are mostly gram-negative. Gram-negative bacteria are more resistant to antibodies and antibiotics than gram-positive bacteria because they have a largely impermeable cell wall (Hund-Rinke et al. 2004).

Bacteria have developed several ways to resist the action of tetracyclines. Currently, more than 30 various resistant genes have been described in the scientific literature. These genes encode three mechanisms that endow bacteria with antibiotic resistance: active antibiotic efflux (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(Z)), ribosome protection (*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T)), and antibiotic detoxification (*tet*(X)) (Suzuki et al. 2015; Chopra and Roberts 2001). An adaptation and development of antibiotic resistance in soil microorganisms has already been reported for antibiotic-spiked soil samples (Xie et al. 2017).

Despite the fact that antibiotics and resistance genes are recognized as a serious environmental problem and many studies (Martinez 2009; Xie et al. 2017; Sharma et al. 2016) are devoted to assessing their content in water, bottom sediments, and soil and plants, information on the level of their presence in the natural environment in Russia is practically absent.

In this study, OTC concentration decrease in the soil was estimated over 30 days beginning with an initial antibiotic concentration of 150 mg kg^{-1}. The effect of OTC on the microbial community of the soil was also studied during a model experiment, namely, the changes in the soil substrate-induced respiration, as well as the number of bacterial and fungal strains, were evaluated for dynamics for 35 days. We hypothesized that even after OTC concentration decrease in soil, it "post influences" the soil microbial community in the form of continued resistance patterns as well as altered microbial community structure.

Materials and methods

Luvisol was used as the subject of the study, sampled in the territory of Laishevsky, District of the Republic of Tatarstan. The soil investigated had a pH value of 5.8 and contained 2.9% total organic carbon, 0.18% total nitrogen, 54.5% sand, 41.2% silt, and 4.2% clay. The climate of the study site was temperate continental; soil sampling was carried out in summer. The sampling site was characterized by the absence of any human activity. Soil samples were collected using the "envelope" method; each sample was collected three times. The soil was collected from the top 20 cm soil layer. After delivery to the laboratory, the collected soil was cleaned of roots, passed through a 2–mm sieve and incubated at room temperature for 1 week while maintaining a moisture capacity of 60%.

To determine the time of OTC concentration decrease upon insertion into the soil, OTC was introduced into the preincubated soil weighing 3 kg at a concentration of 150 mg kg^{-1}. Contaminated soil was incubated for 30 days at room temperature (20–25 °C) with the maintenance of a 60% moisture capacity. The content of the antibiotic in the soil was measured on the 1st, 7th, 10th, 15th, 20th, 25th, and 30th day of incubation. Each measurement was performed in three repetitions.

To assess the effect of antibiotics on the soil microbial community, OTC was added to the preincubated soil weighing 3 kg to make the following final concentrations: 50, 150, and 300 mg kg^{-1}. Soil without antibiotic application was used as a control. The pot experiment was carried out for 35 days. The level of substrate-induced respiration (SIR) and the number of bacteria and fungi were estimated on the 1st, 3rd, 5th, 7th, 14th, 21st, 28th, and 35th day of incubation. Each measurement was performed in three repetitions. Soil samples were stored at a temperature of 20–25 °C with the maintenance of a 60% moisture capacity.

In this work, OTC was used in a solution form for injection. The antibiotic was introduced into the soil as follows: OTC was mixed with 50 ml of ethanol and added to a small amount of calcined sand; after ethanol evaporation, the sand, evenly contaminated with antibiotic, was added to the soil.

OTC was extracted using the extraction method with some minor modifications (Li et al. 2009). Each sample (0.5 g sample) was ultrasonically extracted for 15 min in polypropylene centrifuge tubes with 2 ml of 1/1 (v/v) methanol/EDTA-McIlvain buffer (pH = 4.0) and centrifuged at 4000 rpm for 10 min. This procedure was repeated three times. Instrumental determinations were performed using an Agilent 1200 LC-MS System (Agilent, USA) equipped with an additional UV detector (365 and 265 nm wavelength). OTC was detected using a Zorbax SB C18 column and Chemstation software. Water was mobile phase A and acetonitrile was mobile phase B with a total flow rate of 0.4 ml min^{-1}. The solvents were mixed as follows: 0–7 min, 15% A; 7.01–9 min, 85% A; 9.01–12 min, 15% A. The % recoveries of the analyzed antibiotic were between 52 and 56. The limits of quantification ranged from 20 μg L^{-1} to 100 μg L^{-1}. Concentrations of antibiotics in the soil samples were calculated by an external standard method based on the peak area of the monitored production (Li et al. 2009).

The evaluation of substrate-induced respiration was conducted according to ISO 14240-1. First, 0.02 g of D-glucose was added to 0.02 g of soil, and then the moistened soil was placed in three sacks. A 20 ml solution of sodium hydroxide (0.05 M) was poured into the laboratory flask, and a sack with soil was placed inside the flask. The flask was sealed and incubated for 24 h at 25 °C. After 24 h, the sack was carefully removed, and 2 ml of barium chloride solution (0.05 M) added to precipitate adsorbed CO_2 as barium carbonate. Three to four drops of indicator solution (phenolphthalein) were added, and the residual sodium hydroxide content was diluted with dilute hydrochloric acid (0.1 M).

Extraction of DNA from soil samples was carried out using the FastDNA Spin Kit for Soil (MP Bio, Germany) according to the manufacturer's instructions. DNA purification was carried out using the QIAquick PCR Purification Kit (Qiagen, Germany). Detection of the ratio of bacterial and fungal strains was carried out by real-time PCR (ISO 17601:2016) using two pairs of primers 947f/1349r and ITS1f/ITS2r with the following sequences: F: AACGCGAAGAACCTTAC, R: CGGTGTGTACAAGG CCCGGGAACG (for bacterial strains); F: TCCGTA GGTGAACCTGCGG, R: GCTGCGTTCTTCATCGAT GC (for fungal strains) (Zhang et al. 2013). The master mix reaction mixture (25 μl) contained the following components: DNA template 1 μl, forward and reverse primers (10 μM) 0.5 μl each, dNTPs (10 μM) 2.5 μl, 10× Buffer with SYBR Green 2.5 μl, $MgCl_2$ (25 μM) 2.5 μl, SynTaq polymerase (5 U μl^{-1}) 0.2 μl, and ddH_2O 15.3 μl. Amplification was performed on a BioRad CFX–96 cycler (BioRad, Germany) using the following temperature program: primary denaturation at 95 °C for 5 min, then 39 three-step cycles at 62–60 °C for 45 s, at 95 °C for 15 s, and at 72 °C for 30 s. DNA standard curves were constructed using bacteria *Pseudomonas putida* and micromycete *Penicillium notatum.*

Detection of two *tet* genes, *tet*(M) and *tet*(X), was carried out by real-time PCR (ISO 17601:2016) using two pairs of primers with the following sequences for *tet*(M): F: GGTTGGAATGTGACGGACTG, R: ATCGTTGTAT GCTCGTGAAAGA and *tet*(X) F: GAAAGAGACA ACGACCGAGAG, R: ACACCCATTGGTAA GGCTAAG. In detail, the PCR products of *tet*(M) and *tet*(X) were first cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA). Then, the plasmids carrying *tet*(M) and *tet*(X) were extracted and purified using a PureLink Quick Plasmid Miniprep Kit (Invitrogen Corporation, Carlsbad, CA) (Zhang et al. 2009). The master mix reaction mixture (25 μl) contained the following components: DNA template 1 μl, forward and reverse primers (10 μM) 0.5 μl each, dNTPs (10 μM) 2.5 μl, 10× Buffer with SYBR Green 2.5 μl, $MgCl_2$ (25 μM) 2.5 μl, SynTaq polymerase (5 U μl^{-1}) 0.2 μl, and ddH_2O 15.3 μl. Amplification was performed on a BioRad CFX–96 cycler using the following temperature program: primary denaturation at 95 °C for 5 min, then 39 three-step cycles at 62–60 °C for 45 s, at 95 °C for 15 s, and at 72 °C for 30 s.

The significances in data were repeatedly tested by ANOVA in R with Tukey Honest Significant Differences test or Student test as post hoc tests. Differences between values at $p < 0.001$ were considered statistically significant.

Results

In the first stage, the time of OTC half-life in soil was investigated. The initial concentration of OTC added to the soil at 150 mg kg^{-1} was selected on the basis of a

literature search. The other authors used 1 to 300 mg kg^{-1} of tetracycline in their studies (Chen et al. 2013; Molaei et al. 2017; Duan et al. 2017). During the 30-day incubation of soil contaminated with antibiotic, the concentration of OTC content was measured by liquid chromatography. The concentration dynamics for OTC is shown in Fig. 1. The OTC concentration on day 30 was approximately 98% less than that on day 1.

In the second stage of the study, the effect of OTC at concentrations of 50, 150, and 300 mg kg^{-1} on the SIR of the soil and the number of bacterial and fungal strains were evaluated. The model experiment was carried out for 35 days because it was previously established that the time of destruction of OTC in the soil during incubation at room temperature and with 60% moisture is 30 days. The change in the SIR of the soil contaminated with an antibiotic is shown in Fig. 2.

The level of SIR after antibiotic addition significantly decreased from the 1st to the 5th day of the experiment in all three contaminated samples compared with the control. The greatest decrease (7.2 times) was observed in a sample contaminated with 300 mg kg^{-1} OTC. On the 7th day, the SIR level increased again; in the sample with 50 mg kg^{-1} OTC, it remained comparable to the control until the end of the experiment and in the samples with 150 and 300 mg kg^{-1} OTC, it was 1.3–2.3 times higher ($p < 0.001$) and 1.6–2.6 times higher ($p < 0.001$) than in the control, respectively.

It was suggested that the changes of SIR described were connected with various dynamics of bacterial and fungal communities after addition of the antibiotics. Because OTC is an antibacterial drug, it was assumed that OTC addition, at least in the short term, would lead to a decrease in the bacterial counts in contrast with fungal counts that were expected to maintain or even grow in conditions without high bacterial competition. To verify this assumption, the bacterial and fungal 16S and ITS rRNA gene copy numbers were estimated using the real-time PCR method. The results obtained are shown in Fig. 3 a, b.

The number of bacterial genes in the control soil was 4.15×10^7–2.14×10^8 copies g^{-1}, and the number of micromycete genes was 1.64×10^4–6.02×10^4 copies g^{-1}. It was revealed that the addition of OTC at a concentration of 50 mg kg^{-1} had practically no effect on the bacterial counts, while concentrations of 150 and 300 mg kg^{-1} significantly affected this parameter ($p < 0.001$). Between the 3rd and 14th days, bacterial counts were 2–3 orders of magnitude lower than in the control ($p < 0.001$). Furthermore, an increase in bacterial counts was observed, and on the 28th ($p < 0.001$ for 150 mg kg^{-1}; $p < 0.001$ for 150 mg kg^{-1}) and 35th ($p < 0.001$ for 150 mg kg^{-1}; $p < 0.001$ for 150 mg kg^{-1}) days counts exceeded the control by 1–2 orders of magnitude. The antibiotic addition did not affect the fungal counts in the soil samples except for the 150 mg kg^{-1} on the 14th day ($p < 0.001$) and 300 mg kg^{-1} on the 7th ($p < 0.001$) and 14th ($p < 0.001$) days in the experiment contaminated samples.

At the next stage of the study, the quality of *tet*(M) and *tet*(X) genes was determined using the real-time PCR method. The results obtained are shown in Fig. 4 a, b.

As shown in Fig. 4, *tet*(M) and *tet*(X) tetracycline-resistant genes were detected in soil samples at all three concentrations of OTC ($p < 0.001$). The *tet*(M) gene is responsible for the ribosomal protection of the cell from tetracyclines, and the *tet*(X) deactivates them if they have penetrated into the cell.

Interestingly, various OTC concentrations introduced into the soil did not cause significant differences in the number of resistant genes *tet*(M) and *tet*(X). On the 5th and 7th day of the experiment, the amount of the *tet*(M) and *tet*(X) genes decreased in soil samples with 150 and 300 mg kg^{-1} OTC. Most likely, the microbial

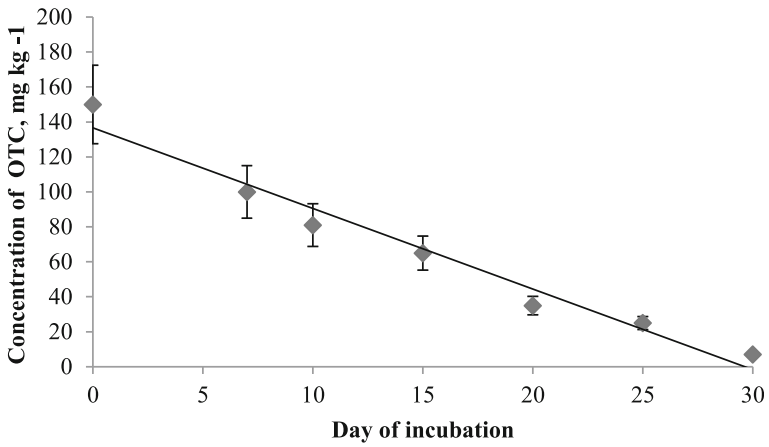

Fig. 1 Concentration of OTC in soil during 30 days of incubation

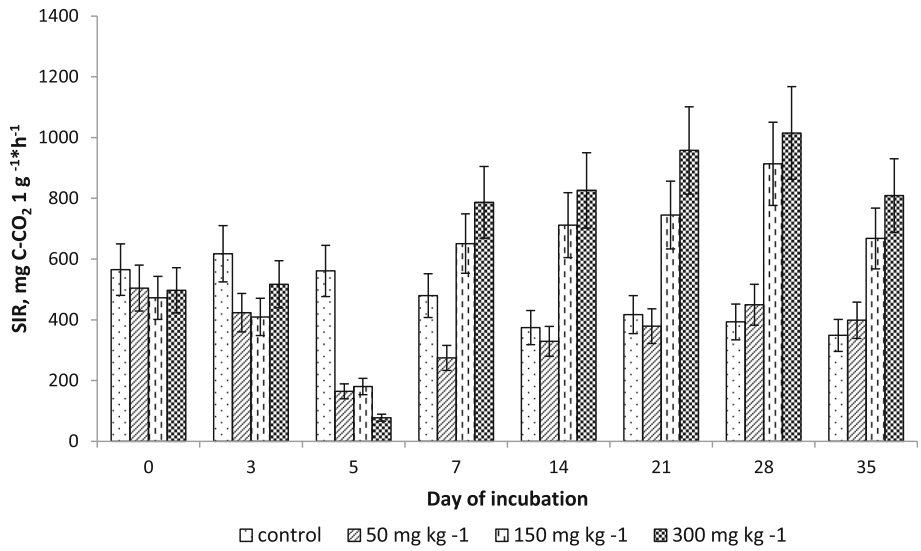

Fig. 2 Change of SIR in soil treated with various concentrations of OTC

community underwent changes during this period in the presence of an antibiotic added into the soil, associated with a decrease in the level of SIR in these samples.

Discussion

Due to various physicochemical properties, antibiotics have different half-lives in the soil from several days to several months (Sarmah et al. 2006). According to previous studies, the half-life of OTC in soil ranged between 10 and 79 days (Kay et al. 2004; Wang and Yates 2008; Sarmah et al. 2006). Various rates of antibiotic destruction in soil may depend on its concentration, soil type, quantity of organic matter content, and microbial activity (Sarmah et al. 2006).

When introduced into the soil, antibiotics can disrupt natural processes in soil ecosystems, inhibiting the functioning of microbial communities in soils (Halling-Sorensen et al. 1998). In this case, shifts in the ratio of bacterial and fungal number and changes in soil microbial activity and biomass can occur. Such changes ultimately can affect the fertility of agricultural soils in general (Sarmah et al. 2006).

To assess the effect of antibiotics on the functions of microbial communities of the soil, parameters such as microbial activity, biomass and respiration are measured. It is considered that substrate-induced respiration represents the total microbial biomass (Blagodatskaya and Kuzyakov 2013).

Most likely, during the first 5 days of the experiment, soil microbes that were sensitive to the antibiotic died, which resulted in a decrease in the overall level of SIR. Furthermore, the released ecological niches were taken by species resistant to OTC that multiplied in the absence of competitors, which resulted in an increase in the level of SIR.

The dose-dependent inhibitory effect of the antibiotic on SIR in soil was observed at the initial stage of incubation; however, further recovery of SIR values to the level of the control soil was observed, which may be associated with the formation of resistance to OTC in bacteria and a decrease in the bioavailability of the antibiotic (Thiele-Bruhn and Beck 2005; Speer et al. 1992; Alonso et al. 2001; Roberts 2005; Martinez 2009; Aydin et al. 2015; Munita and Arias 2016; Zhang et al. 2019). In another study, the suppressing effect of tetracycline on SIR was found only at a high dose of antibiotic (500 mg kg^{-1}); the weak effect of low doses (5 mg kg^{-1} and 50 mg kg^{-1}) was interpreted by a decrease in bioavailability as a result of high sorption of tetracycline in the soil (Hund-Rinke et al. 2004).

The bioavailability of OTC is the highest during the first several days after spiking; therefore, its inhibitory effect is the most pronounced on these days (Molaei et al. 2017). Different authors observed non-linear effects of OTC on soil respiration and biomass. Thus, low (1–15 mg kg^{-1}) and moderate (20–50 mg kg^{-1}) concentrations of OTC were non-inhibitory for these parameters since and OTC was significantly sorbed by the soil particles and even used as a carbon source, while high concentrations (150 mg kg^{-1} and over) led to a decrease in microbial biomass and respiration (Chen et al. 2014; Molaei et al. 2017). Sorption intensity highly depends on soil texture, pH, and other characteristics (Liu et al. 2015). Effects of OTC also may depend on the presence of initial resistance patterns of the soil microbial community. OTC introduced into soil acted as a selective factor for

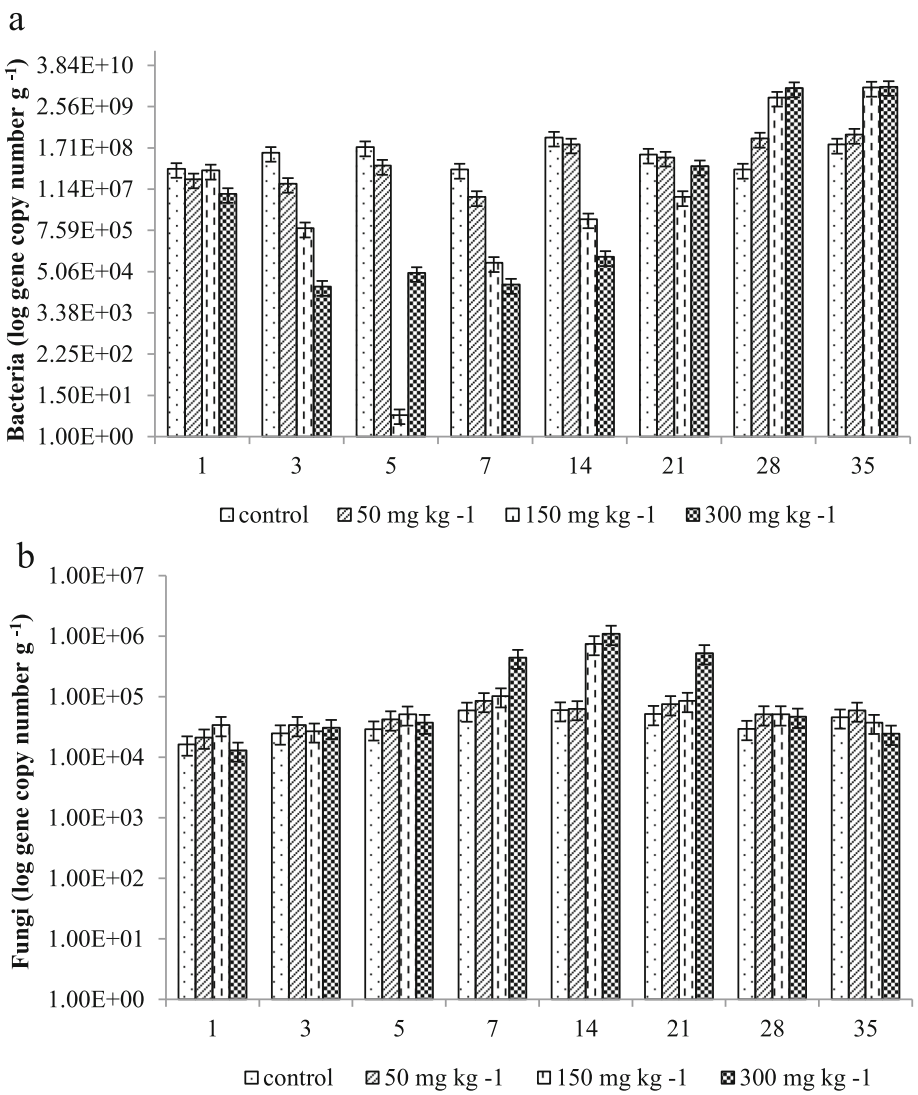

Fig. 3 Number of copies of bacterial (**a**) and fungal (**b**) genes in soil contaminated with OTC

resistant microbes and intensified horizontal gene transfer. When the number of resistant bacteria was high, the influence of OTC on soil microbial activity and biomass was negligible (Ma et al. 2016).

In general, the dynamics of the microorganism counts determined by the real-time PCR method correlated with the dynamics of the SIR level. In the first stages, the antibiotic was present in the soil at high concentrations and it negatively affected the sensitive bacterial species; as a result, the counts of bacteria decreased. When the antibiotic concentration decreased, bacterial populations recovered. The number of micromycetes was not directly affected by OTC. In those samples where there was a significant decrease in the bacterial counts, the fungal population grew as a result of weak competition. After the recovery of bacterial populations, the fungi counts returned to the control values. Interestingly, those bacterial counts not only recovered to the control values but even exceeded them. This could be partly explained by the growth of antibiotic-resistant populations (Cheng et al. 2016).

Excessive use of antibiotics in animal husbandry and the subsequent use of livestock waste introduces large amounts of antibiotics and resistant genes into the soil environment (Du and Liu 2012). Many studies point to the formation of resistant properties in soil microorganisms when antibiotics enter the soil (Xie et al. 2017; Jechalke et al. 2014; Heuer et al. 2011). There are at least three ways in which antibiotics in manure or compost can increase the abundance of resistant genes in the soil. First, the organic matter of manure or compost can contribute to the

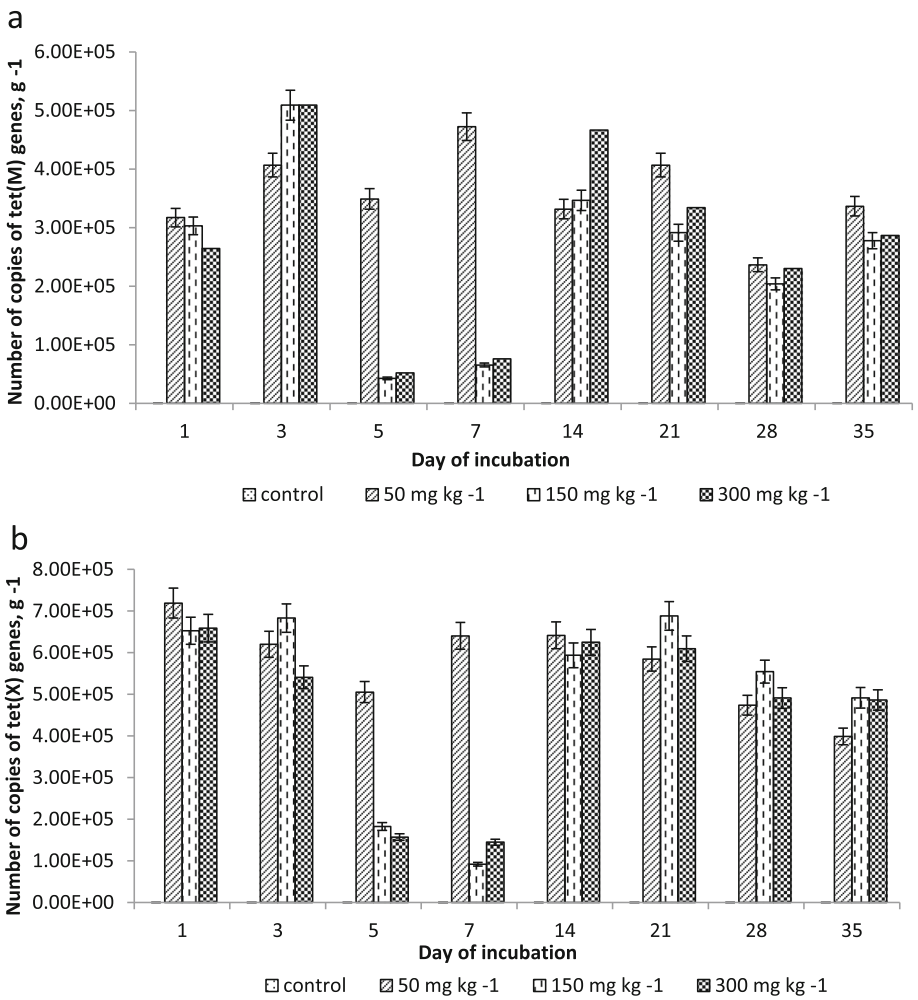

Fig. 4 Number of copies of *tet*(M) genes, g^{-1} in soil treated with various concentrations of OTC (***a***); Number of copies of *tet*(X) genes, g^{-1} in soil treated with various concentrations of OTC (***b***)

increase in intrinsic resistant genes that are initially present in soil bacteria. Second, resistant genes from manure or compost are introduced directly into the soil and stored in the soil by host bacteria, spreading through horizontal gene transfer. The third way is de novo mutations in the soil by selection for antibiotic resistance due to manure or soil (Xie et al. 2016).

Our results obtained also confirm that soil bacteria can acquire resistance in soil contaminated with antibiotics. In addition, another factor that may indirectly contribute to the spread of antibiotic resistance among microbial communities is the heavy metals that accumulate in the soil after manure treatment (Heuer et al. 2011). Recent studies have shown an increase in the level of antibiotic-resistant genes in soils contaminated with antibiotic-spiked manures and composts or irrigated with water containing antibiotics (Heuer et al. 2011; Wang et al. 2015; Tien et al. 2017).

Conclusions

In the laboratory experiment, it was shown that OTC concentration, upon introduction into the soil, decreases rather quickly. At an initial concentration of 150 mg kg^{-1} in the soil, the quantity of the drug reduced by 98% during the 30-day incubation. The introduction of the antibiotic into the soil at concentrations of 50, 150, and 300 mg kg^{-1} was accompanied by a partial elimination of bacteria. On the 5th day of the model experiment, the level of microbial biomass decreased by 26%, 68%, and 90% in soil with a concentration of 50, 150, and 300 mg kg^{-1}, respectively, compared to the reference value. On the 14th day, growth of microbial biomass in contaminated samples was observed. The survival and spread of tetracycline-resistant genes could contribute to bacterial survival in contaminated soil. With a concentration 150 and 300 mg kg^{-1} of OTC, from the 3rd to the 14th day, a decrease in the number of bacteria was observed by 2–3 orders of

magnitude in comparison with the control. OTC had practically no negative effect on the number of fungi in the soil. Simultaneously, with the decrease in the number of bacterial strains, there was a short-term increase in the number of micromycetes, possibly because the fungi occupied the newly vacated ecological niches. Soil contamination with OTC led to the formation of resistance in soil microorganisms. Tetracycline-resistant genes *tet*(M) and *tet*(X) were present in soil containing 50, 150, and 300 mg kg^{-1} OTC. However, there were no significant differences in the number of copies of the *tet*(M) and *tet*(X) genes at various OTC concentrations. We conclude that the effects of OTC introduced into the soil should be estimated based only on the half-life of antibiotics and on the maintenance of the resistance patterns of the microbial community as well as the presence or absence of fungal and bacterial counts. As we demonstrated in this study, the effects of OTC on the microbial community remain longer than the presence of antibiotics.

Abbreviations
OTC: Oxytetracycline; SIR: Substrate-induced respiration.

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Authors' contributions
ND and PG participated in the design of the study and conducted field work. ND, PG, and SS analyzed and interpreted the data. ND and PG wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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