



Tetracycline resistance genes persist in soil amended with cattle feces independently from chlortetracycline selection pressure



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ARTICLE INFO

Article history:

Received 17 February 2014

Received in revised form

6 October 2014

Accepted 18 November 2014

Available online 29 November 2014

Keywords:

Antibiotic resistance

Cattle feces

Chlortetracycline

Grassland soil

Tetracycline resistance genes

int1 gene

ABSTRACT

Antibiotic residues and antibiotic resistance genes originating from animal waste represent environmental pollutants with possible human health consequences. In this study, we addressed the question whether chlortetracycline (CTC) residues in soils can act as selective pressure enhancing the persistence of tetracycline (TC-r) resistance genes in grassland soils receiving cattle feces. We performed a soil microcosm experiment, using 3 grassland soils with different management history, which were incubated with feces from conventionally raised dairy cows. The microcosms included treatments with a low dose (0.2 mg kg⁻¹), a high dose (100 mg kg⁻¹) and no CTC. The presence and abundance of TC-r genes *tet(O)*, *tet(Q)* and *tet(W)* and the *int1* gene coding for class 1 integrase were assessed with real-time PCR after 0, 14, 28, 56 and 86 d of incubation. The genes *tet(Q)* and *int1* persisted in all feces-containing treatments for at least 28 d, and *tet(W)* and *tet(O)* for at least 86 d, though they went close to limits of quantification after 14–28 d in most cases. The soil, but not the dose of CTC, significantly affected the gene persistence. Concluding, certain TC-r genes originating from cattle feces may persist in soil for several months independently from antibiotic selection pressure.

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1. Introduction

The treatment of infectious diseases is currently threatened by increasing antibiotic resistance in pathogenic bacteria. The use of antibiotics in agriculture has been recognized as an important contributor to this problem (Smith et al., 2005). Indeed, antibiotics are commonly administered to farm animals to treat and prevent diseases, and, in many non-EU countries, to promote animal growth. The digestive tract of farm animals may thus represent an important reservoir of antibiotic resistance genes (Durso et al., 2011), which are excreted in the form of intra- and extracellular DNA (Zhang et al., 2013). In addition, a great part of administered antibiotics leave animal bodies in their active form (Chee-Sanford

et al., 2009). Antibiotic residues and antibiotic resistance genes enter soil through manure application to land and their high levels in manured soils have been reported on many occasions (Knapp et al., 2010; Qiao et al., 2012; Jechalke et al., 2013). Environmental pollution with antibiotic resistance genes may have consequences for human health, because the antibiotic resistome may be shared between environmental bacteria and human pathogens (Forsberg et al., 2012).

Tetracyclines are one of the most commonly used antibiotics in animal production (Chee-Sanford et al., 2009). At the same time, they remain important drugs in human medicine. The tetracycline resistome comprises at least 45 different tetracycline resistance (TC-r) gene classes. These encode efflux pumps, ribosomal protection proteins and enzymes inactivating the antibiotic (Roberts, 2005). TC-r genes are commonly associated with mobile genetic elements such as integrons, transposons and plasmids, which allow for their spread to unrelated bacteria, including those from different environmental niches (Chopra and Roberts, 2001; Forsberg et al., 2012). Indeed, virtually the same TC-r genes were

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found in soil, manure and humans (Kobashi et al., 2007; Forsberg et al., 2012).

The presence of antibiotic residues may enhance the persistence and horizontal transfer of antibiotic resistance genes in manured soils (Jechalke et al., 2013; Kopmann et al., 2013). In detailed microcosm studies (Heuer and Smalla, 2007; Heuer et al., 2011), sulfadiazine enhanced the persistence of sulfonamide-resistance genes in soils treated with manure, and this effect was in certain cases dose dependent. In the case of tetracyclines, however, the effect of antibiotic residues on TC-r gene persistence in soil is not clear. A previous microcosm study showed that oxytetracycline from the concentration of 15 mg kg⁻¹ could enhance the persistence of certain TC-r genes in soils amended with pig slurry, differences in gene abundance were, however, not assessed (Schmitt et al., 2006). Moreover, the high sorption potential of tetracycline antibiotics (Figueroa-Diva et al., 2010) would suggest that effects of tetracyclines only occur at concentrations not representative for normal agricultural practice. Indeed, no differences in the presence or abundance of 8 TC-r genes were found when comparing soils amended with oxytetracycline-rich (500 mg kg⁻¹) and oxytetracycline-free cattle manure (Kyselková et al., 2013). In the latter study, however, we assessed only endpoint differences in the presence or abundance of TC-r genes after 12 weeks incubation, without monitoring the gene dynamics.

In the present study, we addressed the question whether the presence of chlortetracycline (CTC) may increase the TC-r gene persistence in soils amended with cattle feces in a dose-dependent manner over the course of 3 months. We performed a soil microcosm experiment, using 3 grassland soils mixed with cattle feces and 2 different doses of CTC. The low dose (0.2 mg kg⁻¹) represented a realistic dose found in manured soils (Hamscher et al., 2005; Qiao et al., 2012), while the high dose (100 mg kg⁻¹) was applied to achieve an extreme antibiotic selection pressure (this concentration can be found in manure samples but was also reported in soil; Pan et al., 2011; Huang et al., 2013). Treatments without CTC and without feces were also included. We chose grassland soils, because these may be regularly exposed to inputs of fresh feces from grazing cattle. The 3 soils represented a gradient of previous cattle impact, increasing from meadow (no impact) to pasture (medium impact) and winter pasture (high impact). Due to a high animal density during winter seasons in the previous years, the winter pasture soil was enriched with cattle excrements, but had low levels of TC-r genes. Compared to the pasture and meadow soil, the winter pasture soil contained more nutrients and bacterial species related to cattle microflora (Radl et al., 2007; Chroňáková et al., 2013). We expected, therefore, an increased chance for horizontal gene transfer in this soil due to the presence of suitable acceptors of mobile genetic elements originating from cattle feces, and abundance of nutrients (Sørensen and Jensen, 1998). We hypothesized that (i) the TC-r gene persistence, in terms of gene presence or abundance over the course of time, will be higher and dose-dependent in CTC treatments, and (ii) the gene persistence will be the highest in the winter pasture soil.

The presence and abundance of TC-r genes *tet(O)*, *tet(Q)* and *tet(W)* in total DNA from soil microcosms was monitored with real-time PCR for up to 3 months. In addition to TC-r genes, we quantified *int1* gene for class1 integrase, because integrons are often associated to, and may be also co-transferred with, TC-r genes (Agersø and Sandvang, 2005; Forsberg et al., 2012). The abundance of integrons may therefore indicate the potential of horizontal gene transfer in feces-amended soils. To monitor the sorption of CTC in soil microcosms, extractable CTC was assessed by HPLC for up to 1 month.

2. Material and methods

2.1. Sampling of soil and cow feces

Composite soil samples were taken in May 2011 near Český Krumlov, in the South Bohemia region of the Czech Republic, as described previously (Radl et al., 2007; Elhottová et al., 2012). Winter pasture soil (W) and pasture soil (P) were sampled at the Borová farm (latitude 48°52' N, longitude 14°13' E). This farm holds about 90 beef cows certified BIO, with a restricted administration of antibiotics (injections of penicillin/streptomycin and ceftiofur, and intrauterine tetracycline are seldom applied). Cattle assemble at a winter pasture from November till May and are then moved to a large pasture for the summer period. W soil was severely impacted by cattle (destroyed vegetation, damaged soil structure) and saturated with cattle excrements (Fig. S1A) at the time of sampling (i.e., one week after cattle had moved out). Pasture soil (P) with a moderate cattle impact and preserved vegetation was sampled at about 100 m distance from W (Fig. S1B). Meadow soil (M) was taken at *Provázková louka* (protected natural monument) located at about 2 km from Borová farm (48°53' N, 11°13' E, Fig. S1C). The meadow has a restricted access for people and farm animals have had no access to the meadow for at least 30 y. Sieved soil (5-mm) was stored at 4 °C until set-up of the microcosm experiment.

Feces were collected at an anonymous medium size (200 cows) dairy farm located in South Bohemia. Typical animal diet at the dairy farm includes 18 kg hay, 18 kg maize silage, 7 kg grain mixture including mineral additives (30 g Ca, 6 g P, 14 g Na, 14 g Mg, 150 mg Cu, 750 mg Zn, 675 mg Mn, 3 mg Co and 4 mg Se) per day. Adult cows regularly (approx. once per year) receive Metricycline (chlortetracycline hydrochloride, 1 g, intrauterine suppository) prophylactically against bacterial infections after calving. According to the product leaflet, CTC should not enter the animal digestive tract. Other antibiotics (e.g., amoxicillin) are occasionally administered to cows to treat bacterial infections. During 2011, 57 fecal samples from cows at different times (0 d up to several months) after the Metricycline application were taken for preliminary PCR screening of TC-r genes. Feces used in the microcosm experiment were taken as an anal grab from 3 cows (Česká straka or Česká straka/Red Holstein crossbred, 4–6 y old) during one week following the Metricycline application. Fecal samples were pooled and stored at 4 °C until set-up of the microcosm experiment (within 1 week). Physicochemical properties of soil and feces are in Table S1.

2.2. Isolation of total and extracellular DNA

Total DNA was extracted from 0.5 g soil or 0.4 g feces using the FastDNA SPIN Kit for Soil (MP Biomedicals Europe, Illkirch, France) according to the manufacturer's protocol with one modification as follows. DNA bound to silica matrix was washed in 1 ml guanidine thiocyanate (Sigma–Aldrich, Prague, Czech Republic; 5.5 M). This was done 1–3 times until the silica matrix returned to its original color. After the last wash with guanidine thiocyanate, the silica was applied to SPIN filter modules (MP Biomedicals Europe) and DNA purification further continued according to the manufacturer's protocol. Extracellular DNA (eDNA) was isolated from 0.25 g pooled cattle feces (in 6 technical replicates) according to Chroňáková et al. (2013).

2.3. Preliminary PCR screening for TC-r genes in cow feces and soils

Prior to the microcosm experiment, the presence of TC-r genes *tet(A)*, *tet(B)*, *tet(C)*, *tet(L)*, *tet(V)*, *tet(Z)* coding for tetracycline efflux pumps and *tet(M)*, *tet(O)*, *tet(Q)* and *tet(W)* coding for ribosomal

protection proteins was assessed in total DNA from feces collected at the dairy farm and from soil samples taken at the pasture and winter pasture, using PCR. The genes *tet(A)*, *tet(B)*, *tet(C)*, *tet(Z)* were amplified according to Aminov et al. (2002), *tet(L)* according to Ng et al. (2001), *tet(V)* according to Kyselková et al. (2012), and *tet(M)*, *tet(O)*, *tet(Q)* and *tet(W)* according to Aminov et al. (2001). The presence of TC-r genes *tet(O)*, *tet(Q)* and *tet(W)* in eDNA isolated from pooled feces from the dairy farm was assessed with PCR using the same protocols.

2.4. Microcosm experiment

Soil microcosms were set up in plastic boxes (radius 8 cm, *h* 6 cm) with a lid. Each soil (M, P, or W) received 4 different treatments, as follows. Control treatments contained the wet weight equivalent of 100 g dry soil, treatment F additionally contained the wet weight equivalent of 10 g dry feces, and treatments FL and FH additionally received feces and a low dose CTC (0.2 mg kg⁻¹ dw) and a high dose CTC (100 mg kg⁻¹ dw), respectively (Fig. S2). Soils were thoroughly mixed with feces and CTC solution (where appropriate) in individual microcosms. Each combination (3 soils × 4 treatments) was prepared in 20 replicates at the beginning of the experiment. Four replicates from each combination were sampled immediately after mixing soil with feces and antibiotics (sampling time point 0). The rest of the microcosms were incubated at 20 °C in the dark, and their water content was held constant at their original values (Table S1). At each of following sampling time points (14, 28, 58 and 86 d), 4 replicates from each combinations were sampled. Approximately 2 g soil from each replicate was immediately used for the assessment of dry matter content. The rest of soil was divided into aliquots that were stored at -80 °C for DNA extraction, at -20 °C for CTC analysis, and at 4 °C for other chemical analyses.

2.5. Real-time PCR

Purified DNA (see the Section 2.2.) was checked for the presence of PCR inhibitors as follows. First, 50×, 100×, 200×, 400×, 800× and 1600× diluted DNA samples representing each soil-treatment combination were run with universal 16S rRNA primers (using 5 µl template per reaction, as in Kyselková et al., 2013) to assess at which dilution a linear real-time PCR response started. Second, we performed the “SPUD” assay (Nolan et al., 2006) to ensure that the chosen sample dilution, when spiked to a standard DNA, did not affect the expected threshold cycle. The dilutions showing no inhibition (i.e., 200× for M a P soils, 400× for W soil and 800× for excrement) were further used for quantification of *tet(W)*, *tet(Q)*, *tet(O)*, *intI1* and 16S rRNA genes.

tet(W), *tet(Q)* and 16S rRNA were quantified with the StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA) as described previously (Kyselková et al., 2013). *tet(O)* and *intI1* were quantified by using probes and primers as described in Aminov et al. (2001) and Barraud et al. (2010). Q-PCR reactions were performed with the CFX384 Real-Time System (Bio-Rad Bio-Rad Laboratories, Hercules, CA) in 10 µl reaction mixture (3 µl of template DNA, 0.3 µM of each primer (for *tet(O)* and *intI1*), 0.2 µM of probe (for *intI1*) and 5 µl of iQ SYBR Green Supermix or iQ Supermix) with a temperature program of 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and annealing for 45 s at 60 °C. 16S rRNA genes were quantified in 3 and the other genes in 2 technical replicates per sample. If the two replicates' threshold cycle differed by more than 0.5, the measurement was repeated.

Limits of quantification (LOQ) were set as follows. Two-fold dilutions of standards were prepared containing 10⁴ to <1 copies in 6 replicates (Martineau et al., 2010). LOQ was calculated with the

program Genex Ver. 5.0.2 (MultiD Analyses AB, Goteborg, Sweden). Using the 90% confidence interval, the LOQ of *tet(W)*, *tet(Q)*, *tet(O)* and *intI1* were 2.79, 2.08, 1.04 and 1.51 log copies per reaction, respectively. The presence of specific PCR products was checked by (i) inspection of melt curves for all samples and (ii) analyzing PCR product size using agarose gel (3% w/v) electrophoresis for selected samples.

2.6. Assessment of chlortetracycline

CTC was extracted from 3 g of fresh (soil, feces) or frozen (microcosms soil) samples with mixture of acetone, 4 M HCl, and deionized water (13:1:6, v/v/v) according to Wang et al. (2010). CTC concentration in the extracts was assessed using HP 1050 HPLC instrument (Hewlett Packard, Palo Alto, USA) equipped with Agilent G1315B diode array detector (DAD; Agilent, Santa Clara, USA) on a 3 µm, 150 mm × 2 mm, Luna C18 (2) column (Phenomenex, Torrance, USA) as described in Kyselková et al. (2013). The limits of detection (LOD) and quantification (LOQ) of the instrument under selected settings were determined based on the Signal-to-Noise Approach as recommended by The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH; www.ich.org), using the signal to noise ratio of 3:1 and 10:1, respectively. The LOD was 0.21 µg mL⁻¹ extract, which on average corresponded to 1.3, 1.0, 1.1 and 5.2 mg kg⁻¹ dw M, P and W microcosms and cow feces, respectively. The LOQ was 0.46 µg mL⁻¹ extract, which corresponded to 2.9, 2.1, 2.4 and 11.3 mg kg⁻¹ dw M, P and W microcosms and cow feces, respectively.

2.7. Statistical evaluation

The effect of time, treatment and soil (where possible) on individual gene abundance and CTC quantity in soil microcosms were compared with permutational ANOVA in 2-way or 3-way crossed design using PERMANOVA+ for PRIMER (Primer-E, Ltd, Plymouth, UK; Anderson, 2001; Clarke and Gorley, 2006). Permutational ANOVA was employed, because the data did not meet the criteria of normality required for conventional parametric ANOVA. Gene abundance was normalized to gram dry soil and expressed in log units. Values under LOQ were replaced by the corresponding LOQ value before normalization. Bray–Curtis similarity was used as the resemblance measure for gene abundances, and Euclidean distance for CTC quantities. Permutation (up to 9999 permutations) of residuals under a reduced model was used for the main and pair-wise tests to assess significant differences among the compared groups at *P* = 0.05. Linear models in R (Wilkinson and Rogers, 1973; Chambers, 1992; R Core Team, 2013) were also used to study the effect of soil, time and treatment on the absolute abundance of *tet(W)* per gram dry soil, and on the decrease of *tet(W)* in 14-day time intervals. Linear models were deemed appropriate as the residuals of the final model were sufficiently normally distributed as judged by qq plots.

3. Results

3.1. Presence and abundance of TC-r and *intI1* genes in cow feces and soils

The preliminary screening of 57 fecal samples from the anonymous dairy farm for the presence of 10 TC-r genes showed that *tet(W)*, *tet(Q)* and *tet(O)* were a stable part of the cattle intestinal metagenome. These genes, together with *intI1*, were thus monitored throughout the microcosm experiment. No or faint signals were obtained with these genes when pasture and winter pasture

soils were screened. The initial abundances of *tet(W)*, *tet(Q)*, *tet(O)* and *intI1* in pooled feces used for the microcosm experiment were 9.9, 9.2, 8.0 and 5.8 log copies per gram dw. The genes *tet(Q)* and *tet(O)*, but not *tet(W)*, were detected in the eDNA from the pooled feces.

3.2. Presence of TC-r and *intI1* genes in soil microcosms

The genes *tet(W)*, *tet(Q)* and *intI1* were detected (in all replicates) in untreated W soil (Fig. S3–S6) at the beginning of the experiment (0 d; see asterisks in Fig. S3–S6). After feces addition, *tet(W)*, *tet(Q)*, *tet(O)* and *intI1* genes were detectable in all 3 soils, and all could be found in all treated soils for at least 28 d. In the case of *tet(Q)* and *intI1*, however, only 1–2 replicates out of 4 were positive in many cases (Fig. S3–S6). These genes were not, therefore, followed beyond 28 d. *tet(W)* and *tet(O)* were detectable in all soils and treatments until the end of experiment, i.e., 12 weeks. *tet(O)*, however, was found only in 1–2 replicates in PFH, WFL and WFH treatments after 28 d.

3.3. Abundance of TC-r and *intI1* genes in soil microcosms

The average initial abundances of *tet(W)*, *tet(Q)* and *tet(O)* in microcosms treated with cow feces (with or without CTC addition)

were approx. 7.7–8.2, 6.9–7.2 and 6.1–6.5 log copies per gram dw, corresponding to a ratio to 16S rRNA genes of 0.1–0.5%, 0.01–0.08% and 0.002–0.01%, respectively (Fig. S3–S5). The initial abundance of *intI1* in soil W was 6.8 log copies per gram dw and it spiked to 7.0–7.1 log copies per gram dw (0.01–0.02% when normalized to 16S rRNA gene) in WF, WFL and WFH treatments (Fig. S6). Roughly 10% of the resistance genes applied with manure could be thus recovered from the soils directly after manure amendment, at 0 d. The abundances of *tet(O)* and *tet(Q)* in P and W soil microcosms, and of *intI1* in M and P soil microcosms had mostly dropped below LOQ at 14 d (Fig. S3–S6). Differences in gene abundance between treatments and time points could be thus assessed for *tet(O)* and *tet(Q)* in M soil microcosms, for *intI1* in W soil microcosms and for *tet(W)* in all 3 soils up to 28 d (Fig. 1, Table S2–S3).

3.3.1. Differences in *tet(O)* abundance between treatments and time points in M soil

There was a significant effect of treatment and time, and also of the interaction between the 2 factors, on the *tet(O)* abundance in M soil microcosms (Table S2). The treatments containing feces (MF, MFL and MFH) differed significantly from the control at all time points, with the exception of MF treatment at 14 d where this difference was marginal ($P = 0.053$; Table S3). The treatments containing feces usually did not differ from each other, with 2

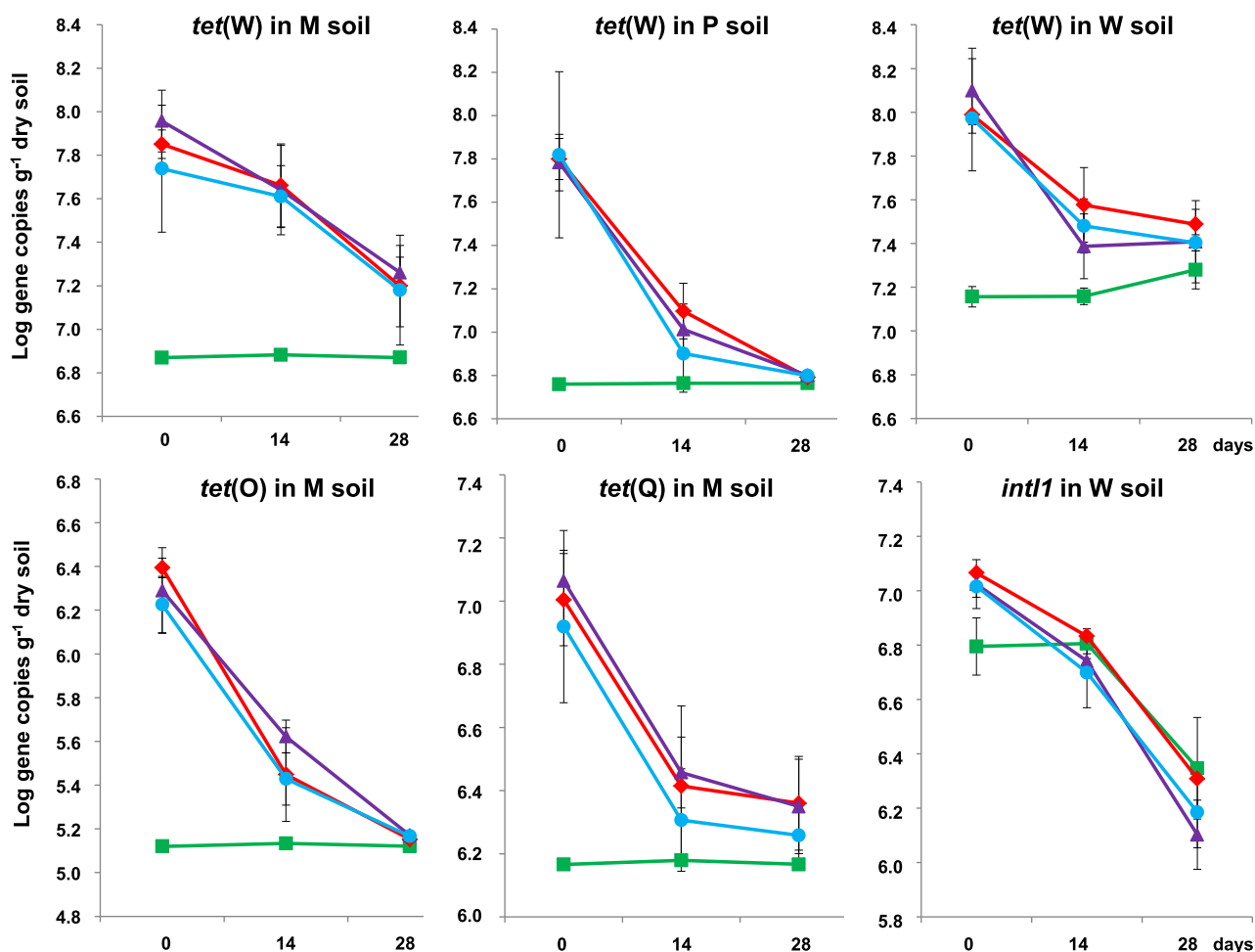


Fig. 1. Abundance of TC-r genes and *intI1* in meadow (M), pasture (P) and winter pasture (W) soils, 0, 14 and 28 days after cow excrement addition. Symbols represent mean log gene copies g⁻¹ dry soil and error bars represent standard deviations ($n = 4$). Green square: control soils, red diamond: soils mixed with feces, blue circle: soils mixed feces and low dose of CTC (0.2 mg kg⁻¹), violet triangle: soils mixed feces and high dose of CTC (100 mg kg⁻¹). Gene abundances were assessed with real-time PCR and values under the limit of quantification (LOQ) were replaced by the value of LOQ.

exceptions, i.e., MF contained significantly more *tet*(O) than MFL at 0 d ($P = 0.030$) and MFL contained less *tet*(O) than MFH at 14 d ($P = 0.030$). *tet*(O) decreased significantly in MF, MFL and MFH treatments between 0 and 14 d, and in MFL and MFH also between 14 and 28 d.

3.3.2. Differences in *tet*(Q) abundance between treatments and time points in M soil

As in the case of *tet*(O), there was a significant effect of treatment, time and the interaction between the 2 factors, on the abundance of *tet*(Q) in M soil microcosms (Table S2). The treatments containing feces (MF, MFL and MFH) differed significantly from the control at all time points, with the exception of MF at 14 d ($P = 0.099$). In contrast, there was no difference between MF, MFL and MFH treatments at any time point. The *tet*(Q) abundance in MF, MFL and MFH decreased significantly between 0 and 14 d (Table S3).

3.3.3. Differences in *int1* abundance between treatments and time points in W soil

The abundance of *int1* in W soil was significantly affected by time and by interaction of time with treatments, while the effect of treatment alone was marginal ($P = 0.066$; Table S2). In the control W soil, *int1* remained on the same level between 0 and 14 d and then it decreased significantly between 14 and 28 d. In contrast, the decay of *int1* in WF, WFL and WFH was significant at both 14 and 28 d (Fig. 1, Table S3). The levels of *int1* in W soil were significantly elevated right after feces addition (treatments WF, WFL and WFH at 0 d). After 14 d incubation, WF and WFL treatments had the same levels of *int1* as the control W soil, while the abundance of *int1* in WFH was even lower than in the control ($P = 0.028$) and WF treatment ($P = 0.025$). At 28 d, only WHF and WF treatments significantly differed (WFH having less *int1* than WF; $P = 0.031$), while the other treatments were indistinguishable from the each other.

3.3.4. Differences in *tet*(W) abundance between soils, treatments and time points

According to linear models, soil and time had a significant influence on *tet*(W) abundance, and significant differences were found for the effect of time in P and W soil as compared to M soil (significant interaction between soil and time). For the treatments, only the control treatment was significantly different from all treatments with feces, regardless of whether CTC was added or not. The effect of soil and treatment on *tet*(W) decay was studied in more detail by linear models using the values of *tet*(W) decrease in 0–14 d and 14–28 d time intervals, instead of absolute *tet*(W) abundance. It showed that the decay was faster in P and W soil, as compared to M soil. Again, FL and FH treatments did not differ from F treatment.

Results of PERMANOVA were in accordance with linear models (Table S2), showing that soil, treatment and time had all a significant effect on *tet*(W) abundance. In addition, there were significant interactions between each pair of factors. The generally higher levels of *tet*(W) in W soil were probably caused by the background occurrence of *tet*(W) in W soil (Fig. S3). The higher levels of *tet*(W) in control M soil, compared to P soil, were obviously due to normalization to gram dw, because both soils had undetectable levels of *tet*(W) before feces addition, while M soil had lower dry matter content. In contrast, the differences between feces-amended soils should be considered as real, as *tet*(W) was above the LOQ and the differences in dry matter content were adjusted by applying the same ratio of dry feces per dry soil. A significant decrease of *tet*(W) abundance between 0 and 14 d, and between 14 and 28 d was noted for all treatments with feces, whether or not

they contained CTC. In addition, the *tet*(W) abundance did not differ between the treatments F, FL and FH throughout the soils and time points, indicating that CTC at any dose had no effect on *tet*(W) persistence in the 3 soils (Table S3).

3.4. Quantity of chlortetracycline in cow feces and soil microcosms

CTC was under the LOD in the pooled fecal sample and in the control soils. Right after addition of $100 \text{ mg kg}^{-1} \text{ dw}$ CTC to soil microcosms (FH treatments at 0 d), we could recover as little as 5–32 (min–max) $\text{mg kg}^{-1} \text{ dw}$ CTC, depending on soil (Fig. 2). There was significantly more extractable CTC in M soil than in P and W soil. The effect of time on the quantity of CTC was insignificant. CTC was not assessed in FL treatments, because the low dose of CTC applied to ($0.2 \text{ mg kg}^{-1} \text{ dw}$) was below the calculated LOQ.

4. Discussion

In this study, we conducted a soil microcosm experiment in order to assess the persistence of tetracycline resistance (TC-r) genes after soil amendments with cow feces and the influence of chlortetracycline (CTC) content on this persistence. We have shown that certain TC-r genes may be introduced to grassland soils via application of cattle feces and persist there for several months. For example, *tet*(W) and *tet*(O) were detectable in all 3 feces-amended soils for at least 86 days (Fig. S3–S4), though their abundance decreased by one order of magnitude after 28 d incubation. Obviously, the fate of TC-r genes may differ from our study under complex field conditions, but the long-term persistence of TC-r genes in manure-amended soils was recently confirmed in an *in situ* study (Hong et al., 2013). The long gene persistence increases the risk of the further spread of the genes in the environment. Several mechanisms may be involved in the gene persistence in soil, e.g., survival of fecal microflora in soil (which is possible on a several-month term, Jiang et al., 2002), horizontal gene transfer to indigenous soil microflora (Heuer and Smalla, 2007), or stability of eDNA in soil (Nielsen et al., 2007).

The gene *tet*(W) was the most abundant in cow feces and was detected in most feces-amended soil samples at the end of the experiment. This is consistent with previous reports on its ubiquity in cow and pig manure and manure-impacted soils (Santamaría et al., 2011; Zhu et al., 2013). In this study, *tet*(W) was not detected in fecal eDNA suggesting that it was mainly present in living bacterial cells and mechanisms such as bacteria survival or

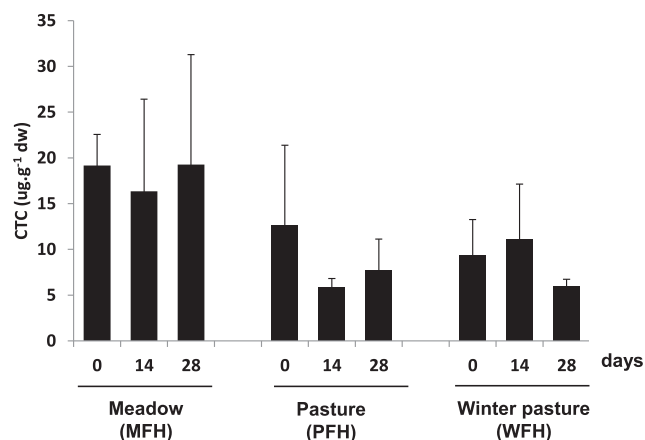


Fig. 2. Extractable CTC (mean \pm SD, $n = 4$) in soil microcosms where $100 \mu\text{g g}^{-1} \text{ dw}$ CTC was added together with cow excrements at 0 (right after CTC addition and mixing), 14 and 28 d of incubation.

conjugational transfer could thus be involved in its maintaining in soil. Unfortunately, no *tet(W)* harboring isolate was obtained from these microcosms in a parallel study where we screened CTC-resistant isolates for the presence of TC-r determinants (not shown). It thus remains unknown which species or mobile genetic elements contribute to the persistence of this gene in manured soils. Class 1 integrons can capture multiple resistance genes on their gene cassettes, and can be mobilized when located on plasmids or transposons (Gillings et al., 2008). However, class 1 integrons do not seem to play a dominant role in the transfer of the monitored tetracycline resistance genes, because the *int1* abundance is lower than the abundance of all genes in manure as well as in M and P soils and could thus explain a small part of the resistance gene abundance at most.

The gene persistence was not enhanced by the addition of CTC into soil microcosms, even at a high (100 mg kg⁻¹) concentration. Indeed, the difference in the abundance of TC-r and *int1* genes between soil microcosms with feces containing CTC or not was, if any, marginal or even opposite to our expectation (i.e., lower abundances in treatments containing feces and CTC than in treatments containing only feces). Our study has shown that CTC was quickly and strongly bound to soil (about 5–30% could be recovered using acid acetone right after CTC addition to soil), suggesting that its bioavailability was low. The strong sorption of tetracyclines in soil was already reported elsewhere (Teixidó et al., 2012) and is likely the reason why tetracyclines had a limited effect on the functioning of soil bacteria, as compared to sulfonamide type of antibiotics (Heuer and Smalla, 2007; Liu et al., 2012). These results corroborate our previous findings (Kyselková et al., 2013) that antibiotic selection pressure may not be needed for the persistence of certain TC-r genes in manure-amended soils. The increased abundance of some TC-r or integrase genes may indicate, therefore, that a soil has a history of fecal pollution but not necessarily of antibiotic pollution. It remains unknown which selection pressure, if any, helps keeping the TC-r genes in soil in the long term. Co-selection of antibiotic resistance with heavy metal resistance is one possibility (Baker-Austin et al., 2006), supported by the high concentrations of heavy metals that enter soil with feces from conventionally raised dairy cattle (Table S1).

The kinetics of gene decay was soil-dependent, as shown with *tet(W)*. The 3 soils used in this study were chosen because they represented a gradient of previous cattle feces impact, increasing from meadow to winter pasture soil. In contrast to our expectation, the meadow soil with no previous cattle impact showed the highest *tet(W)* persistence. Likewise, *tet(O)* and *tet(Q)* were detectable for a longer time in meadow soil than in both pasture soils. We suggest that this may be due to a higher competition of the introduced fecal microflora with the fecal microflora already present in pasture soils due to previous presence of the bio-cattle. This is further supported by a slower decay of the 'indigenous' *int1* vs. *int1* introduced with feces in our experiment in winter pasture soil (Fig. 1). Other possible explanation might be the differences in soil abiotic composition (e.g., micro and macronutrient availability, soil moisture, texture, oxygen content and pH; Table S1), which affect the stability of eDNA a mobile genetic elements (Van Elsas and Bailey, 2002; Nielsen et al., 2007). The design of our study does not allow distinguishing between these effects. Based on the current results we suggest that the comparative effects of soil management and abiotic composition on the antibiotic gene persistence in soil merit further studies.

5. Conclusions

Our study has shown that TC-r and class 1 integrase genes occur in cattle feces at farms where the use of antibiotics is restricted only

to prophylaxis and/or disease treatment. These genes may persist in feces-amended soils for several months, and this persistence is not enhanced by even high concentrations of CTC in soil. The results suggest that the increased abundance of TC-r and integrase genes in soil may be an indicator of fecal pollution but not necessarily of antibiotic pollution. The mechanisms behind the persistence of TC-r genes in soil without antibiotic selection pressure merit further attention, because they may contribute to the problem of the spreading of resistance to antimicrobials.

Acknowledgments

This study was supported by the Czech Science Foundation (P504/10/2077), by the project Postdok_BIOGLOBE – CZ.1.07/2.3.00/30.0032 co-financed by the European Social Fund and the state budget of the Czech Republic, and by the project CzechGlobe – Centre for Global Climate Change Impacts Studies, Reg. No. CZ.1.05/1.1.00/02.0073. We thank MVDr. S. Kollar for feces sampling and discussion, and Mr. and Mrs. Kamr from the Borová farm and CHKO Blanský les for enabling us the soil sampling. P. Havlíčková, L. Dymáčková and K. Kopejtka are thanked for technical help.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2014.11.018>.

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