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**Veterinary Medicines**

**DISSIPATION AND EFFECTS OF CHLORTETRACYCLINE AND TYLOSIN IN TWO AGRICULTURAL SOILS: A FIELD-SCALE STUDY IN SOUTHERN DENMARK**

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**Abstract**—Presently, there is a basic lack of information concerning the accumulation of antibacterial agent residues in agricultural soils. In this field study, performed in southern Denmark, we assess the dissipation of chlortetracycline (CTC), and tylosin A (TYL A) as a function of time. Field soils were classified as a sandy loam soil (field A) and a sandy soil (field B) and each field was sampled on six occasions during the 155-d experimental period from May to October 2000 for chemical analysis and counts of colony-forming units (CFU) detecting the level of aerobic bacteria surviving antibiotic exposure. Colony-forming units and TYL A were detected throughout the entire sampling period, with respective starting soil concentrations of 30 and 50 µg kg⁻¹ soil declining to 1 and 5 µg kg⁻¹ soil, on day 155. Compound half-lives (95% confidence limits in parentheses) were estimated for both fields and T₅₀ for CTC was 25 d (20–34) and 34 d (28–42) in fields A and B, respectively, and T₅₀ for TYL A was 67 d (54–86) and 49 d (40–64) in fields A and B, respectively. No significant difference was determined between compound half-lives on the two fields. The level of aerobic antibiotic-resistant bacteria in the soil over time and soil fauna community was assessed in relation to application of manure containing antibacterial agents to the agricultural fields. The level of both CTC- and TYL-resistant bacteria was affected in the soil by amendment of manure, but declined during the study to the same level as observed at the beginning.

**Keywords**—Antibacterial agents  Dissipation  Chlortetracycline  Tylosin  Field study

**INTRODUCTION**

Several studies recently have assessed the biodegradation or chemical stability of antibiotics on a laboratory-scale level, using different experimental conditions leading to different redox conditions in various matrices [1–5]. However, information on a field-scale level, to our knowledge, is very sparse (e.g., [6–7]). Reports of soil concentrations in the range of a few to even hundreds of µg/kg soils [6,8] have been reported, however only a few studies [6,7] to date actually have followed the decline in substance concentration over time in soils.

The purpose of this 155-d study, therefore, was to follow the disappearance of two veterinary antibiotic agents (i.e., chlortetracycline [CTC] and tylosin [TYL]) in the topsoil (20 cm) of two different soil types. Furthermore, the level of surviving CTC- and TYL-resistant bacteria amended by manure to the soil was assessed over time. Tetracyclines and macro- lides represent two of the major groups of antibiotics widely administered to farm animals to control infections and have a range of different physicochemical characteristics and soil sorption capacities. The chemical structure, physicochemical parameters, and soil–water distribution coefficients (Kₐ) of these compounds are listed in Table 1. The field experiment was conducted on two agricultural fields located near Askov Experimental Station in southern Denmark (Fig. 1), named Askov and Lundgaard. The two fields represent two different soil types, a loamy sand soil (field A, Askov) and a sandy soil (field B, Lundgaard). Both fields were operated using traditional Danish cultivation systems and antibacterial agents were introduced to the soil in accordance with Danish regulation and in association with liquid manure. After collection from both fields, soil samples were analyzed for CTC and TYL. Tylosin contains the active compound TYL A and some biological impurities in the formulation used, therefore, we analyzed all soil samples for TYL A, B, C, and D.

**MATERIALS AND METHODS**

**Antibacterial agents and chemicals substances**

Chemicals were purchased from the following companies: Oxytetracycline hydrochloride ([OTC], lot 28555, 95.7%; Unikem, Copenhagen, Denmark), 4-epi-chlortetracycline hydrochloride ([EECTC], A013639601, 97%; Aceros Organics, Geel, Belgium), chlortetracycline hydrochloride (lot 27H7703, 79%), tylosin tartrate (lot 85H10165), and erythromycin ([ERY], lot 31K1683; Sigma-Aldrich, Hamburg, Germany). J. Hoogmartens (Facultait Farmaceutische Wetenschappen, Leuven, Belgium) kindly donated TYL A, TYL B, TYL C, and TYL D that were used for identification of chromatograms. Formic acid, citric acid monohydrate, and NaOH pellets were from Merck (Darmstadt, Germany). Methanol of high-performance liquid chromatography (HPLC) grade was obtained from the company KEBO (Albertslund, Denmark).

**Field experiments**

Two agricultural fields in an area used for intensive livestock farming were selected for the field study (Fig. 1). The soil at the Askov site (field A) was a loamy sand soil and the soil at the Lundgaard site (field B) was a sandy soil. Physi-
Table 1. Chemical structure and selected physicochemical parameters (e.g., molecular weight [MW], pK<sub>a</sub>, log K<sub>sw</sub>, and the distribution coefficient between soil and water [K<sub>d</sub>]) for the antibacterial agents studied in the field study. ND = no data available; CTC = chlortetracycline; ECTC = 4-epi-chlortetracycline; TYL A = tylosin A; TYL B = tylosin B; TYL C = tylosin C; TYL D = tylosin D.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Structure</th>
<th>MW (g/mol)</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
<th>Log K&lt;sub&gt;sw&lt;/sub&gt; (L/kg)</th>
<th>Log K&lt;sub&gt;d&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTC</td>
<td><img src="image" alt="CTC structure" /></td>
<td>478.9</td>
<td>3.30; 7.44; 9.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>2.9 [2.8–2.9]&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ECTC</td>
<td>Epimer of CTC (see above)</td>
<td>478.9</td>
<td></td>
<td>2.4 [2.3–2.5]&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>TYL A</td>
<td>Same structure as TYL</td>
<td>916.1</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>TYL C</td>
<td>Same structure as TYL</td>
<td>902.1</td>
<td>2.20</td>
<td>2.50 ± 0.84&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>TYL D</td>
<td>Same structure of TYL</td>
<td>918.2</td>
<td></td>
<td>2.17 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>TYL B</td>
<td>771.9</td>
<td></td>
<td></td>
<td>1.66 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Park et al. [18].  
<sup>b</sup> Calculated from the shift in pK<sub>a</sub>-value in the presence of rapidly stirred 1-octanol [19].  
<sup>c</sup> Estimated for uncharged molecule [20].  
<sup>d</sup> Measured on Lundgaard soil (field B) and expressed as logarithm to the distribution coefficient K<sub>d</sub> with 95% confidence intervals.  
<sup>e</sup> Distribution coefficients, K<sub>d</sub> measured on both soils (field A and B) as the ratio between the concentrations of antibiotic bound to the soil and the concentration in solution measured indirectly. The values are means of three replicate with 95% confidence intervals (Rabolle et al. [21]).

cochemical properties of the two soils are presented in Table 2. On each of the two fields, three replicate plots, each with an area of 56 m<sup>2</sup> (14.0 × 4.0 m) were amended with manure obtained from a nearby piglet-breeding farm. Animals on this farm were treated with CTC and TYL; according to information collected from veterinary reports on treatments within the study period, the level of antibacterial agents in the manure were estimated initially to be within the range of 100 to 300 mg/kg dry weight manure. Manure was preserved for three months before amendment on April 30, 2000 (day 0). A distance of 1 m was introduced between each of the plots on fields to avoid crosscontamination. Control plots were fertilized with identical type of manure not containing antibiotics. All plots were fertilized following Danish standard regulation with manure at a level of 100 kg N/ha/year. The amended manure was ploughed to a depth of 20 cm on the day of application. The fields were seeded with spring barley (Hordeum vulgare) in May 2000.

All other plots on the two fields were used in other experiments as the sites took part in a soil-monitoring project on sustainable land use in Denmark. The soil properties and land use of the two fields have been well-documented since the 1970s [9]. Climatic data for the Askov site (field A) for the study period, together with average climatic data for the period 1961 to 1990, are listed in Table 3, and it is seen that the climatic conditions during spring and summer 2000 were consistent with the average climatic data. The climatic conditions are assumed to be equivalent for both field sites as the fields are located within a distance of 2 km.

Field sampling procedure and preparation

Soil samples for chemical analysis were collected on seven occasions, both before and after amendments with manure within the period of May to October 2000. The first sampling day after manure amendment was on May 3, 2000 (day 0). The following samples were taken on days 9, 21, 68, 128, and 155 after manure application, corresponding to May 9, May 21, July 10, September 5, and October 2. Soil samples were
collected to a depth of 20 cm using a soil auger (diameter of 25 cm). Three to five cores were sampled per plot. All samples were stored at $-20^\circ$C until analysis. Prior to analysis, the soil samples were air-dried to a water content of approximately 5% and sieved through a 2-mm sieve.

Soil samples to be used for the detection of antibiotic resistance were sampled on five occasions at the same sampling sites and stored for a maximum of 48 h at $4^\circ$C before extraction of bacteria.

**Extraction of antibacterial agents from soil**

Pressurized liquid extraction (PLE) of the antibacterial agents from soil samples was performed using an accelerated solvent extractor (ASE 200) system from Dionex (Sunnyvale, CA, USA). Approximately 10 g soil, in accurately measured lots, was mixed with 10 g Ottawa Sand Standard (Fisher Scientific, Fair Lawn, NJ, USA) and the mixture was added to a 33-ml ASE extraction cell. The sand was added to the soil sample to avoid blockage of the extraction cell during extraction.

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**Table 2. Soil characteristics for fields A (Askov, Denmark) and B (Lundgaard, Denmark) of the field study**

<table>
<thead>
<tr>
<th>Location</th>
<th>Soil depth (cm)</th>
<th>Clay (&lt;2 μm)</th>
<th>Silt (2–20 μm)</th>
<th>Fine sand (20–200 μm)</th>
<th>Coarse sand (200–2,000 μm)</th>
<th>Organic C</th>
<th>pH (CaCl₂)</th>
<th>CEC* (meq/v/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field A (loamy sand)</td>
<td>0–20</td>
<td>11.3</td>
<td>10.7</td>
<td>37.9</td>
<td>37.5</td>
<td>1.6</td>
<td>6.1</td>
<td>10.0</td>
</tr>
<tr>
<td>Field B (sand)</td>
<td>0–20</td>
<td>5.2</td>
<td>4.8</td>
<td>24.4</td>
<td>63.2</td>
<td>1.4</td>
<td>5.6</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*Data for the Askov and Lundgaard soils were obtained from the Danish Institute of Agricultural Sciences, Research Centre Foulum, Tjele, Denmark.
*CEC* = Cation exchange capacity.
tion and to increase sample-solvent contact surface by filling the extraction cell volume. Before extraction, the soil samples were fortified to soil content equivalent to 300 µg kg⁻¹ soil with OTC and ERY as surrogate internal standards for CTC and TYL, respectively. Blank soil samples containing no fortifications were analyzed to determine the background content levels of the internal standards. Extraction solvent consisted of 50% methanol and 50% 0.2 M sodium acetate. The SAX cartridges were standardized with 2 ml 0.2 M sodium acetate. The SAX cartridges were analyzed using method B. For both methods, the HPLC system was operated with a 100-mm Xterra MS-C18 column from Waters, with particle size 3.5 µm and ID 2.1 mm, using a flow of 250 µl min⁻¹. Gradient elution was applied with mobile phases consisting of 0.08 M formic acid solutions containing 5% and 95% methanol, respectively, for method A, and 20% and 95% methanol, respectively, for method B. Injection volume was 5 µl and calibration standards were analyzed for each series of 15 samples. All samples were analyzed in duplicate. The MS detection was operated in the positive-ion mode using multiple-reaction-monitoring detection; the precursor-product ion combinations are listed in Table 4, together with all MS/MS parameter settings for both methods.

**Validation of the soil-extraction method**

The limit of quantification (LOQsoil) and limit of detection (LODsoil) for the entire soil-extraction method, based on the standard error (s_soil) and the slope for the calibration curve

<table>
<thead>
<tr>
<th>Month</th>
<th>Precipitation (mm)</th>
<th>Potential evaporation (mm)</th>
<th>Soil temperature 10 cm (°C)</th>
<th>Soil temperature 30 cm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>May</td>
<td>74.8</td>
<td>103.4</td>
<td>13.5</td>
<td>13.0</td>
</tr>
<tr>
<td>June</td>
<td>54.8</td>
<td>93.1</td>
<td>15.0</td>
<td>14.4</td>
</tr>
<tr>
<td>July</td>
<td>51.1</td>
<td>88.1</td>
<td>16.1</td>
<td>15.6</td>
</tr>
<tr>
<td>August</td>
<td>70.0</td>
<td>77.7</td>
<td>16.0</td>
<td>15.8</td>
</tr>
<tr>
<td>September</td>
<td>54.3</td>
<td>44.7</td>
<td>14.1</td>
<td>14.2</td>
</tr>
<tr>
<td>October</td>
<td>133.5</td>
<td>23.7</td>
<td>12.0</td>
<td>12.3</td>
</tr>
</tbody>
</table>

*Table 4. Climatic data for field A (Askov, Denmark) for the experimental period May to October 2000, and corresponding average values for the period 1961 to 1990 [22]*
were determined for the antibacterial agents as $LOQ_{\text{soil}} = (10 s_{x/y})/\text{slope}$ and $LOD_{\text{soil}} = (3 s_{x/y})/\text{slope}$) of these compounds and because, this solution also was used for calibration curves and spiking of soil samples, the recovery for TYL A was estimated by comparing the content in spiked soil with the standard at the known spiking level for TYL A. Recoveries with corresponding 95% confidence levels are shown in Table 6.

### Survival of CTC- and TYL-resistant bacteria in soil

Colonizing unit counts of aerobic-cultivable bacteria recovered from soil were made on Luria Bertani agar [11], used to assess the level of CTC and TYL aerobic–resistant bacteria over time in the soil plots. Both the plots treated with manure containing CTC and TYL and the corresponding control plot (same as the one analyzed with chemical analysis) were assessed in both field A and field B. Samples were taken at five occasions on the following dates: April 20 (before amendment), May 3, May 21, July 10, and October 2 (corresponding to days 3, 21, 68, and 155). For specific selection of Gram-positive bacteria, the Luria Bertani medium was supplied with 3.2 $\mu\text{g} \text{ml}^{-1}$ polymyxin B sulfate and 15 $\mu\text{g} \text{ml}^{-1}$ nalidixic acid. For selection of antibiotic-resistant bacteria, the medium was supplied with 8 $\mu\text{g} \text{ml}^{-1}$ tetracycline hydrochloride. Erythromycin was used as selective agent to measure macrolide (includes TYL) resistance because TYL and ERY crossresist. In this case, the Luria Bertani medium was supplemented with 8 $\mu\text{g} \text{ml}^{-1}$ ERY, 3.2 $\mu\text{g} \text{ml}^{-1}$ polymyxin B sulfate, and 15 $\mu\text{g} \text{ml}^{-1}$ nalidixic acid. The two latter antibiotics were added to limit the growth of Gram-negative bacteria [12], which are resistant to ERY in the concentrations tested here. All Luria Bertani agars were amended with 25 $\mu\text{g} \text{ml}^{-1}$ Natamycin (Pimaricin) to inhibit fungal growth [13]. Agar plates were incubated at 25°C for 3 d.

### Data treatment

**Soil analysis.** After LC-MS/MS analysis of soil sample extracts, the concentration of CTC and TYL in the soil samples were corrected using the recovery of the internal standards (OTC and ERY), determined for each sample. First-order kinetic degradation rates for all antibacterial agents, degradation products, and impurities were estimated using duplicate samples from each of the three soil plots. Samples from day 0 were excluded from estimations, due to high standard deviations probably caused by the heterogenic distribution of compounds in soil. It was not possible to determine half-life of the compounds in the Askov soil plot 3 (field A), due to high standard deviations.

**Antibiotic resistance.** For determination of the level of CTC or TYL bacterial resistance, soil samples with resistant bacteria below the detection limit (1 CFU per 0.01 g of soil) were omitted. The results were reported as the average of three observations from the three plots and significance found by pair-wise comparison (Student’s t-test) between treated and untreated control plots. Using the Student’s t-test, $p < 0.05$ was considered significant. The statistical treatment is explained in detail in Sengeløv et al. [12].
RESULTS AND DISCUSSION

LC-MS/MS procedure

The sensitive and selective quantification of the investigated antibacterial agents in soil was achieved in this study using LC-ESI-MS/MS. Soil contents were in the range of 1 to 50 μg kg⁻¹ soil and the system was sensitive within the scope of this study as demonstrated by the estimated limits of quantification (LOQsoil) and limits of detection (LODsoil) for the entire method (Table 5). Linearity was demonstrated in the complete concentration range tested (1–500 μg L⁻¹), which covers the necessary range for this study. Method validation parameters are listed in Tables 5 and 6.

Mean recoveries and corresponding confidence levels for the extraction of six replicate soil samples fortified with antibacterial agents and concentrated using SPE, are listed in Table 6. Recoveries for all antibacterial agents are satisfactorily high and comparable recoveries are found for both soil types. For some day-to-day variation experiments, the 95% confidence intervals (CI) are not overlapping, indicating significant difference between recoveries obtained on different days. However, due to the low standard deviations obtained for six replicate samples, the confidence levels are narrow and the day-to-day variation generally is below 30%.

Occurrence, distribution, and dissipation of CTC and TYL in soil

The dissipation of CTC, ECTC, and TYL A, B, C, and D were followed in both the loamy sand soil (field A) and a sandy soil (field B) over time. The first sampling day was 3 d after manure amendment and the concentrations were surprisingly low on the first day of sampling. Furthermore, these results were associated with high standard deviations. This may be explained by sample heterogeneity, possibly resulting from the initial manure handling method at the time of fertil-
Fig. 2. Soil content of antibacterial agents on the loamy sand soil (△ field A, Askov, Denmark) and the sandy soil (■ field B, Lundgaard, Denmark) measured during the experimental period of 155 d. For each soil, a point on the curves represents the mean value for replicate measurements from three soil plots and the error bars are the standard deviation. CTC = chlortetracycline; ECTC = 4-epi-chlortetracycline; TYL A = tylosin A; TYL B = tylosin B; TYL C = tylosin C; TYL D = tylosin D.

Sampling day

Fig. 2. Soil content of antibacterial agents on the loamy sand soil (△ field A, Askov, Denmark) and the sandy soil (■ field B, Lundgaard, Denmark) measured during the experimental period of 155 d. For each soil, a point on the curves represents the mean value for replicate measurements from three soil plots and the error bars are the standard deviation. CTC = chlortetracycline; ECTC = 4-epi-chlortetracycline; TYL A = tylosin A; TYL B = tylosin B; TYL C = tylosin C; TYL D = tylosin D.

Chlortetracycline and its major epimer, ECTC, were detected throughout the entire sampling period in both the sandy soil and the sandy loam soil. The average contents of CTC and ECTC on the two soils are plotted as a function of time in Figure 2. The level of CTC quickly was reduced from 20- to 30-μg kg⁻¹ soil to 2- to 5-μg kg⁻¹ soil in both soils within the first few weeks and this is consistent with a recent Italian study [7]. This fast decline primarily may be attributed to the strong sorption of CTC to the organic fraction of soils, though degradation processes are thought to play a minor role in this disappearance [14]. In addition, stronger sorption with increased residence, possibly has sequestered the compound within the matrix and may have rendered CTC nonextractable with time, thereby resulting in lower detected concentrations.

Contrary to the decline in CTC content, the level of the epimer ECTC was fairly constant throughout the entire experimental period (Fig. 2B).

First-order half-lives and corresponding 95% CIs for CTC were estimated for each of the three soil plots on each field, excluding sampling day 3 because of heterogeneous samples.
Table 7. Degradation half-lives ($T_{1/2}$) for chlortetracycline (CTC) and tylosin A (TYL A), determined for each soil plot and the complete field, respectively, in the field study, assuming first-order degradation kinetics. CI = Confidence interval

<table>
<thead>
<tr>
<th>Soil</th>
<th>CTC</th>
<th></th>
<th></th>
<th>TYL A</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil plot</td>
<td>$T_{1/2}$ (days)</td>
<td>95% CIs</td>
<td>$T_{1/2}$ (days)</td>
<td>95% CIs</td>
<td></td>
</tr>
<tr>
<td>Field A (Askov, Denmark)</td>
<td>1</td>
<td>26</td>
<td>18–46</td>
<td>58</td>
<td>44–85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25</td>
<td>18–39</td>
<td>76</td>
<td>65–91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>58</td>
<td>32–362</td>
<td>ND$^a$</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>All (–3)</td>
<td>25</td>
<td>20–34</td>
<td>67</td>
<td>54–86</td>
<td></td>
</tr>
<tr>
<td>Field B (Lundgaard, Denmark)</td>
<td>1</td>
<td>28</td>
<td>21–40</td>
<td>45</td>
<td>35–62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>44</td>
<td>31–77</td>
<td>52</td>
<td>37–88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>33</td>
<td>27–42</td>
<td>54</td>
<td>35–113</td>
<td></td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>34</td>
<td>28–42</td>
<td>49</td>
<td>40–64</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Half-life not determined for this plot.

(see Table 7). For each soil type, the 95% CI for each soil plot overlapped, indicating no significant difference in dissipation between plots. Therefore, half-lives for the entire fields (excluding Askov plot 3, because of high standard deviation) were estimated to 25 d (20–34) for field A and 34 d (28–42) for field B (95% CI in parentheses). Because the confidence intervals of the half-lives for the loamy sand and sandy soils overlapped, it may be conclude that there was no influence of soil type on the disappearance rate (see Table 2 for soil characteristics).

Tylosin content in both soils also was followed over time. Soil samples were analyzed for both the active compounds TYL A and the degradation products TYL B, C, and D. The content of TYL A in the two soils as a function of time is plotted in Figure 2C. Within the 155-d study period, TYL A concentrations decline from approximately 50-μg kg$^{-1}$ soil to 10-μg kg$^{-1}$ soil in field A and from approximately 25-μg/kg to 3-μg/kg, in field B. The half-lives for TYL in the two soils were estimated for the entire fields. Half-lives of 67 (54–86) and 49 (40–64) d for fields A and B, respectively, were estimated and equivalent to CTC estimations, the 95% CIs overlap for the two soil types, indicating no significant difference between disappearance rates. Figure 2 also shows the content of TYL B, C, and D as a function of time, demonstrating that the content of TYL B and TYL D declines slightly throughout the experimental period, whereas the content of TYL C is constant. Half-lives (95% CIs in parentheses) for TYL B are estimated to 114 (70–314) d and 84 (60–143) d for fields A and B, respectively, and for TYL D to 79 (61–113) d and 82 (58–140) d. It was not possible to determine the half-life of TYL C because the content does not decline during the experimental period and, hence, the degradation rate is infinitely low. These values indicate that the degradation products are more persistent in the soil than the parent compound TYL A.

Levels of aerobic antibiotic-resistant bacteria in the soil

Figure 3 shows the ratios between resistant bacteria and total count on nonselective plates for the antibacterial agents (performed as CFU counts). Erythromycin-resistant counts were divided by Gram-positive total counts, because Gram-negative bacteria intrinsically are resistant to macrolides in the concentrations used. Erythromycin was used as selective agent to measure macrolide (includes TYL) resistance. The results were reported as the average of three observations from the three plots and significance found by pair-wise comparison (Student’s $t$-test) between treated and untreated control plots (results not shown).

Levels of tetracycline hydrochloride resistance in soil increased after application of manure to the soil as compared to the level of resistance before application. This increase was significant ($p < 0.05$) for all samplings after manure amendment. This also was the case for ERY (TYL) resistance, although it must be considered that only one of the soil samples from field A (Askov) sampled before manure amendment con-
tained ERY-resistant bacteria (data not shown). Erythromycin resistance levels were low at all sampling times. Thus, the initial increase in the resistance levels were observed for both the antibacterial agents; however, as seen from Figure 3, the proportion (%) of resistant bacteria of total CFU drops to a level near the starting-point after a more pronounced increase shortly after spreading. This decrease in resistance levels after spreading previously has been observed [12], and probably is caused by the limited survival potential of intestinal bacteria in the soil [15–17]. The high number of tetracycline hydrochloride–resistant bacteria present shortly after manure amendment mainly is due to addition of large numbers of resistant bacteria with the manure, but gene transfer to indigenous soil bacteria also may have occurred. Whether the significant increase in tetracycline hydrochloride and ERY resistance observed at the last sampling time (day 155) is due to a permanent accumulation of resistance in soil caused by amendment of manure or due to variations in the heterogeneous soil environment cannot be determined from the present data. Measurements of resistance levels from soil sampled outside the test area of the fields would indicate whether or not the resistance levels measured in the test fields were within the range of a nonmanured soil. Previous experiments showed that resistance levels five months after spreading were within the range of resistance in a nonmanured control soil [12]. It is important to realize that, in this study, only the level of aerobic CTC- and TYL-resistant bacteria were reported, whereas the level of resistant anaerobic bacteria remains unknown.

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