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Determination of Persistent Tetracycline Residues in Soil Fertilized with Liquid Manure by High-Performance Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry

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Little is known about the occurrence and the fate of veterinary drugs in the environment. Therefore, a liquid chromatography/tandem mass spectrometry method was developed and employed to investigate in detail the distribution and persistence of the frequently used tetracyclines and tylosin in a field fertilized with liquid manure on April 2000 and April 2001; soil sampling was performed in May 2000, November 2000, and May 2001. We detected 4.0 mg/kg tetracycline and 0.1 mg/kg chlortetracycline in the liquid manure of April 2000, as well as comparable amounts in the liquid manure of April 2001. In the soil samples of May 2001, the highest average concentrations of 86.2 (0−10 cm), 198.7 (10−20 cm), and 171.7 μg/kg (20−30 cm) tetracycline and 4.6−7.3 μg/kg chlortetracycline (all three sublayers) were found. At soil depths between 30 and 90 cm, as well as in soil or groundwater, tetracyclines could not be detected. In addition, oxytetracycline and tylosin could not be detected in any sample investigated. We conclude that tetracyclines enter the environment in significant concentrations via repeated fertilizations with liquid manure, build up persistent residues, and accumulate in soil. Therefore, tetracyclines may have a potential risk and investigations on the environmental effects of these antibiotics are necessary.

Pharmaceuticals in the environment are of growing scientific interest worldwide.[1] In both human and veterinary medicine, a large number of drugs are used extensively. Substance classes with a possible environmental impact include antibiotics, antiparasitic agents, and hormones. Estimations of the European Federation of Animal Health (FEDESA) reveal that, in 1999, approximately 8500 tons of antibiotics in human medicine and 4700 tons in veterinary medicine were used in the European Union (including Switzerland).[2] After excretion, these drugs and their metabolites can enter the environment via several pathways.[3] In certain instances, drugs used in human medicine are most frequently released by passing sewage treatment plants and reaching surface waters. Investigations performed during the last two decades show that more than 40 different drugs can be found in river water, in groundwater, and even in drinking water sources from the nanogram per liter to the microgram per liter range (for extensive review, see refs 1, 4, and 5). Currently, intense work has been undertaken toward the development of analytical methods especially for the detection of various antibiotics (originating from human and veterinary medicine) in the aquatic environment including urban wastewater.[6,7] These methods and the obtained data are urgently needed for a sound risk assessment of drugs in the environment.

Veterinary drugs can also enter the environment directly by spreading liquid manure used as fertilizer. Recently, it was shown that tetracycline and sulfadimidine are present in liquid manure at concentrations up to 20 and 40 mg/L, respectively, after application of these drugs in recommended dosages.[8−9] Despite these observations, there is still very little known about the concentrations and the fate of these veterinary drugs in soil. Chlortetracycline has been shown to persist in soil; however, this was dependent on temperature. In one particular study, a standard solution of chlortetracycline was mixed with chicken feces to obtain a final concentration of 5.6 mg/kg in soil, and after 30 days, 44% remained at 30 °C, 88% at 20 °C, and no degradation occurred

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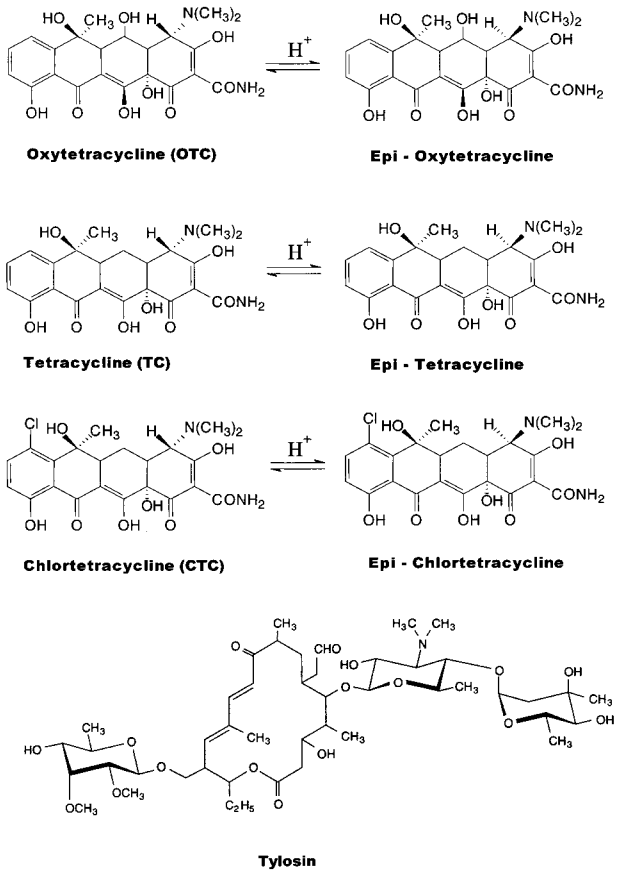

Figure 1. Molecular structures of the three investigated tetracyclines, their corresponding reversible epimers, and tylosin.

at 4 °C.[10] In laboratory studies, it was demonstrated that oxytetracycline is strongly adsorbed to soil and thus leaching of this compound into deeper soil segments seems to be unlikely.[11]

At present, there are no existing field studies that have observed the accumulation of antibiotics such as tetracyclines in soil and the possible contamination of groundwater after fertilization with liquid manure. One reason for this lack of knowledge is that there is no reliable method available to selectively detect these drugs in liquid manure, soil, and soil water in the microgram per kilogram range. Therefore, a method was developed for the measurement of tetracyclines and tylosin (molecular structures shown in Figure 1) in these compartments. In a pilot study using the newly developed high-performance liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC−ESI-MS−MS) methodology (the latter was recently demonstrated as a very useful method for the detection of various tetracyclines[12]), tetracycline and chlortetracycline were detected in agricultural fields of several so-called "long-term soil monitoring areas" fertilized with liquid manure.[13,14] The samples were obtained in February, about 4−5 months after the last application of liquid manure. These preliminary findings revealed that tetracyclines occur in the environment and that the detected amounts in several areas were higher than the so-called "phase I trigger value" of 10 μg/kg soil as recommended by the European Agency for the Evaluation of Medicinal Products (EMEA) until May 2001. According to this recommendation, ecotoxicological tests must be carried out prior to the final registration of a new veterinary drug, when this trigger value is exceeded.[15]

In the present paper, we provide a description of the methods developed for the extraction and determination of frequently used drugs oxytetracycline, tetracycline, chlortetracycline, and tylosin in various matrixes including soil, liquid manure, soil water, and groundwater. Another aim of this work involved the detailed and repeated investigation of an agricultural field that was mainly fertilized with liquid manure originating from a pig-fattening farm. In addition, the persistence of tetracycline and chlortetracycline in the soil stored for up to 12 months under defined conditions was determined. In the last part of this investigation, analysis of tetracyclines in air-dried liquid manure aggregates was undertaken. This was primarily achieved in order to observe whether higher concentrations of antibiotics could be expected in these aggregate samples compared to bulk soil.

EXPERIMENTAL SECTION

Chemicals. Acetonitrile (J.T. Baker, Griesheim, Germany) and ethyl acetate (Aldrich, Munich, Germany) were of HPLC grade, and ammonium acetate, citric acid, formic acid, sodium hydroxide (all obtained from Merck, Darmstadt, Germany), and disodium ethylenediaminetetraacetate (Sigma, Munich, Germany) were of analytical reagent grade. Water was prepared in-house by a Milli-Q system (Millipore, Eschborn, Germany). Oxytetracycline, tetracycline, and chlortetracycline (as their hydrochlorides) and tylosin tartrate were obtained from Sigma. Stock solutions of all standards were prepared by dissolving 1 mg of each drug in 1 mL of methanol. The stock solutions were stored at −80 °C and were stable for at least 2 months. Working dilutions were prepared freshly on the day of use.

Liquid Chromatography−Tandem Mass Spectrometry and Liquid Chromatography−MS$^{(3)}$ Spectrometry and Quantification. Mass spectrometry was carried out using a LCQ ion trap with an electrospray ionization source (Finnigan Mat, San Jose, CA). Standard compounds (10 ng/μL) were infused through an integrated syringe pump at a flow rate of 10 μL/min for tuning the mass spectrometer and optimizing capillary temperature, sheath gas, and auxiliary gas flow rates. The source polarity was set positive for all compounds; the spray needle voltage was 5 kV. Drying gas was nitrogen generated from pressurized air in an Ecoinert 2 ESP nitrogen generator (DWT-GmbH, Gelsenkirchen, Germany). The optimized conditions were as follows: sheath gas flow was set at 100 units, the auxiliary gas was turned off, and the capillary temperature was 150 °C. LC, MS−MS, and MS$^{(3)}$ parameters are summarized in Table 1. MS$^{(3)}$ is a technique that can be exclusively performed on ion trap mass spectrometers. Briefly, a product ion resulting from a MS−MS experiment can be trapped, once more fragmented, and the obtained ions are registered.

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Table 1.

(A) Retention Times (RT) and Optimized MS−MS Parameters for the Determination of Various Tetracyclines and Tylosin in Liquid Manure, Soil, Soil Water, and Groundwater

compound	RT (min)	precursor mass (m/z)	collision energy (%)	product ions (m/z) (rel abundance in %)
oxytetracycline	7.06	461	20	426 (8), 443 (100), 444 (8)
tetracycline	7.39	445	20	410 (6), 427 (100), 428 (5)
chlortetracycline	8.35	479	27	444 (70), 461 (56), 462 (100)
tylosin	9.47	917	28	772(100), 773 (4)

(B) Optimized LC−MS$^{(3)}$ Parameters for the Confirmation of Various Tetracyclines in Liquid Manure and Soil

compound	precursor mass 1 (m/z)	collision energy (%)	precursor mass 2 (m/z)	collision energy (%)
oxytetracycline	461	45	426	27
tetracycline	445	45	410	27
chlortetracycline	479	45	444	27

The HPLC system employed was a gradient system consisting of a Thermoquest P4000 pump, an AS3000 autosampler (San Jose, CA, and a Puresil C18 column (5 μm, 4.6 × 150 mm, Waters Corp., Milford, MA) operated at 23 °C. The flow of 1 mL/min was split 1:10 before entrance into the mass spectrometer. The mobile phase consisted of 0.5% formic acid in water with 1 mM ammonium acetate (solvent A, pH 2.5), and acetonitrile (solvent B). The gradient run was 0−50% B in 9 min and then held at 50% B for 1 min. After each run, the column was rinsed for 3 min with 99% acetonitrile and reequilibrated for 12 min with solvent A. The injection volume was 8 μL for soil and water samples and 1−2 μL for liquid manure samples. The autosampler was rinsed after each injection with 3 mL of 90% methanol/10% 10 mM oxalic acid in water.

Calibration curves constructed for oxytetracycline, tetracycline, chlortetracycline, and tylosin ranged from 0.1 to 10 ng per injection and were linear, with $r^2 > 0.99$ for the MS−MS procedure. Quantification was obtained by comparing the peak areas of the sample with that of the external calibration curves, and all data were corrected for recovery.

Sampling of Soil, Dried Liquid Manure Aggregates, Soil Water, and Groundwater. In May 2000, November 2000, and May 2001, soil samples were collected from an agricultural field in an area with intensive livestock farming in northern Germany. The site is part of the soil monitoring project of Lower Saxony, and soil properties and land use are well documented since 1993.[13] The sandy soil has sand, clay, and organic carbon contents of 91.6, 2.4, and 1.8%, respectively. The $pH_{(CaCl_2)}$ is 4.5. Corn for silage was grown in the years from 1999 to 2001. The field is regularly fertilized with liquid manure at a rate of 30−50 m^3/ha per year. A few weeks prior to the spring samplings, the field was fertilized with approximately 40 and 30 m^3 liquid manure, respectively. Between April 2000 and April 2001, no liquid manure fertilization occurred. Samples were collected in four specifically marked areas of 16 × 16 m (see Figure 2) at depths of 0−10, 10−20, 20−30, 30−60, and 60−90 cm below the soil surface. For subsoil sampling (30−90 cm), the material of the plough layer was removed to avoid contamination of the samples with antibiotic-containing topsoil. After an intensive soil survey of the field in a 25 × 25 m grid, subplots were chosen with similar soil properties, especially concerning the soil profile, and were positioned at least 100 m away from the field border.

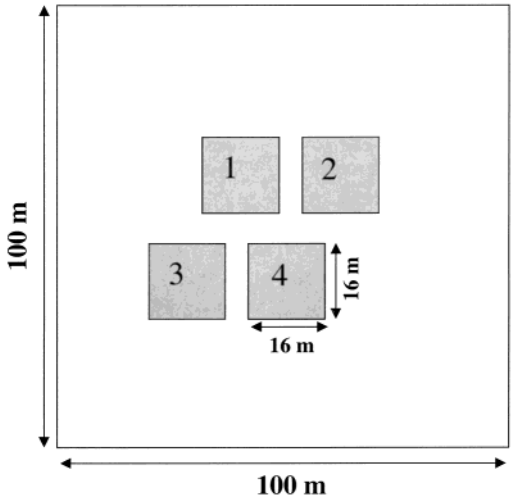

Figure 2. Diagrammatical representation of the soil-monitoring plot (100 × 100 m) within a field of ∼5 ha. The marked areas indicate the four specific subplots (numbered 1−4) of 16 × 16 m from which samples were collected for analysis.

Samples were immediately transported under cooled conditions to the laboratory and stored in the dark at 4 °C prior to analysis. In addition, samples of the liquid manure from April 2000 and 2001 were analyzed for antibiotics. To investigate the possible degradation of the antibiotics under laboratory conditions, the soil sampled in May 2000 was stored for 6 and 12 months at 4 °C in the dark before analysis.

In May 2000, dried liquid manure aggregates from the soil surface, as well as soil samples underneath the aggregates at 0−10-, 10−20-, and 20−30-cm depths, were also obtained as described above. However, these samples were from another three fields recently fertilized with liquid manure originating from pigs (areas A and B) or cattle (area C) in the neighborhood of the soil-monitoring plot.

Groundwater was sampled at a depth of 200−240 cm below the surface using a PTFE cylinder (outer diameter 20 mm, inner diameter 8 mm, and 150-mm length with slits at a width of 0.5 mm on the sides, closed on the bottom, and with a tube connection on top) and a PTFE tube (300-cm length, 4-mm inner diameter) connected to a vacuum pump. Groundwater was sampled in May 2000, November 2000, and June 2001. The brown glass bottles

contained 2.5 mL of 5 M ammonium acetate. The initial fraction of 250 mL was rejected before sampling 250 mL for analysis.

Soil water was sampled at a depth of 80 cm below the surface using P80 ceramic suction samplers (Staatliche Porzellan Manufactur, Berlin, Germany). The samplers were placed in the field in autumn. In May and October 2000, 100 mL of soil water was pumped into glass bottles containing 1 mL of 5 M ammonium acetate using a vacuum of 200−400 hPa.

Extraction Procedures. General Remarks. Tetracyclines are well known to form chelate complexes with metal ions and bind to proteins and silanol groups.[16] Therefore, all glassware used was heated for 2 h at 450 °C, cooled, rinsed with 2.5 mL of a saturated methanolic EDTA solution, and air-dried prior to analysis. Sample preparation and measurement were performed on the same day because the solubility of the extracted tetracyclines during freezing and thawing was variable.

Extraction of Soil and Liquid Manure Samples. Prior to the extraction of soil, the dry weight was investigated as follows: 20 g of each soil sample was incubated at 100 °C for ∼24 h until constant weight was reached. The extraction procedure for the soil and liquid manure samples was developed based on a method for the extraction of tetracyclines from food.[17] One gram of soil or liquid manure was intensively vortexed with 1.2 mL of 1 M citrate buffer (pH 4.7) in a 10-mL glass tube for 1 min. Six milliliters of ethyl acetate was added, again intensively vortexed for 1 min, and then kept for another 15 min in an automatic shaker (KS 125 basic, IKA Labortechnik, Staufen, Germany). The sample was centrifuged for 10 min at 1000*g*, and the organic phase was transferred into a 25-mL flask. The extraction procedure was repeated with another 6 mL of ethyl acetate, and the combined organic phases were evaporated to dryness under vacuum at 40 °C. The dry residue was redissolved in 200 μL (1 mL for liquid manure samples) of 90% acetonitrile/10% 100 mM ammonium acetate in water. Prior to LC−MS−MS analysis, the samples were kept at 10 °C in the autosampler.

Recovery studies were carried out with control sandy soil spiked at the 5, 10, 25, 50, and 100 μg/kg level. The control soil was prepared from 10 antibiotic-free sandy soil samples taken at depths of 0−10, 20−30, and 20−30 cm. Therefore, aliquots of 50 g of each sample were carefully mixed. The mean total organic content of the control sandy soil was 2% and close to that of the areas investigated. The recovery rate was calculated as an average of eight experiments at each concentration.

Day-to-day variation of the method was tested with eight sandy soil samples containing tetracycline (average concentrations from 9.1 to 203 μg/kg) and chlortetracycline (average concentrations from 6.0 to 69.9 μg/kg). The day-to-day variation was calculated using five to six independent experiments.

Recovery studies for liquid manure were carried out with a residue-free liquid manure at the 0.2 and 1 mg/kg level originating from a pig-fattening farm. The recovery rate was calculated as an average of eight experiments at both concentrations.

Extraction of Water Samples. Sample preparation was performed on a Baker column processing system SPE-12G using 200 mg of Baker SDB 1 solid-phase cartridges (Baker, Griesheim, Germany). The cartridges were conditioned with 10 mL of methanol, pH 2.5 (the pH was adjusted with acetic acid), followed by 5 mL of methanol and equilibrated with 10 mL of 0.5 M citric acid buffer. A 25−50-mL aliquot of soil water or groundwater samples was diluted with the same volume of 1 M citric acid buffer (pH 4.7), and the resultant mixture was stirred for 1 min. The loaded cartridge was washed with 10 mL of distilled water followed by 2 mL of methanol. The elution of the retained antibiotics was performed with 10 mL of acidified methanol. The eluate was evaporated to dryness and the residue dissolved in 200 μL of methanol. Recovery studies were carried out with a mixture of 10 antibiotic-free soil water samples at the 0.2 and 1 μg/L level. The recovery rate was calculated as an average of eight experiments at each concentration.

Statistical Analysis. Two comparisons were carried out to investigate the potential influence of soil depth and sampling or storage time on the antibiotic concentration in the soil. First, the soil samples of May 2000, November 2000, and May 2001 were compared between the varying soil depths and sampling times. Second, the soil samples of May 2000 were compared after storage for 6 and 12 months. All comparisons were tested with two-way analysis of variance (ANOVA) using the Sigma Stat software (Program version 2.0, Build 2.0173, Jandel Corp., San Rafael, CA). The Student−Newman−Keuls method was employed as a post-ANOVA test with the level of significance set at $P < 0.05$.

RESULTS AND DISCUSSION

Accuracy, Precision, Quantification, and Detection Limits for LC−MS−MS. HPLC employing a simple gradient system combined with ESI-MS−MS allowed the sensitive and selective determination of the investigated antibiotics in soil, liquid manure, and water samples. The mean recoveries and the standard deviations for the various matrixes tested are given in Table 2.

The limit of quantification based on a signal-to-noise ratio greater than 6 was 5 μg/kg for all compounds in soil, and the limits of detection based on a signal-to-noise ratio greater than 3 were approximately 2 μg/kg for chlortetracycline and 1 μg/kg for the other compounds. In Figure 3a and b, LC−MS−MS chromatograms and tandem mass spectra of a standard sample (representing 0.5 ng of each compound on column) and a soil sample respectively containing tetracycline and chlortetracycline are shown. There were no soil samples from our field studies where oxytetracycline or tylosin could be detected. Consequently, the variations from day to day were determined in eight soil samples containing tetracycline and chlortetracycline only (Table 3). The relative standard deviations (RSDs) calculated from these experiments ranged from 10.1 to 49.7% and were independent of the concentration range. It should be noted that soil is not such a homogeneous matrix compared to water, and due to the adsorptive nature of tetracyclines, local peak concentrations in the soil could not be excluded. Taking this into account, four independent samples from four marked areas within the field were investigated for each data point in Table 4. The RSDs obtained with this sampling procedure for the field samples were in a comparable range when directly measured.

In water samples (including groundwater and matrix-rich soil water), the limit of quantification based on a signal-to-noise ratio greater than 6 of 0.1 μg/L for all compounds was obtained. The pre-elution of the SDB-1 cartridges with methanol led to very clean extracts for the final LC−MS−MS analysis. During sample

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Table 2. Recoveries ± Standard Deviations and the Corresponding Relative Standard Deviations of the Studied Compounds in Soil, Water, and Liquid Manure[a]

	oxytetracycline		tetracycline		chlortetracycline		tylosin	
concn	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)
				In Soil				
5 μg/kg	66.7 ± 19.2	28.8	33.8 ± 9.7	28.6	76.0 ± 15.8	20.8	63.9 ± 8.2	12.9
10 μg/kg	74.2 ± 15.1	20.3	33.7 ± 8.0	23.6	57.3 ± 11.0	19.2	67.4 ± 8.4	12.4
25 μg/kg	85.9 ± 14.7	17.1	46.5 ± 5.1	11.1	66.4 ± 10.0	15.0	63.2 ± 9.6	15.2
50 μg/kg	71.2 ± 6.1	8.5	33.2 ± 2.5	7.5	72.1 ± 4.7	6.5	66.1 ± 2.5	3.8
100 μg/kg	73.0 ± 8.4	11.6	41.2 ± 3.5	8.6	75.7 ± 5.8	7.7	60.3 ± 4.8	7.9
				In Water				
0.2 μg/L	90.0 ± 17.4	19.3	98.2 ± 11.0[b]	11.2	86.6 ± 15.2[b]	17.6	68.6 ± 11.1	16.2
1 μg/L	76.9 ± 9.5	12.4	86.1 ± 14.2[b]	16.5	89.7 ± 9.2[b]	10.3	72.0 ± 13.0	18.1
				In Liquid Manure				
0.2 mg/kg	87.3 ± 6.0	6.9	82.3 ± 5.9[b]	7.2	94.1 ± 8.5[b]	9.1	*c*	*c*
1 mg/kg	100.2 ± 2.1	2.1	104.2 ± 3.4[b]	3.3	127.1 ± 9.7[b]	7.6	*c*	*c*

[a] Values are the mean of eight independent determinations at each concentration. [b] Including their 4-epimers. [c] The extraction procedure applied for the analysis of tetracyclines is not suitable for the analysis of tylosin in liquid manure.

Table 3. Day-to-Day Variation of the LC−MS−MS Method in Soil Samples Containing Tetracycline and Chlortetracycline in Various Amounts

sample	n	tetracycline (μg/kg)	RSD (%)	sample	n	chlortetracycline (μg/kg)	RSD (%)
S0062	6	9.1 ± 4.4	48.9	S0016	6	6.0 ± 1.4	23.4
S0106	6	9.4 ± 3.5	36.9	S0015	6	7.4 ± 2.3	31.0
S0107	5	12.8 ± 6.1	47.2	S0023	5	7.7 ± 0.8	10.1
S0082	6	35.5 ± 10.9	30.8	S0030	6	8.1 ± 0.8	10.4
S0015	6	54.8 ± 23.5	43.0	S0062	6	13.9 ± 4.5	32.2
S0016	6	66.2 ± 13.6	20.5	S0106	6	27.3 ± 7.7	28.1
S0030	6	135.6 ± 53.9	39.7	S0107	5	38.8 ± 19.3	49.7
S0023	5	203.0 ± 60.5	29.8	S0082	6	69.9 ± 26.7	38.2

preparation, a significant epimerization of tetracycline and chlortetracycline occurs; therefore, these epimers were taken into consideration when the water was analyzed. The limit of detection based on a signal-to-noise ratio greater than 3 was 0.05 μg/L for all compounds in the water samples. Recently, comparable methods based on solid-phase extraction (as a powerful sample pretreatment step) were developed for the analysis of tetracyclines and sulfonamides in groundwater and surface water employing LC−MS[18] and for the analysis of tetracyclines in groundwater and lagoon water employing LC−MS−MS.[19] Lindsey et al.[18] obtained a limit of quantitation of 0.1 μg/L for each tetracycline, and Zhu et al.[19] reported method detection limits for the tetracyclines from 0.2 to 0.28 μg/L.

The limit of quantification for liquid manure based on a signal-to-noise ratio greater than 6 was 0.05 mg/kg for the tetracyclines and the limit of detection was ∼0.02 mg/kg. In addition to tetracycline, 15−20% of the epimer 4-*epi*-tetracycline was found in the liquid manure samples (Figure 4). Due to the very low recoveries in liquid manure, the sample pretreatment for tetracyclines cannot be recommended for tylosin in this matrix.

In the case of liquid manure, an example is shown for the employment of the highly selective LC−MS$^{(3)}$ procedure as further confirmation of the findings from the MS−MS method (Figure 4). As a result of the analytical system, LC−MS$^{(3)}$ is ∼7-fold less sensitive than a comparable MS−MS technique and is only recommended, for example, for the confirmation of tetracycline concentrations greater than 40 μg/kg in soil.

Optimization and Applicability of the Method. The high recovery rates of about 60−70% for oxytetracycline, chlortetracycline, and tylosin in such a difficult matrix as soil, in combination with RSDs of less than 30% in spiked soils, makes this method very useful for the analysis of antibiotics in soil even in the low-microgram per kilogram range. A mean recovery rate for tetracycline of 38% may be the result of a much stronger or a more specific adsorption of this compound to the soil matrix compared to oxytetracycline and chlortetracycline.

During method development, it was established that agents such as EDTA or strong acids such as hydrochloric acid—which destroy the chelate-binding properties of tetracycline to metallic ions—did not affect the recovery rates in soil. The final modified extraction procedure (using a high molar citric acid buffer at pH 4.7) was based on a method recently developed for the analysis of tetracyclines in food.[17] It may therefore be that tetracyclines—like sulfonamides[20]—are mainly adsorbed to organic constituents of soil, e.g., to humic materials as demonstrated by Sithole and Guy.[21] This specific binding in soil would also explain the generally very good recoveries of tetracyclines in liquid manure. Further

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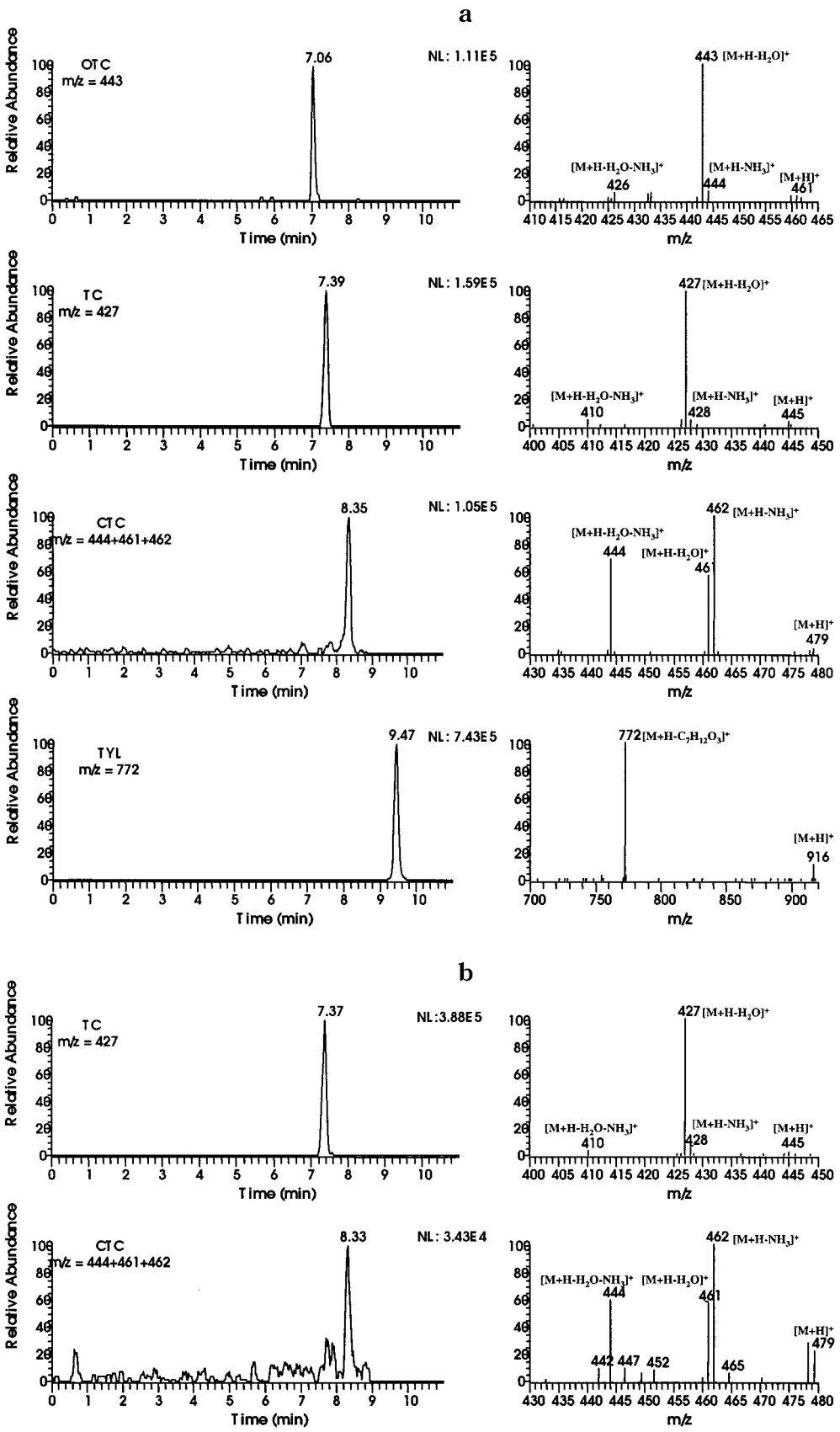

Figure 3. (a) Left panel: reconstructed ion chromatograms of oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and tylosin (TYL) in a standard solution with 0.5 ng of each compound on column analyzed with LC−MS−MS (NL, normalized level). Right panel: the corresponding tandem mass spectra of OTC, TC, CTC, and TYL. (b) Left panel: reconstructed ion chromatograms of TC and CTC in a soil sample containing 92.7 μg/kg TC and 5.4 μg/kg CTC, where oxytetracycline and tylosin were not detectable, analyzed with LC−MS−MS. Right panel: the corresponding tandem mass spectra of TC and CTC.

Table 4. Investigation of a Long-Term Soil Monitoring Area with LC−MS−MS after Repeated Fertilization with Liquid Manure Containing 4 mg/L Tetracycline and 0.1 mg/L Chlortetracycline in April 2000 and 3.2 mg/kg TC and 0.09 mg/kg CTC in April 2001[a,b]

	sample collection				
soil depth (cm)	in May 2000	in Nov 2000	in May 2001	in May 2000, stored for 6 months at 4 °C	in May 2000, stored for 12 months at 4 °C
			Tetracycline (μg/kg)[c,d]		
0−10	56.4 ± 20.1	43.4 ± 22.2	86.2 ± 15.3	49.8 ± 11.3	73.6 ± 19.7
10−20	100.5 ± 68.8	94.2 ± 40.3	198.7 ± 84.0	61.8 ± 22.6	119.4 ± 86.5
20−30	90.5 ± 35.4	59.0 ± 21.7	171.7 ± 65.4	101.5 ± 51.5	141.3 ± 82.6
30−60	nd[f]	nd	nd	nd	nd
60−90	nd	nd	nd	nd	nd
			Chlortetracycline (μg/kg)[e]		
0−10	4.6 ± 1.1	5.1 ± 1.4	6.1 ± 2.0	3.7 ± 1.0	6.6 ± 1.4
10−20	4.7 ± 0.3	6.0 ± 1.2	7.1 ± 3.4	4.4 ± 1.0	6.8 ± 1.5
20−30	4.8 ± 1.2	5.0 ± 1.0	5.8 ± 3.2	4.2 ± 1.4	7.3 ± 3.2
30−60	nd	nd	nd	nd	nd
60−90	nd	nd	nd	nd	nd

[a] The values (μg/kg of dry soil) represent the means ± standard deviations of four samples taken from four specifically marked areas, which have been corrected for mean recovery (37.7% for TC, 69.2% for CTC). Oxytetracycline and tylosin were not detectable in any sample. [b] Results of the two-way ANOVA analysis for the factors of time (May 2000, November 2000, and May 2001) and soil depth (0−10, 10−20, and 20−30 cm) followed by the post-ANOVA Student−Newman−Keuls method. Mean values and the standard error of the mean (in μg/kg) are given in parentheses in footnotes *c*−*e*. TC and CTC at depths of 30−90 cm were not detectable and not included into the calculations. [c] The concentrations of TC in samples collected in May 2001 (152.2 ± 13.7) at 0−10, 10−20, and 20−30 cm were significantly higher ($p < 0.05$) than for those samples collected in May 2000 (82.3 ± 13.7) and November 2000 (65.5. ± 13.7). [d] The concentrations of TC in samples collected in May 2000, in November 2000, and in May 2001 at 0−10 cm (62.0 ± 13.7) were at all times significantly lower ($p < 0.05$) than in the soil depths of 10−20 (131.0 ± 13.7) and 20−30 cm (107.1 ± 13.7). [e] The concentrations of CTC in samples of May 2000 stored for 12 months (6.9 ± 0.4) at 0−10, 10−20, and 20−30 cm were significantly higher ($p < 0.05$) than that of May 2000 without storage (4.7 ± 0.4) and with storage for 6 months (4.1 ± 0.4). [f] nd, not detected.

experiments will be performed to validate these findings and to observe the mechanisms by which tetracyclines may be specifically desorbed. Nevertheless, the RSDs for the analysis of tetracyclines in soil are at an acceptable level over a broad concentration range. Suitable sample preparation was also developed for soil water and liquid manure. Good recovery rates (with the exception of tylosin in liquid manure) and clear MS−MS spectra were obtained in the analysis of these environmental samples (Figures 3b and 4). Further confirmation of the concentrations of tetracycline and its epimer can be obtained in ion trap mass analyzers with LC−MS$^{(3)}$ procedures. Although the spectra for tetracycline and its epimer look very similar in the MS−MS mode, very selective information about the compounds is available in the LC−MS$^{(3)}$ mode (Figure 4b). This impressive tool for the further confirmation of tetracycline residues in various environmental samples should also have applicability to other sample matrixes, e.g., food samples.

Occurrence, Distribution and Fate of Tetracyclines and Tylosin in Liquid Manure and Soil. The data presented here demonstrate, for the first time, that tetracyclines not only occur in significant amounts in soil after repeated fertilizations with liquid manure but also persist and accumulate in the environment.

Oxytetracycline and tylosin could not be detected in any liquid manure or soil sample investigated. The liquid manure sampled in April 2000 contained 4.0 mg/kg tetracycline and 0.1 mg/kg chlortetracycline, which was similar to the concentrations of the liquid manure sampled in April 2001 (i.e., 3.2 mg/kg tetracycline and 0.09 mg/kg chlortetracycline).

Our investigations show that the antibiotics are spread evenly over the whole field: the RSDs between the four subplots varied between 17.7 and 72.4% for tetracycline and 6.4 and 55.2% for chlortetracycline (calculated from Table 4). This was similar to the day-to-day variation between analyses of comparable samples from the same subplot, which ranged from 21 to 49% for tetracycline and 10 to 50% for chlortetracycline (Table 3). Considering the crude method of applying and mixing the liquid manure into the soil by the farmer, these variations in the field are surprisingly low.

There was no statistically significant difference between the tetracycline concentrations in the field in May and November 2000, indicating that there had been no observable degradation of this compound during the summer months, when no liquid manure fertilization had occurred. After the liquid manure fertilization in April 2001, the tetracycline concentrations were significantly higher than in November 2000 and May 2000 ($p < 0.05$). The highest mean concentrations of tetracycline in the soil of up to 198.7 μg/kg were also detected in May 2001 (Table 4). The maximum concentration in one of the specifically marked areas was 307.4 μg/kg.

At all three sampling dates, the antibiotics were only found in the first 30 cm of the topsoil. This indicates that the compounds, spread on the soil surface by liquid manure amendment, are incorporated into the topsoil and that no tetracyclines are transferred into the subsoil, e.g., by infiltration water. The chlortetracycline concentrations were up to 50-fold lower than the tetracycline concentrations and did not differ between the three sampling dates when the samples were immediately analyzed.

Storage for 12 months at 4 °C had no statistically significant effect on the tetracycline concentrations. However, this was not the case for chlortetracycline concentrations ($p < 0.05$). There was an obvious but nonsignificant increase in tetracycline concentration of samples stored for 12 months at 4 °C compared to those obtained immediately after sampling or after storage for 6 months. In light of this observation, a release of tetracyclines

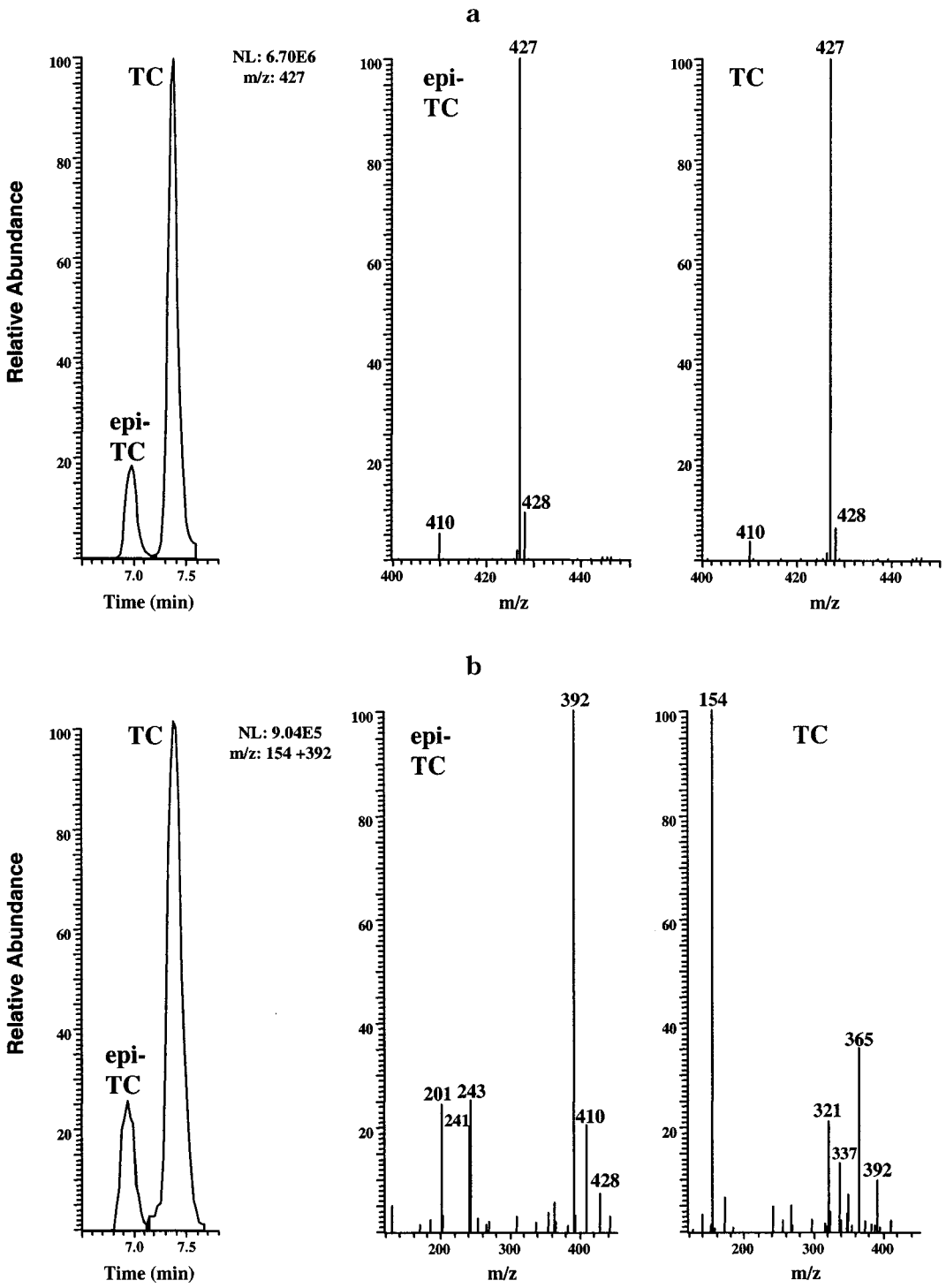

Figure 4. (a) Investigation of liquid manure with LC−MS−MS. Left panel: Reconstructed ion chromatograms of 4-*epi*-tetracycline (epi-TC) and TC. Central and right panel: the corresponding tandem mass spectra of 4-epi-TC and TC, respectively. (b) Investigation of liquid manure with LC−MS$^{(3)}$. Left panel: Reconstructed ion chromatograms of epi-TC and TC. Central and right panels: the corresponding MS−MS−MS spectra of 4-epi-TC and TC, respectively.

during sample storage cannot be excluded and may be due to the further degradation of the organic material in the soil, shifts in the microorganism population, or variations of the pH and redox potential. Furthermore, the adsorption of tetracyclines to the soil may be reversible. This is of ecotoxicological importance, since the possibility of "bound" antibiotics being released under natural soil conditions (e.g., by acidification or by a reduction of the redox potential after water saturation of the soil) is apparent. If this release of bound tetracycline residues can also occur in nature, part of the higher concentration found in May 2001 could be attributed to this effect.

The ecological impact of antibiotics widely distributed over a whole field in subtherapeutic concentrations over a long period with regard to the development of antibiotic resistance of bacteria cannot be estimated at present. On one hand, no effect on soil respiration was found at high concentrations (50 mg/kg of soil)

of chlortetracycline.[22] On the other hand, tetracyclines were found highly toxic with EC_{50} values of 0.05 mg/L for chlortetracycline and 0.09 mg/L for tetracycline when the freshwater cyanobacteria *M. aeruginosa* was tested.[23] Even if a general link between antibiotic use and percentage of resistant strains of soil bacteria is supposed,[24] it is unclear at which threshold concentration in soil a shift toward an increase in the percentage of tetracycline resistant strains has to be expected.

One important tool for the risk assessment of veterinary drugs in the soil is the estimation of the predicted environmental concentrations based on guidelines recently published by EMEA.[15] The detected tetracycline concentration in liquid manure of 4 mg/kg and the amount of liquid manure added (i.e., 40 m^3/ha) were used for these calculations for the year 2000. When certain factors are taken into account such as ploughing of soil and dilution of the compounds—the liquid manure is usually mixed into a depth of 30 cm—the mean concentration in the plough layer should be ~36 μg/kg. In comparison, the data from the field samples and the calculations in Table 4 reveal that the tetracyclines are quantitatively transferred into the soil. Furthermore, antibiotic residues from past liquid manure fertilizations were still present in the soil and, thus, were only partly degraded. This observation is strengthened by the results from May 2001; further fertilization with liquid manure containing amounts of tetracycline similar to the previous year led to significantly higher tetracycline concentrations in the soil layers at 0−30 cm. The observed increase of 40−110 μg/kg between November 2000 and May 2001 was even higher than the expected increase from the liquid manure application (30 m^3 with 3.2 mg/kg tetracycline incorporated into the 30-cm soil corresponds to 21 μg/kg of soil). One possible reason for this deviation in the field samples could be the additional release of tetracyclines (as already mentioned above) from November 2000 until the sampling time. Nevertheless, an accumulation of the drug in soil occurs as evidenced by the overall data and the fact that the annual supply rate of tetracyclines by organic fertilization is higher than the degradation rate in the soil.

For all three sampling times, the mean concentration of tetracycline in the upper soil layer (0−10 cm) was significantly lower than in the two layers below ($p < 0.05$). From these findings, it was concluded that a degradation of tetracycline occurs. Tetracycline has been shown to be readily photodegraded in water,[25] and this may also occur at the soil surface. Nevertheless, photodegradation could only be effective in the first millimeter of the topsoil, where the light can penetrate.[26] Therefore, a complete degradation of the drug is not likely and was not shown in the field samples.

In an initial study, we screened several samples for the possible reversible degradation product, 4-*epi*-tetracycline. Significant concentrations of 4-*epi*-tetracycline were found in the soil amounting to 10−15% of the parent drug (data not shown). An equal ratio of the drug and its epimer was obtained in the analysis of liquid manure (Figure 4). Therefore, we conclude that the 4-epimer is transferred from the liquid manure into the soil. To elucidate the formation of other degradation products of tetracycline, laboratory studies employing soil enriched with the pure drugs will be performed.

Table 5. Tetracycline and Chlortetracycline Concentrations Determined by LC−MS−MS in Dried Liquid Manure Aggregates and in the Underlying Soil Obtained from Three Different Areas[a]

dried liquid manure/ soil depth (cm)	tetracycline (μg/kg)	chlortetracycline (μg/kg)
area A[b]	349.3	1435.0
0−10	33.2	59.9
10−20	50.1	12.0
20−30	30.3	14.9
area B[b]	117.1	4.9
0−10	4.0	1.7
10−20	2.6	2.6
20−30	2.6	2.9
area C ($n = 4$)	6.6 ± 3.2	10.9 ± 3.9
0−10	5.0	7.2
10−20	4.6	6.6
20−30	2.3	3.3

[a] All values are μg/kg of dry soil and corrected for mean recovery. [b] There was only one dried liquid manure aggregate found in the field.

Acute problems may arise with the high concentrations of antibiotics found in the dried liquid manure aggregates at the soil surface where 0.35 mg/kg tetracycline and 1.44 mg/kg chlortetracycline were detected (Table 5). These aggregates can remain intact after incorporation into the soil and create spots with high antibiotic concentration in the soil matrix. Consequently, soil bacteria exposed to these concentrations, which fall within the minimal inhibitory concentration (0.5−2 mg/L[27]) for various bacteria, may cause acute toxic effects and lead to a reduction in the biodiversity of soil microorganisms.

In June 2001, the Steering Committee of the Veterinary International Committee on Harmonization (VICH) increased the trigger value for antibiotic concentrations in soil from 10 to 100 μg/kg.[28] Our investigations show that, for the "old" veterinary drug tetracycline, the new and 10-fold higher trigger value is still exceeded in soils that are regularly fertilized with liquid manure. Furthermore, the rate of tetracycline application to soils exceeds the degradation rate of this compound such that further accumulation is expected.

Several soils had tetracycline concentrations between 10 and 100 μg/kg (Tables 3 and 4).[14] Even if these concentrations fall below the actual trigger value for risk assessment, it cannot be presently ruled out that the microbial soil community is affected and antibiotic resistance is occurring at these subtherapeutic concentrations. Thus, tetracyclines have a potential risk for the environment and additional investigations on the environmental effects of this drug are urgently needed.

Antibiotics in Soil Water and Groundwater. Neither tetracyclines nor tylosin was detected in any water sample (i.e., soil water obtained in May and October 2000 at a depth of 80 cm or groundwater sampled in May 2000, November 2000, and June 2001

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(28) VICH Environmental impact assessments (EIAs) for veterinary medical products (VMPs): Phase I. VICH 2000, available at: www:\\vich.eudra.org.

at a depth of 200−240 cm below soil surface). Rabølle and Spliid[11] did not find any oxytetracycline in the leachate of soil columns, and our results from the various field studies support these findings for tetracycline and chlortetracycline; even with the specific and sensitive analytical procedures, antibiotics below 30 cm in the soil and residues in soil water and groundwater could not be detected. Further confirmation of our findings are supported by Lindsey et al.[18] and Zhu et al.[19] Both groups did not detect any tetracyclines in groundwater sites in areas of animal husbandry within the United States.

CONCLUSIONS

We have demonstrated that LC−MS−MS, combined with a rather simple but very effective sample cleanup, is the method of choice for the detection of tetracyclines in various environmental samples. The application of the newly developed methods made the detection of significant amounts of persistent tetracyclines in liquid manure and in the soil possible. We detected these compounds not only in subtherapeutic concentrations but also in the range of the minimum inhibitory concentration for relevant bacterial species in the environment. Ecotoxicological studies especially on soil microorganisms should be performed, to estimate the risk for the soil flora and the spread of antibiotic resistance.

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