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Stability of Tylosin A in manure containing test systems determined by high performance liquid chromatography

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Abstract

Tylosin is a widely used antibiotic for the treatment of infections in swine. Tylosin consists of a mixture of Tylosin A, Tylosin B, Tylosin C and Tylosin D. All components contribute to the potency of tylosin but Tylosin A is by far the major component (usually about 90% and not less than 80%). A fast, robust and easily performed HPLC method has been developed for determination of Tylosin A in the presence of tylosin residues; Tylosin B, Tylosin C and Tylosin D in manure containing incubation media. The separation was performed using a YMC-Pack ODS-AQ column (250×4.6 mm i.d., 5 μm particle size) operated at 35°C. The mobile phase consisted of 2.25% (w/v) sodium perchlorate pH 2.5 – acetonitrile (60:40 v/v). Detection was performed by measuring the UV absorption at a wavelength of 290 nm. Calibration curves of tylosin made in the incubation medium containing 6.4% manure were linear in the range from 0.375 to 128.0 mg/l ($R^2 = 0.999$). The limit of quantitation (at the RSD 20% level) for Tylosin A was found to be 0.4 mg/l in incubation media containing 6.4% manure. The recovery of Tylosin A was in the range from 100% to 108% depending on the concentration of manure. The reproducibility was good as the relative standard deviation ($n = 4$) in each matrix tested was in the range from 0.7 to 1.9 at the 25 mg/l level. The stability of Tylosin A was studied under methanogenic conditions and the half-life was found to be less than two days. Studies under aerobic conditions showed that the degradation rate was found to increase with increasing concentrations of manure particles in the incubation medium. It is, however, not clear whether the decrease in the concentration of Tylosin A is caused by sorption, abiotic or biotic chemical degradation. The major degradation product of Tylosin A in methanogenic as well as aerobic incubation media has a UV-spectrum and a retention time corresponding to Tylosin B. Furthermore, Tylosin D is believed to be a minor degradation product.

1. Introduction

Even though pharmaceuticals are designed to possess biological activity, the fate of these substances in nature have not been given much interest. Tylosin, is produced by fermentation of Streptomyces strains, and is one of the most extensively used feed additive in pig production (ca. 62 tons/year in Denmark in 1997; 14 tons/year in 1998) (Bager, 1999, DANMAP 98). Tylosin (Fig. 1) consists of a mixture of the macrolides Tylosin A, Tylosin B, Tylosin C and Tylosin D. All four components contribute to the potency of tylosin. Tylosin A is by far the major component (usually about 90% and not less than 80%) (Horie et al., 1998; European Pharmacopoeia, 1999). After excretion, urine and faeces from pigs produced in intensive farming are kept in a manure tank in average for 90 days prior to dispersal on arable land. A substantial amount of tylosin and its metabolites may therefore be dispersed with manure unless degradation (biotic or abiotic) takes place in the manure tank.

Tylosin is used by veterinarians for the treatment of infections caused by gram-positive and some gram-negative bacteria. It is widely used for treating pigs

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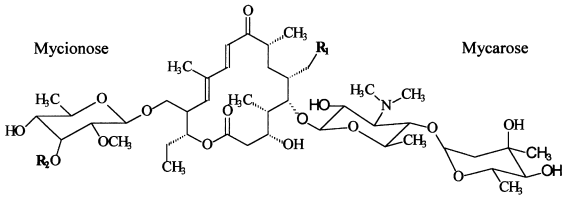

Substance	R_1	R_2	Mycarose	Mycinose
Tylosin A	CHO	CH_3	+	+
Tylosin B	CHO	CH_3	-	+
Tylosin C	CHO	H	+	+
Tylosin D	CH_2OH	CH_3	+	+
Lactenocin	CHO		-	+
OMT	CHO		-	-
DMT	CHO		+	-

"+" = sugar present "-" = sugar not present

Fig. 1. Structures of the macrolide antibiotic Tylosin A and related compounds.

infected by *Treponema hyodysentheriae*, swine dysentery. It mechanism of action is inhibition of the protein synthesis in the bacteria. Tylosin A fed to pigs is extensively metabolised. Residues found in faeces include Tylosin A, Tylosin B, Tylosin D and dihydrodesmycosin (Horie, 1995). Although tylosin is extensively metabolised, no single metabolite appears to be present in a higher concentration than the parent compound. Thus it is relevant to study the degradation of the latter in manure.

The persistance of xenobiotics in the environment is dependent of several parameters such as redox conditions, temperature, and bacterial community. Similarly, the degradation of antibiotics in the manure tank is dependent on several parameters. In a manure tank biodegradation takes place under methanogenic conditions (Boopathy, 1996). The biodegradation may therefore be studied in an incubation medium consisting of manure, nutrient salts and water under methanogenic conditions. Such a study could give data of the possible degradation and/or sorption of Tylosin A which would enable the calculation of the amounts of antibiotics and recidues in the manure before it is spread on arable land. These data can help to estimate predicted environmental concentrations (PEC) in different field scenarios.

The basis of such stability studies is a simple, robust and sensitive method for detection of low concentrations of Tylosin A in the presence of potential residues and decomposition products in very complex matrices like manure. Aerobic test systems are more easily handled and may be used in recovery and sorption studies if the stability and degradation pattern of tylosin under aerobic conditions not is to different from the stability in methanogenic test systems.

Recently, a review on the methods for separation and detection of macrolides have appeared (Kanfer et al., 1998). High performance liquid chromatographic (HPLC) methods for separation are the most widely used. Due to the amino group that is concealed in Tylosin A, tailing is often a problem on silica based column packings. Thus addition of substances that will form an ion pair with the amino group e.g. the perchlorate ion (Van der Heyden et al., 1999) or additives like tetrabutylammonium salts (De Liguoro et al., 1998; Roets et al., 1993) that will shield the interaction with the residual silanol groups is required. Alternatively separations are performed using polymer based packing materials (Paesen et al., 1995a). Recently, two HPLC methods for determination of tylosin impurities in tylosin tatrate were compared in an interlaboratory study (Van der Heyden et al., 1999). A method using silica-based reversed phase columns with a mobile phase containing 22.5% (m/v) sodium perchlorate was preferred over separation methods using a styrene divinyl benzene polymer column with respect to selectivity and reproducibility.

Analysis of tylosin and tylosin recidues in matrices like meat, tissue, muscle, kidney and liver has been performed (De Liguoro et al., 1998; Chan et al., 1994; Horie et al., 1998) but determination of Tylosin A in manure containing samples has not been described previously.

The aim of our work was to study the fate of Tylosin A in manure containing test systems under aerobic as well as methanogenic redox conditions. To achieve this an HPLC method for the determination of Tylosin A in presence of the major tylosin residues (Tylosin B, Desmycosyltylosin (DMT) and Tylosin D) in the manure containing test systems has to be developed.

2. Materials and methods

2.1. Chemicals

Tylosin tartrate (CAS no. 74610-55-2) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Tylosin A, Tylosin B, Tylosin D, and DMT (desmycosyltylosin) were kindly donated by professor Hoogmartens (Katholieke Universiteit Leuven, Leuven, Belgium). Acetonitrile (HiPerSolv for HPLC) was purchased from BDH (Poole, England), and sodium perchlorate was purchased from Riedel-de Haën (Seelze, Germany).

In the preparation of all solutions, degassed Milli-Q water was used, if not otherwise mentioned. Stock solution of tylosin was prepared to contain 200 mg/l. This was consecutively diluted to the concentrations used in the experiments.

2.2. Manure

The manure used for the experiments was obtained from a farm in the northern part of Zealand, Denmark. On this farm antibiotics had never been used in the production of pigs, thus the manure collected did not contain tylosin. The raw manure used had been filtered through a 1 mm sieve into 1 l serum bottles. The manure that was filtered through the 1 mm sieve is characterised as raw manure. Nitrogen was bubbled through the raw manure for 15 min before the bottles were closed. The manure was then stored for six months at 4°C, and for five months at −20°C.

2.3. Apparatus

A Waters 2690 Alliance system equipped with a sample cooler and a column oven was used in combination with a Waters Photodiode Array Detector (DAD) for UV detection (Milford, Massachusetts, USA). Data collection and data treatment was done by use of Millennium software v.2.15.01.

2.4. Chromatography

A stainless steel YMC-Pack ODS-AQ column (250×4.6 mm i.d.) particle size 5 μm, 120 Å (YMC Europe GmbH, Weselerwald, Germany) was used. The mobile phase consisted of sodium perchlorate (2.25% m/v) adjusted to pH 2.5 with hydrochloric acid) – acetonitrile (60:40 v/v). The flow rate was 1.0 ml/min. The column was operated at 35°C. The injection volume was 100 μl. The detection was performed at a wavelength of 290 nm.

2.5. Standards and samples

Freshly prepared standard solutions, made in methanol–water (1:1 v/v), were used. Samples were analysed immediately after preparation or stored at −15°C until analysed. During analysis, all samples were protected from light and kept at 5°C in order to prevent hydrolysis and epimerization. Calibration standards was prepared by adding increasing amounts of Tylosin A to filtered manure incubation medium (MIM)-methanol (1:1 v/v) as well as to methanol–water (1:1 v/v) in order to compare the calibration curves. No difference was observed, but to prevent any kind of chemical decomposition of tylosin in the standard all standards were made in methanol–water (1:1 v/v).

Buffer solutions used were 50 mm potassium phosphate made in Milli-Q-water. pH was adjusted to 6.0, 7.0 and 8.0 with potassium hydroxide.

2.5.1. Procedures for preparation of aerobic manure containing test systems

Raw manure was either mixed directly or centrifuged and filtered through a 0.45 μm filter (Minisart, Sartorius, Göttingen, Germany) and then mixed with degassed Milli-Q water giving three different amounts of manure 0.1%, 0.5%, 3.0% or 6.4% (v/v) in the final solution. These solutions were characterised as MIM. Samples containing raw manure that was filtered through a 0.45 μm filter will be referred to as "filtered manure". Samples containing raw manure that was not filtered through a 0.45 μm filter will in the following be referred to as "non-filtered manure". Mixing of test solutions was done in closed dark glass flasks. An aliquot of the stock solution was added to each flask resulting in a final concentration of tylosin of 25.0 mg/l. The total volume of sample per flask was 81.6 ml. The flasks were protected from light and kept at room temperature (ca. 20°C) during the experiments. All experiments were made in four replicates unless other wise stated.

2.5.2. Procedures for preparation of methanogenic manure containing test systems

The experiments were set up according to the ISO standard "11734" (1995), with the exception that all volumes were multiplied by fifty in order to achieve a total test volume of 680 ml. Incubation media for the abiotic study was autoclaved three times 30 min at 120°C. The following four experiments were made in four replicates: (1) media containing 6.4% activated manure incubated with tylosin; (2) control with 6.4% activated manure; (3) media containing 6.4% inactivated manure (autoclaved) incubated with tylosin; (4) control with 6.4% inactivated manure. Initial concentrations of tylosin was 5 mg/l. The incubation temperature was 20°C. Samples of 5–10 ml were taken using sterile syringes through a rubber septum every second day (activated manure) or every fourth day (autoclaved manure) during the first two weeks after that with longer intervals.

2.5.3. Blanks

Controls from the respective test systems served as blanks.

2.6. Sample preparation

Manure samples were prepared for HPLC analysis by mixing 1.50 ml of sample with 1.50 ml of methanol in plastic test tubes. Subsequently, the manure-methanol mixture was centrifuged at ca. 600 g for 10 min. Prior to the HPLC analyse the supernatant was filtered through a 0.45 μm filter (Minisart, Sartorius, Göttingen, Germany) into 1.5 ml glass vials (Kontram, Olarinkioma, Espoo, Finland). Samples that could not be analysed

immediately were stored for less than 24 h in glass vials at −20°C.

3. Results and discussion

3.1. HPLC method

The HPLC-method developed for analysis of Tylosin A in presence of several tylosin recidues was based on the chromatographic method recommended by Van der Heyden et al. (1999). These authors recommended a very high concentration of sodium perchlorate in the mobil phase but in the current study it was reduced by a factor of 10 in order to minimise the risk of precipitation of salt in the chromatographic system. No major changes in selectivity were observed compared to the original method. Thus the final mobile phase consisted of 2.25% (w/v) sodium perchlorate pH 2.5 – acetonitrile (60:40 v/v).

Samples containing manure may have a high content of particles as well as germs. Methanol was therefore added to the samples in equal volume in order to flocculate the particles and to extract the analytes from any collide phase that may be present in the sample prior to filtration. Moreover, methanol inhibits biotic degradation of tylosin during storage prior to analysis. The prepared sample contains a higher percentage of organic solvent than the mobile phase and is furthermore injected in a relative high volume. This may cause a disturbance of the chromatographic system leading to a decrease in the resolution and resulting in an invalidating loss of selectivity. Signs of this phenomenon were observed but the loss of resolution was only minor. In UV-spectrometry loss of analyte due to incorporation in a colloid phase is a well-known error. As calibration curves prepared from standard solutions of tylosin in a mixture of manure and methanol corresponded to the calibration curves from standard solutions prepared in a mixture of methanol and water colloid-dependent loss of tylosin was concluded to be of no major importance.

The precision and error of the final method is given in Table 1. The recovery of Tylosin A was 100–108%. This is a much higher recovery than reported for other methods (De Liguoro et al., 1998; Chan et al., 1994; Horie et al., 1998). Thus there was no sign of instant sorption of tylosin to the particles from the manure. Likewise sorption to glass was excluded as the same amount of tylosin was found in standards having been through various numbers of glass containers.

3.2. Selectivity of the method

The selectivity of the method was evaluated by comparing the chromatograms of blank manure samples with samples spiked with Tylosin A, Tylosin B, Tylosin C , Tylosin D as well as DMT. A number of minor peaks interferring with Tylosin A were detected in samples with 3% of raw manure. However, the peak area did not exceed the value corresponding to 0.3 mg/l of Tylosin A. These interfering peaks were thus without major relevance to the overall picture of the disappearance of Tylosin A in the test flasks. However, development of a more selective sample clean-up method is necessary to obtain lower limits of detection and quantification especially if authentic manure from pig production is to be analysed. From Fig. 2 it is clearly seen that neither Tylosin B, Tylosin C nor Tylosin D interfere with the determination of Tylosin A. It is also obvious that it is difficult to determine the exact concentrations of the major degradation products Tylosin B and Tylosin D in samples with high concentration of manure. There is a

Table 1
Validation parameters for the analytical method

Range (mg/l)	0.375–128.0
LOQ (mg/l)	0.4
Correlation coefficient (R^2)	0.999

Sample matrix manure (%)	Added[a] (mg/l)	Found[a] (mg/l)	Error[a] (%)	Precision (RSD in %, $n=4$)	Recovery (%)
0.1% non-filtered	25.0	25.9	3.6	1.6	103.6
0.1% filtered	25.0	25.3	1.2	1.9	101.2
0.5% non-filtered	25.0	26.9	7.6	1.5	107.6
0.5% non-filtered	125.0	134.9	7.9	0.6	107.9
3.0% non-filtered	25.0	26.3	5.2	0.7	105.2
3.0% filtered	25.0	26.0	4.0	0.9	104.0
6.4% non-filtered	25.0	25.2	0.8	1.1	100.8
6.4% autoclaved[b]	25.0	25.7	2.8	0.8	102.8
6.4% non-autoclaved[b]	25.0	25.2	0.8	1.3	100.8

[a] Average of four replicates.
[b] These solutions were made in ISO-medium and one of them was autoclaved before storage for later use.

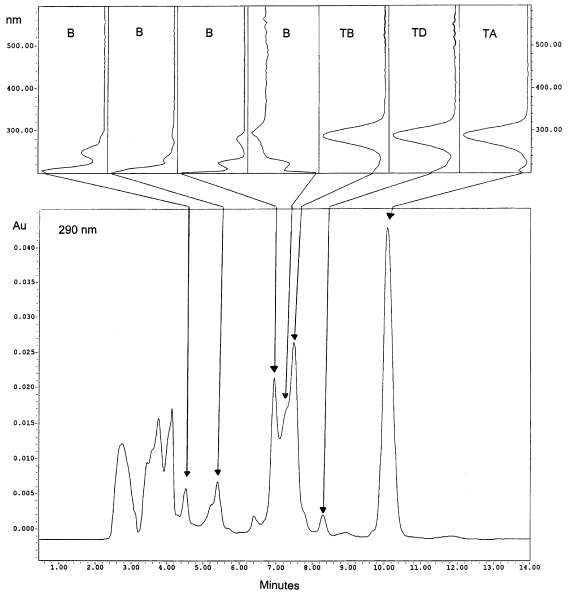

Fig. 2. Chromatogram of 3.0% non-filtered manure after 4 h incubation with tylosin (25 mg/l) under aerobic conditions. Other conditions please see Section 2. Diode array detection (DAD) enables the identification of substances with the same chromophore system as Tylosin A. TA: Tylosin A, TB: Tylosin B, TD: Tylosin D, B: Blank peak.

noticeable difference between the amount and heights of "blank-peaks" in chromatograms from filtered and non-filtered blank samples (Fig. 3).

3.3. Chemical stability

Standards of tylosin kept at 5°C and analysed during two days showed no sign of degradation. Tylosin A was stable during three days at 20°C in potassium phosphate buffers having pH values of 6.0, 7.0 or 8.0, respectively. No major degradation products were detected. This corresponds with the results published by Paesen et al. (1995b). The pH in the manure samples was ranged from 6 to 8. Thus chemical degradation is unlikely to occur at the pH found in the manure tank.

3.4. Stabilty of tylosin in manure containing test systems

The developed HPLC method was used to determine the concentration of tylosin the aqueous phase in activated as well as autoclaved manure kept under methanogenic conditions. Samples were taken at the onset (with-in the first hour) of the study and every second day (activated manure) or every fourth day (autoclaved manure) with-in the first week of the experiment. Tylosin was only detected in the samples taken at the onset of the experiment. A peak with similar retention time and

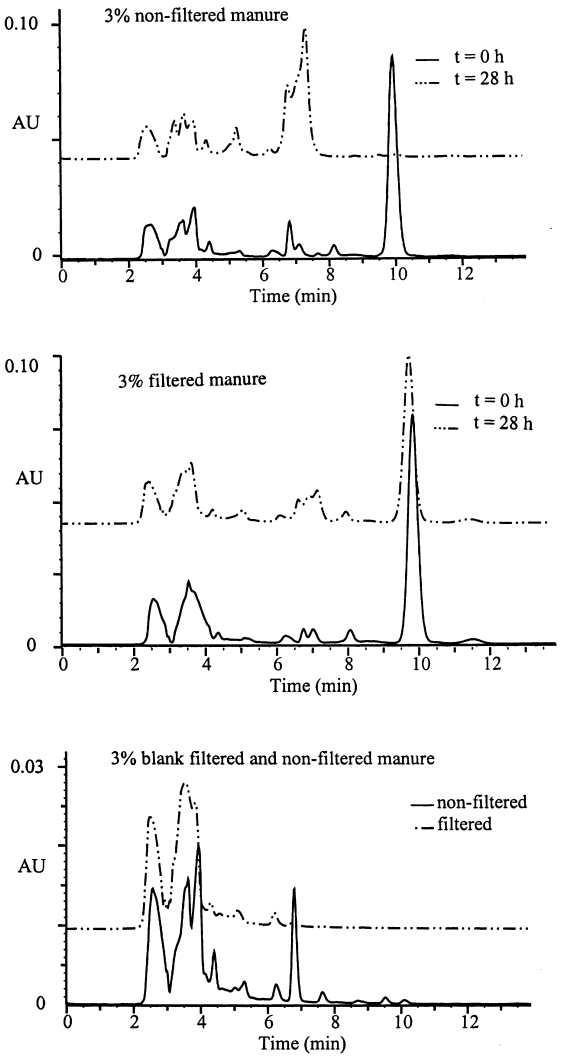

Fig. 3. Chromatograms of: (1) 3.0% blank manure, filtered and non-filtered. (2) 3.0% of filtered manure spiked with 25.0 mg/l tylosin and (3) 3.0% of non-filtered manure spiked with 25.0 mg/l tylosin incubated under aerobic conditions in the time indicated.

UV-spectrum as Tylosin B was detected in samples drawn on the second day but not on the fourth day of the study. Thus the half-life of Tylosin A in the aqueous phase in manure is less than two days.

The rapid decrease in the concentration of Tylosin A in the aqueous phase, especially for the inactivated samples, where degradation was believed to be abiotic, the disappearance of Tylosin A was much faster than would have been expected, according to data on the chemical stability of tylosin. Photolytic degradation was found to be very unlikely as the test flasks were protected from light. Sorption of tylosin to soil has been reported (Rabølle and Spliid, 2000). Thus it is possible

that sorption to particles from the manure contribute to the decreasing aqueous concentration of Tylosin A.

Physical–chemical properties of methanogenic and aerobic test systems are quite similar. The influence of manure particles on fate of Tylosin A was therefore studied in filtered manure systems as well as non-filtered manure systems under aerobic conditions. A decrease in the Tylosin A concentration in filtered manure reflects the chemical stability in the test system, as the filtering process removes a major part of the bacteria but leaves salts and proteins. In the non-filtered manure a decrease in the Tylosin A concentration may be caused by sorption or abiotic degradation, provided no or very little biological activity is left in the manure after the storage at −80°C.

Fig. 3 illustrates the stability of Tylosin A during the experiments. Throughout the experiment with 0.1% manure almost no degradation was detected neither in flasks containing filtered nor non-filtered manure and only a minor increase in the degradation was observed with increase in filtered manure. In contrast, the degradation rate was found to increase dramatically with increasing concentrations of non-filtered manure in the test system containing 3.0% of raw manure. The concentration of salts is expected to be the same in filtered and non-filtered samples containing the same percentage of raw manure. This indicates that the concentration of particles in the solution strongly influences the concentration of Tylosin A in the aqueous phase.

The chromatograms in Fig. 4 illustrate the pronounced difference between the degradation of Tylosin A in non-filtered and filtered manure. In the flasks containing 3.0% and 6.4% of non-filtered manure a peak indicating a major metabolite arose as the peak from Tylosin A disappeared. This peak was found to have the same UV-spectrum and retention time as Tylosin B. The concentration of the degradation product corresponds to about 70% of the loss of Tylosin A. Other degradation products were found in concentrations that corresponded to less than 5% of Tylosin A. Small peaks descending from Tylosin B, Tylosin D and DMT were also found as minor impurities in the tylosin standards (less than 1%). These results indicate that Tylosin B are a major degradation product and that sorption to the particles in manure in the test systems only are a minor factor in the decrease of the Tylosin A concentration in the test systems. It should, however, be noted that the concentration of manure particles is low in the test systems compared to the manure tank and that importance sorption increase with increasing concentration of manure particles.

The loss of Tylosin A due to pH related hydrolysis in the manure solutions or varying concentrations of salts in the test medium or sorption was excluded. It is, however, possible that Tylosin A is metabolised by bacteria that *did* survive the pre-treatment of the manure. This last case was not expected, because the manure had been stored for consecutively six months at 5°C and five months at minus 20°C. Additionally, further storage of the manure for one week at −80°C did not change the degradation pattern. This last result was believed to support the assumption that the manure taken from −20°C had no biological activity left. However, the similarity of the degradation patterns

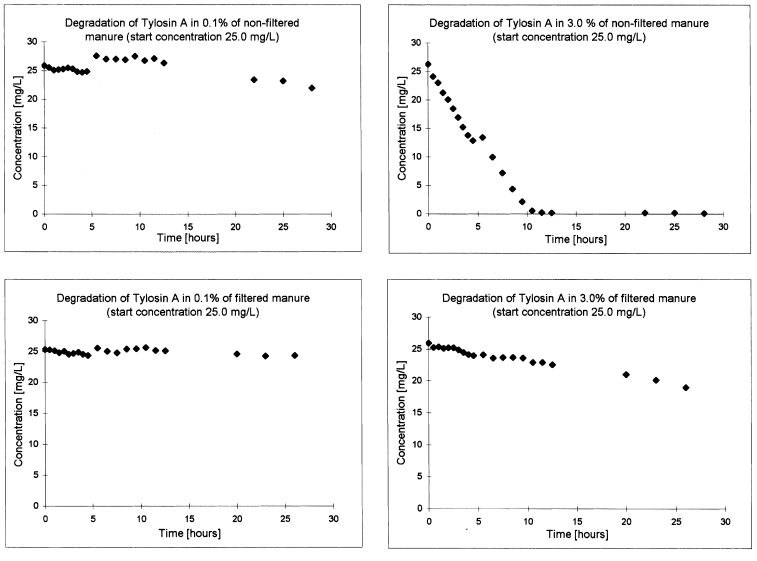

Fig. 4. The decline in the concentration of Tylosin A in test systems as indicated. All samples were initially spiked with 25.0 mg/l of tylosin. Each data point represents the average of four replicates. Samples were kept dark at 20°C during the experiment.

might demonstrate something else, namely that manure from both kinds of storage *still* possesses biological activity, and that not even storage at very low temperatures (−80°C) is enough to eliminate bacteria in manure. Autoclaving of the manure used in the abiotic aerobic degradation tests was not used because it was considered to be a very rough treatment which could lead to an undesirable disruption and change of the manure matrix. In light of the very fast decrease in Tylosin A concentration in the autoclaved methanongenic test systems, it was therefore decided to use another way of deactivating the bacteria in the manure. No test was made to ensure abiotic conditions in the test flasks in the methanogenic test systems and the results may indicate that biomass was present.

4. Conclusion

An HPLC method for the determination of tylosin in the presence of its major residues has been developed and the fate of tylosin manure has been studied under different redox conditions. Aerobic systems were useful for the more physical–chemical studies although the redox conditions in the test systems does not reflect the conditions in a manure tank. The concentration of Tylosin A decrease rapidly under methanogenic as well as aerobic conditions and the half-life in aqueous phase in manure must be expected to be less than two days. Our experiments did not provide us with a final answer to what causes the rapid decrease in Tylosin A. In a manure tank there would be a much higher concentration of particles and colloids in the matrix than in the test systems and sorption to these particles is an important parameter to consider although biotic degradation seams to be most important in the test systems. Thus further studies on sorption of tylosin to the particles in manure are needed. The degradation studies of Tylosin A under aerobic as well as methanogenic conditions indicate that Tylosin B is the major degradation product and that Tylosin D may be a minor degradation product in manure. Further work is in progress to verify the identity of the degradation products of Tylosin A formed in manure.

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