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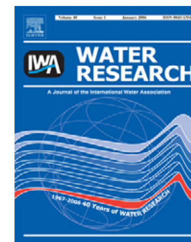

Effect of the presence of the antimicrobial tylosin in swine waste on anaerobic treatment

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ABSTRACT

An anaerobic sequencing batch reactor (ASBR), seeded with a biomass inoculum that previously had not been exposed to the macrolide antimicrobial tylosin (mixture of Tylosin A, B, C, and D), was operated for 3 months with swine waste without Tylosin A and for 9 months with swine waste containing Tylosin A at an average concentration of 1.6 mg/L. When swine waste with tylosin was fed to the ASBR, methane production and volatile solids removal did not appear to be inhibited and a methane yield of 0.47 L methane per gram volatile solids fed to the ASBR was observed. Throughout the operating period, Tylosin A levels in ASBR biomass and effluent were below the detection limit of 0.01 mg/L. However, during the first 3 months of operation, the levels of macrolide–lincosamide–streptogramin B (MLS_B)-resistant bacteria in the ASBR biomass increased substantially as determined by hybridizations with oligonucleotide probes designed to target MLS_B-resistant bacteria. Since no Tylosin A was present in the swine waste during the initial 3 months, the presence of MLSB-resistant bacteria in the swine waste was likely the reason for the increase in resistance. Subsequently, the levels of MLS_B-resistant bacteria in ASBR biomass stabilized with an average of 44.9% for the 9 months of operation with swine waste containing Tylosin A. The level of MLS_B-resistant bacteria in the swine waste fed to the ASBR during this period averaged 18.0%. The results indicate that anaerobic treatment of a waste stream containing tylosin was effective (based on reactor performance) and that the level of resistant bacteria in the ASBR was substantially higher than in the waste stream fed to this system.

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1. Introduction

Antimicrobials have been used extensively during the past 70 years to treat and prevent bacterial infections in humans and animals, and to promote growth in confined livestock animals, such as swine and poultry. As a result of the widespread use of antimicrobials, antimicrobial-resistant microbes have become abundant and the rise in their appearance has become a serious public health concern (Mellon et al., 2001). Antimicrobials and antimicrobial-resistant bacteria are introduced into biological waste treatment systems through their presence in hospital, pharmaceutical, and domestic wastewaters (Ferreira da Silva et al., 2006; Guardabassi et al., 1998) and in animal wastes (Aminov et al.,

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2001; Chee-Sanford et al., 2001). Only a limited number of studies have focused on determining the effects of the presence of antimicrobials and antimicrobial-resistant bacteria on biological waste treatment systems in general and on animal waste treatment systems in particular (Chee-Sanford et al., 2001; Hanzawa et al., 1984; Jindal et al., 2006; Poels et al., 1984; Zilles et al., 2005). In addition, determining the levels of antimicrobials and antimicrobial-resistant bacteria in excess biomass generated by animal waste treatment systems is important since disposal through land application is common, possibly resulting in the contamination of ground and surface waters, soils, and crops with antimicrobials and antimicrobial resistance genes (Chee-Sanford et al., 2001; Jindal et al., 2006; Zilles et al., 2005).

The current study focused on the antimicrobial tylosin (a mixture of Tylosin A, B, C and D), a macrolide consisting of a 16-membered lactone ring. Tylosin belongs to the macrolide–lincosamide–streptogramin B (MLS_B) antimicrobials, some of which are used commonly to treat and prevent infection in the livestock industry (Zilles et al., 2005). Antimicrobials within this group also are used at subtherapeutic levels as feed additives, such as lincomycin (lincosamide) and virginiamycin (streptogramin B) for chicken and turkey, and tylosin for swine and feed cattle (Mellon et al., 2001). Although the antimicrobials within the three classes of MLS_B antimicrobials are chemically distinct, their function and resistance mechanisms are similar. Therefore, they are grouped together as MLS_B antimicrobials and their mechanism of resistance is referred to as MLS_B resistance.

The peptidyltransferase loop of the 23S ribosomal RNA (rRNA), a universally conserved region within the molecule, is the target of action for MLS_B antimicrobials (Weisblum, 1995). Binding of the antimicrobials to this site inhibits protein synthesis. A common mechanism of resistance for most macrolide-resistant strains is target site modification, which consists in a posttranscriptional modification (mono- or di-methylation) of the adenine at position 2058 (A_{2058}, *Escherichia coli* numbering system) in the 23S rRNA by the adenine-N_6-methyltransferase (Andersson and Kurland, 1987; Fluit et al., 2001; Vester and Douthwaite, 1994). This methylation in the 23S rRNA blocks the binding of MLS_B antimicrobials (Leclercq and Courvalin, 1991). Genes encoding enzymes responsible for methylation have been designated *erm* (erythromycin ribosomal methylase). In the present study, we relied on this mechanism of resistance to develop a method that allows for the direct (i.e., without bacterial culturing) determination of the levels of MLS_B-resistant bacteria. While methylation of the 23S rRNA is the major cause of macrolide resistance in animals and humans, it is not the only mechanism of resistance to MLS_B antimicrobials (Douthwaite and Vester, 2000; Nash, 2003; Weisblum, 1995).

The objectives of the current study were to evaluate the effect of the presence of the antimicrobial tylosin and MLS_B-resistant bacteria in swine waste on anaerobic biological treatment efficiency and to quantify the levels of tylosin and MLS_B-resistant bacteria in the biomass from these systems. To carry out these objectives, we fed swine waste without tylosin to a laboratory-scale, high-rate anaerobic sequencing batch reactor (ASBR) for 3 months, then fed swine waste containing tylosin for a 9-month period. The performance of the ASBR, the extent of tylosin degradation, and the level of MLS_B-resistant bacteria were monitored.

2. Materials and methods

2.1. ASBR operating conditions and performance

Diluted swine waste with a concentration of 20 g/L as volatile solids (VS) was treated in a 5-L ASBR (Figure S1, Supplementary information) by sequencing through a feed step (1 min), a react step (23.2 h), a settling step (45 min), and a decant step (2–5 min). Gentle, intermittent mixing was performed by biogas recycling (1 min of biogas recycling every hour at a flow rate of 26 L/h). The temperature of the ASBR was maintained at 25 °C by circulating water through a jacket mounted around the reactor with a heating recirculator (Polyscience, model 210, Niles, IL). At the start of operation, 0.25 L of swine waste was fed per day, which resulted in a volumetric loading rate (VLR) of 1.0 g VS per L reactor volume per day and a hydraulic retention time (HRT) of 20 days. The VLR was increased when the biogas production rate had been stable (fluctuations were less than 5% for weekly averages) for a period corresponding to at least three HRTs. For each stable operating period, at least three samples were obtained from the ASBR before the VLR was increased. The final VLR was 4 g VS/L/d (HRT of 5 days).

The reactor performance was assessed by determining the VS removal efficiency and by monitoring the methane production and total VFA concentration in the effluent. The volumetric methane production rate (VMPR) was obtained by correcting the biogas production (measured with a gas meter, Supplementary information) to standard temperature and pressure (STP) using the ideal gas law and converting this with the wet volume of the reactor and the methane percentage that was present in the biogas. Therefore, the VMPR was expressed as volume of methane per reactor volume per day (L/L/d). The methane yield (at STP) was obtained by plotting pseudo-steady-state daily VMPRs versus VLRs. Least-square linear regression allowed estimation of the slope, which represents the methane yield during the operating period, and is expressed as volume of methane produced per g VS swine waste fed to the digester (L/g VS fed).

The ASBR was inoculated at an initial VS concentration of 20 g/L (VS to total solids [TS] ratio of 0.51) with anaerobic digester sludge from the secondary digester operated by the Urbana-Champaign Sanitary District, Northeast Wastewater Treatment Plant (Urbana, IL) and thus had not been in contact with swine waste.

2.2. Swine waste and other environmental samples

Swine waste was collected once every 1–3 months during and immediately after scraping of finisher swine buildings (University of Illinois at Urbana-Champaign [UIUC] research farms, Urbana, IL). The swine waste was screened through a 1.7-mm screen to remove large debris to prevent clogging of tubing feeding the ASBR, diluted with tap water (City of Urbana, IL) to a concentration of 20 g VS/L (VS to TS ratio of 0.77), and stored at −20 °C in 1-L batches to prevent

degradation. Frozen batches were thawed overnight before feeding.

To quantify MLS_B-resistant bacteria in biomass samples that had not been in contact with tylosin, we also obtained swine waste from an organic farm, which did not use any antimicrobials. The organic farm consisted of indoor/outdoor swine pens with concrete floors. The waste mixture had been outdoors for weeks before collection. Biomass samples were collected from a laboratory-scale anaerobic bioreactor fed sucrose, which was seeded with biomass from an anaerobic bioreactor treating brewery wastewater (Angenent et al., 2002), the activated sludge system of the Urbana-Champaign Sanitary District, Northeast Wastewater Treatment Plant, and secondary anaerobic digesters from the Urbana-Champaign Sanitary District, Northeast Wastewater Treatment Plant (i.e., biomass used to inoculate the ASBR) and the City of Ames, IA, Wastewater Treatment Facility.

2.3. Analytical procedures

TS and VS, soluble chemical oxygen demand (SCOD) levels, total volatile fatty acids (VFA) concentrations, and sludge volume index (SVI) were determined as described in Standard Methods (Clesceri et al., 1998). Prior to analysis, samples for SVI measurements were diluted to a maximum of 10 g TS/L to prevent introducing a bias due to settling hindrance of high solids concentrations. Total ammonia (ammonia+ammonium) concentrations were measured by raising the pH to 11 to convert all ammonium to ammonia, and by subsequently measuring the ammonia concentration using an ATI Orion Model 720A Benchtop pH/ISE meter and an ammonia probe (ATI Orion, Boston, MA). Methane levels in the biogas were determined using gas chromatography (Series 58, Gow-Mac Instruments, Co., Bridgewater, NJ, USA) with thermal conductivity detector (column: $2.4\,m \times 3.2\,mm \times 2.1\,mm$ washed molesieve $13 \times 80/100$ mesh; carrier gas: argon).

Swine waste, biomass, and effluent samples were obtained and stored at $-20\,^{\circ}C$ during the study and then processed concurrently for Tylosin A quantification. Tylosin A is a specific chemical form of tylosin, which constitutes the major component of the tylosin mixture (approximately 90% (Loke et al., 2000)). Ethanol (0.5 mL) was added to 0.5 mL of sample in 1.5 mL polypropylene centrifuge tubes. The mixture was incubated for 15 min at $30\,^{\circ}C$ in an ultrasonic bath (Branson Ultrasonics Corp., Danbury, CT), which was operated at a relative power intensity of 75 units. Samples were centrifuged at 10,000*g* and the supernatants were transferred to pyrex test tubes. This step was repeated twice; biomass pellets were resuspended in 1 mL of distilled deionized water (ddH_2O) containing 50% (vol:vol) ethanol plus 3 mM ammonium acetate (pH 5.5). The pooled supernatant fractions were dried under vacuum (−80 kPa). The dried fraction was resuspended in 0.5 ml of 50% acetonitrile:H_2O, which contained 4.5 mM ammonium acetate (pH 5.5). Resuspended samples were analyzed by high-performance liquid chromatography (Thermo Finnigan P4000/AS3000, Waltham, MA) electrospray ionization mass spectrometry (Thermo Finnigan LC-Duo; LC-MS) (Zahn et al., 2001). Each sample was injected three times and the data reported represent the mean values for these injections. Tuning of the mass spectrometer was performed daily with reference standard grade tylosin (lot RS0193, Eli Lilly and Co., Indianapolis, IN) at a concentration of 0.1 mg/L. Analyte concentration was determined in the positive ion mode through integration of peak area across a range from 916 to 917 *m*/*z* for Tylosin A ($916.4 = [M+H]^+$) and 406.5–407.5 *m*/*z* for dehydroxy-tylonolide ($407.1 = [M+H]^+$). This putative tylosin metabolite is composed of a lactone ring that is lacking the mycaminose, mycarose, and mycinose sugar moieties. A calibration curve for Tylosin A was generated using five individual injections of the following concentrations: 20, 10, 0.50, 0.10, 0.05, 0.01 mg/L. The R^2 value for the 30 injections was 0.997, and the plot of the residuals for this analysis showed a normal distribution around the fitted line, indicating that there was no concentration-related bias in the detector response. The detection limit of Tylosin A was 0.010 mg/L.

2.4. Half-life experiment for Tylosin A

To perform anaerobic batch studies, effluent samples (1 mL) of the ASBR (9.1 g VS/L-day 249) were added into capped 5 mL glass serum vials (Supelco, Bellefonte, PA), which were pre-purged with oxygen-free nitrogen gas. The vials were placed in a water-bath shaker (Model G76, New Brunswick Scientific, Edison, NJ) at $25\,^{\circ}C$ with gentle shaking (80 rpm; 2.5 cm stroke length). Ten μL of 10% (vol:vol) dimethylsulfoxide (DMSO):H_2O solution containing 10.0 μg of reference grade tylosin (lot RS0193) was injected into each vial to reach a final concentration of 10 mg/L, and the contents of the vial were mixed. Final concentrations of DMSO of 0.05% and 0.2% (vol:vol) resulted in similar (±4.3%) degradation rates, which indicated that DMSO did not directly impact the rate of degradation. After injecting tylosin, a 50 μL sample was immediately removed from the vial using a glass syringe and injected into a glass 2 mL micro-reaction vessel (Supelco, Bellefonte, PA). Subsequently, 500 μL of ethanol was added to the micro-reaction vessel to stop the reaction, which did not influence the concentration of Tylosin A greatly (<0.8% change). All samples from the half-life experiment were analyzed immediately after the experiment was conducted with a maximum time period to the actual analysis of 8.4 h. Similarly, subsequent samples were removed from the vial at 0.5, 1.0, 2.0, 3.0, 5.0, 10.0, 24.0, and 48.0 h after tylosin addition. The rate constant and half-life of Tylosin A biodegradation were estimated by assuming first-order kinetics.

2.5. Quantitative hybridizations

ASBR biomass samples or environmental samples were added to 2.2 mL screw-cap microcentrifuge tubes, the tubes were centrifuged at 2000*g* at $4\,^{\circ}C$ for 2 min, and the supernatant was removed. Samples were frozen immediately in an ethanol-dry ice bath and stored at $-80\,^{\circ}C$ until RNA extraction. A low-pH hot-phenol extraction method was used to isolate RNA from the biomass (Raskin et al., 1994; Stahl and Amann, 1991).

To detect and quantify the abundance of MLS_B-resistant strains, we developed oligonucleotide probes that can differentiate between the methylated and non-methylated A_{2058} in

the peptidyl transferase loop of the 23S rRNA, and thus between resistant and sensitive strains, respectively. Mono- or di-metlylation of A_{2058} disrupts the ability of thymidine (T) in probe L-*-Bact-2053-a-A-13 (5′-GGGTCTTTCCGTC-3′) to form hydrogen bonds, causing a mismatch in the DNA:RNA hybrid of resistant strains. Thus, this probe detects the 23S rRNA of wild-type or sensitive strains (i.e., 23S rRNA with non-methylated A_{2058}). A second probe L-*-Bact-2053-b-A-13 (5′-GGGTCTT5CCGTC-3′) detects the 23S rRNA of virtually all bacteria (those with a conserved peptidyltransferase loop) regardless of mono- or di-methylation of A_{2058}. In this second probe, the thymidine is replaced by 5-nitroindole, a nucleotide that binds almost indiscriminately with all natural nucleotides as well as with methylated nucleotides (Loakes and Brown, 1994). The two-step hybridization approach consisted in (i) blocking non-methylated target 23S rRNA by hybridizing with the unlabeled probe L-*-Bact-2053-a-A-13 and (ii) quantifying the hybridization signal after hybridization with the ^{32}P-labeled probe L-*-Bact-2053-b-A-13 to measure the abundance of MLS_B-resistant strains exhibiting *erm*-type modifications. The total 16S rRNA was quantified with a ^{32}P-labeled universal probe S-*-Univ-1390-a-A-18 (Zheng et al., 1996). Results were expressed as the percentage of resistant ribosomes in the total community, because a 1:1 ratio of 16S rRNA to 23S rRNA can be assumed.

The hybridization protocol described by Raskin et al. (1994) was changed slightly for the quantification of MLS_B resistance. After pre-hybridization at 40 °C for 12 h, hybridization was performed at 40 °C for 12–18 h with 5 nM of the unlabeled wild-type probe (L-*-Bact-2053-a-A-13). Next, the membranes were washed for 15 min at 35 °C in wash buffer (0.1% sodium dodecyl sulfate [SDS] and 1.67 × SSC [0.20 M NaCl, 0.020 M sodium citrate]). Immediately after washing, hybridization was performed at 35 °C for 12–18 h with 0.5 nM of ^{32}P-labeled probe L-*-Bact-2053-b-A-13. Then, the membranes were washed twice for 1 h each at 35 °C and once for 30 min at 35 °C. Hybridization signals were quantified using an Instant Imager (Packard Instruments, Meriden, CT) and the abundance of resistant strains was expressed as a percentage of the total 16S rRNA. Temperature of dissociation (T_d) and specificity studies for both probes were performed with resistant and non-resistant strains listed in Tables S1 and S2 (Supplementary materials).

3. Results and discussion

3.1. Tylosin A concentration in swine waste fed to ASBR

When Tylan 40 Premix is used as a feed additive for swine finishing operations, it generally is applied at rates between 113 and 227 g of Tylan 40 Premix per 907 kg of dry feed, which results in a tylosin concentration of 10–20 g of tylosin per 907 kg of feed (Editors of Feedstuffs, 1998). When Tylan 40 Premix was used at the UIUC research farm from which swine waste was collected as the substrate for the ASBR, it was used at the upper limit of this range (227 g of Tylan 40 Premix per 907 kg of dry feed, resulting in 20 g tylosin per 907 kg of dry feed). Tylan 40 Premix used by the UIUC research farm contained 88.1% Tylosin A, 6.4% Tylosin C (902.5 = $[M+H]^+$), and 5.5% Tylosin D (918.4 = $[M+H]^+$). The chromatographic retention for Tylosin A and its degradation product dehydroxy-tylonolide were 22.5 ± 0.04 and 18.10 ± 0.03 min, respectively.

Tylosin A and dehydroxy-tylonolide were present in four of the five swine waste batches that were tested and fed to the ASBR. During the first 69 days of ASBR operation, the concentrations of Tylosin A and dehyhroxy-tylonolide in swine waste 1 (SW1) were below the detection limit of the method (0.01 mg/L). The mean concentration of Tylosin A in the swine waste fed during the remainder of the operating period was 1.6 ± 0.5 (standard error [SE]; $n = 4$) mg/L (Table 1). The presence of dehydroxy-tylonolide in the swine waste indicates that Tylosin A was degraded in the intestinal tract of the swine or in the swine waste collection and distribution system of the confinement building.

3.2. Performance of anaerobic treatment of swine waste with and without tylosin

The ASBR was operated for 374 days by feeding swine waste at increasing VLRs (Fig. 1a). Tylosin A was not present in the swine waste (SW1) during the first 69 days of operation, but was present thereafter until the end of the operating period (SW2–SW5). Startup performance was as expected with initial increases in VFA and ammonia concentrations (Fig. 1c and d). The switch from feeding swine waste without Tylosin A to swine waste with Tylosin A at an average concentration of 1.6 mg/L was not noticeable in the performance data and the trends towards stable performance continued when feeding with swine waste with tylosin was initiated (Fig. 1).

A pseudo-steady-state VMPR for the target VLR of 4 g VS/L/d was reached by day 350 (Fig. 1a). Biomass settleability is one of the most important factors for a high-rate anaerobic system, such as an ASBR, in which a much longer SRT than HRT is maintained through the settling step in each ASBR cycle (Dague et al., 1970; Dague and Pidaparti, 1992; Sung and Dague, 1995; Zhang et al., 1997). The ASBR biomass settleability initially was relatively poor as demonstrated by an SVI of 56 mL/g TS at startup (Fig. 1b) and an initial decrease in the VS concentration (Fig. 1b). At the end of the operating period (day 372), the SVI and biomass levels had improved greatly (17 mL/g TS and 26 g VS/L, respectively), because of the ability of the ASBR to select for well-settling biomass.

The ASBR maintained a relatively constant methane yield of 0.47 L CH_4/g VS fed during pseudo-steady-state conditions (Fig. 2) with a VS removal efficiency >50%. Chen (1983) estimated the ultimate methane yields (i.e., methane yield at an infinite HRT) with waste from swine fed a corn-based diet to be between 0.44 and 0.52 L CH_4/g VS fed, regardless of the operating temperature. The methane yield in the current study was close to the ultimate yields determined by Chen, likely because we used high-rate anaerobic digestion with a solids retention time (SRT) much larger than the HRT of 5–20 days. Thus, the feeding of swine waste containing Tylosin A at an average concentration of 1.6 mg/L did not appear to impact the performance of swine waste digestion based on the observed high methane yields and high VS removal

Table 1 – Levels of MLS_B resistance and Tylosin A and its degradation product for swine waste, ASBR, and environmental samples

Samples	MLS_B resistance (% of total 16S rRNA)	Tylosin A conc. (mg/L)	Presence of dehydroxi-tylonolide (Yes [Y] and No [N])
UIUC-farm swine waste (SW1), fed day 0–69	46.5	<0.01	N
UIUC-farm swine waste, not fed to ASBR	20.7	NA	NA
UIUC-farm swine waste (SW2), fed day 70–142	12.9	0.92	Y
UIUC-farm swine waste (SW3), fed day 143–201	15.3	2.10	Y
UIUC-farm swine waste (SW4), fed day 202–213	NA[a]	1.46	Y
UIUC-farm swine waste (SW5), fed day 214–270	16.4	1.82	Y
UIUC-farm swine waste (SW6), fed day 271–331	25.8	NA	NA
UIUC-farm swine waste (SW7), fed day 332–373	19.4	NA	NA
Mean of UIUC-farm swine waste samples	22.4 ± 11.4 (SE; $n = 7$)[b]		
Effluent ASBR, day 203	NA	<0.01	N
Biomass ASBR, day 211	45.2	<0.01	N
Effluent ASBR, day 211	NA	<0.01	N
Biomass ASBR, day 226	47.5	<0.01	Y
Effluent ASBR, day 226	NA	<0.01	Y
Biomass ASBR, day 249	38.7	<0.01	N
Effluent ASBR, day 249	29.8	<0.01	Y
Organic-farm swine waste	24.6	<0.01 [$n = 2$]	N
UIUC-farm lagoon sludge	29.3	NA	NA
Urbana-Champaign digester sludge, Inoculum ASBR	4.3	<0.01 [$n = 2$]	N
Ames digester sludge	12.4	NA	NA
Urbana-Champaign activated sludge	12.8	NA	NA
Urbana-Champaign activated sludge	10.7	NA	NA
Anaerobic granular biomass 1	13.6	NA	NA
Anaerobic granular biomass 2	8.4	NA	NA
Anaerobic granular biomass 3	8.2	NA	NA
Mean of samples without tylosin exposure	10.1 ± 3.3 (SE; $n = 7$)		

[a] NA = not available.
[b] Mean for MLS_B resistance in swine waste samples fed from days 70–373 was 18.0 ± 5.0 (SE; $n = 5$).

efficiencies. This observation is consistent with results obtained in two studies that evaluated the impact of tylosin addition (up to 16.7 mg/L) on anaerobic treatment of swine manure (Massé et al., 2000; Poels et al., 1984). However, a decrease in methane production was reported by Loftin et al. (2005) after addition of tylosin (1–25 mg/L) to anaerobic bioreactors and Shimada et al. (accepted) observed a decrease in the rates of methane production and propionate uptake when tylosin was added to an ASBR at a concentration of 1.7 mg/L, while the total methane production did not change in their study. When tylosin was added at high concentrations (25–250, >400, or 167 mg/L) to anaerobic bioreactors, decreased treatment performance was reported (Chelliapan et al., 2006; Sanz et al., 1996; Shimada et al., accepted).

3.3. *Degradation of Tylosin A during anaerobic digestion*

The Tylosin A concentrations in the biomass and effluent of the ASBR were below the detection limit (Table 1), suggesting that Tylosin A was degraded during anaerobic digestion. In addition, the degradation product dehydroxy-tylonolide was found in three of the seven samples analyzed (Table 1). To estimate the rate constant for the half-life of Tylosin A (Fig. S2, Supplementary information), the Tylosin A concentration was measured to be 5.8 ± 0.4 mg/L immediately after tylosin addition to biomass from ASBR effluent and a brief mixing event. The Tylosin A concentration decreased further during a period of 48 h after which the concentration of Tylosin A was just above the 0.01 mg/L detection limit. The decrease in tylosin concentration during the 48 h incubation was accompanied by an increase in a putative tylosin breakdown product, dehydroxy-tylonolide ($407.1 = [M+H]^+$). Based on the results of this batch experiment, we estimated the half-life for Tylosin A in anaerobic treatment systems to be 2.49 h. Given the relatively short half-life, the removal of Tylosin A in the ASBR was expected since the HRT of the ASBR varied between 5 and 20 days. This is in agreement with Zilles et al. (2005), who were unable to detect tylosin in several swine waste treatment processes despite its subtherapeutic usage at the farms included in their study.

We investigated whether the decrease in Tylosin A concentration was due to abiotic or biotic degradation, or sorption. Tylosin has a high distribution coefficient that favors sorption to organic and clay-containing materials in soils or animal manure (Ingerslev and Halling-Sørensen, 2001; Kolz et al., 2005b; Rabolle and Spliid, 2001). Sorption is reversible,

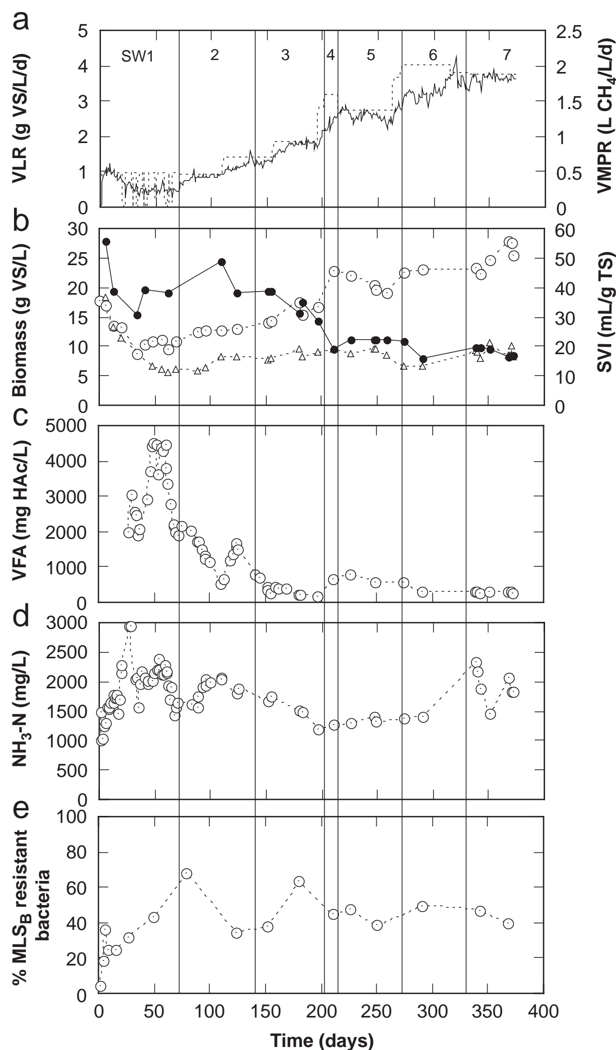

Fig. 1 – Performance and MLS_B resistance levels for ASBR operated for 374 days. Vertical lines separate periods with different batches of swine waste as indicated in Table 1. During the first 70 days of operation, the tylosin concentration in the swine waste fed to the ASBR was below the detection limit. (a) Volumetric loading rate (VLR) (dotted lines) and volumetric methane production rate (VMPR) (straight lines); (b) biomass levels in ASBR (⊙) and effluent (△) and sludge volume index (SVI) of biomass (●); (c) total volatile fatty acid (VFA) level in effluent; (d) total ammonia–N concentration in effluent; and (e) relative levels of MLS_B-resistant bacteria expressed as a percentage of resistant 23S rRNA to the total 16S rRNA.

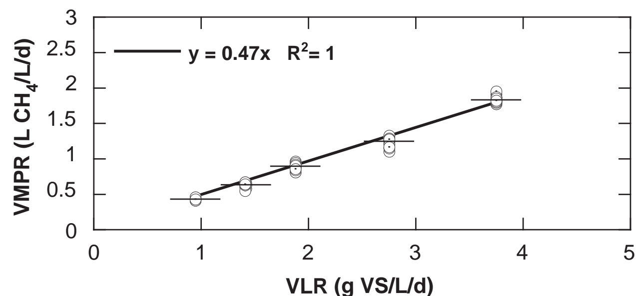

Fig. 2 – Methane yields at pseudo-steady-state conditions. The horizontal bars represent the average daily volumetric methane production rate (VMPR) during pseudo-steady-state conditions.

however, securing mineralization of tylosin in soil columns (tylosin half-life 3.3–8.1 days) (Ingerslev and Halling-Sørensen, 2001). Kolz et al. (2005a) found that 90% of Tylosin A in anaerobic sludge was rapidly sorbed and degraded (either abiotic or biotic) within a period of 5 days. By using a selective and sensitive means of detection using electrospray mass spectrometry, we determined a recovery efficiency of 92±3% and 98±1% for triplicate analyses of ASBR biomass to which 0.1 or 1.0 mg/L of tylosin was added. These high recovery efficiencies, combined with the observed removals of tylosin, indicate that tylosin degradation rather than sorption explained the removal in the anaerobic batch experiment and the ASBR. The appearance of dehydroxy-tylonolide in the anaerobic batch experiment and in the ASBR further support the conclusion that tylosin is degraded in the ASBR. The shorter Tylosin A half-life of ~2.5 h in biomass from our high-rate anaerobic digester compared with the longer half-lives of 2–8 days in soils or passively stored manure (Ingerslev and Halling-Sørensen, 2001; Loke et al., 2000; Rabolle and Spliid, 2001) may indicate enrichment of microbes capable of tylosin degradation in digesters.

3.4. *MLS_B resistance in ASBR biomass*

The relative levels of MLS_B-resistant bacteria in ASBR biomass increased substantially during the first 69 days of operation (Fig. 1e). Since the inoculum (anaerobic digester sludge) contained low levels of MLS_B-resistant bacteria (Table 1), a considerable increase was anticipated simply due to the addition of resistant bacteria from the swine waste. Indeed, this was the likely mechanism by which the MLS_B-resistant bacteria became enriched because during days 0–69 of the operating period the level of MLS_B-resistant bacteria in the swine waste (SW1) fed to the ASBR was determined to be 46.5% ($n = 1$), while the level of Tylosin A was below detection limit. Furthermore, subsequent research with an ASBR fed a synthetic substrate without MLS_B-resistant bacteria at a tylosin concentration of 1.7 mg/L did not result in an increase in the levels of MLS_B-resistant bacteria in ASBR biomass (Shimada et al., accepted). After the initial period of 69 days, the percentage of MLS_B-resistant bacteria in ASBR biomass stabilized to 44.9±8.7% (SE; $n = 9$). These levels of MLS_B-resistant bacteria were considerably higher than those in the swine waste fed during the period between days 70 and 373 of the operating period (18.0±5.0%; SE; $n = 5$). The gradual increase in the VLR did not appear to have an impact on the levels of MLS_B-resistant bacteria. Furthermore, there was no apparent correlation between changes in reactor performance and changes in MLS_B resistance (Fig. 1).

3.5. *Background levels of MLS_B resistance*

To determine background levels of MLS_B-resistant bacteria, we analyzed a variety of biomass samples from biological

waste treatment systems with limited exposure to antimicrobials, including anaerobic granular sludge from a laboratory-scale anaerobic bioreactor fed sucrose, activated sludge from a domestic wastewater treatment system, and biomass from anaerobic digesters from two different domestic wastewater treatment plants. These samples contained lower levels of MLS_B-resistant bacteria (mean = 10.1 ± 3.3%; SE; $n = 7$) compared with biomass from the ASBR fed swine waste with tylosin (Table 1). We also collected swine waste from an organic farm. The level of MLS_B-resistant bacteria in the organic farm sample (24.6%; $n = 1$) was higher than the mean level of MLS_B-resistant bacteria in waste from swine that were fed a diet containing tylosin (22.4 ± 11.4%; SE; $n = 7$; Table 1). However, swine waste from the organic farm did not contain Tylosin A nor its breakdown product dehydroxitylonolide (Table 1).

Since the samples analyzed were taken from systems that were not studied in detail (e.g., concentrations of antimicrobials were not determined, history of the bioreactors was not evaluated), we can only speculate on the reason(s) for the occurrence of relatively high levels of MLS_B resistance. Jindal et al. (2006) and Zhou (2007) also found high levels of MLS_B resistance in some organic farms and attributed this to the co-selection of tetracycline genes (*tet* genes) and MLS_B resistance genes (*erm* genes). Dual resistance for MLS_B antimicrobials and tetracyclines was observed in a prevalent bacterial population in swine manure from an organic farm and several tetracyclines were detected in the same sample (Zhou, 2007). These observations, together with the documented co-localization of *tet* and *erm* genes in common transposons (Cochetti et al., 2007), suggest that the relatively high levels of MLS_B resistance at the organic farm may be due to the presence of antimicrobials other than MLS_B antimicrobials (i.e., tetracyclines). Even though the levels of MLS_B resistance for the other environmental samples were lower than for the swine waste from the organic farm, a similar explanation seems plausible for the biomass samples obtained from domestic wastewater treatment plants since a variety of antimicrobials have been detected in domestic wastewater (Yang et al., 2005).

3.6. *Implications for land application of biosolids*

The levels of MLS_B-resistant bacteria in biomass from an ASBR operated for swine waste treatment were considerably higher than those in the swine waste. Further work is needed to determine if resistant bacteria persist in the environment after biomass from ASBRs or other biological treatment systems is land applied for soil improvement, and if resistance can be transferred in this soil environment. During such studies, it is important to consider that ~55% VS reduction is achieved in ASBR treatment of swine waste. Therefore, the amount of resistant bacteria introduced into the environment after swine waste treatment may be lower than the amount of resistant bacteria disposed during direct application of raw swine waste. On the other hand, MLS_B-resistant bacteria enriched in the ASBR biomass also may have acquired other types of resistance genes (e.g., for other antibiotics, metals, pesticides). Thus, the concern that bacteria from the ASBR biomass would transfer multiple resistance genes to native bacterial populations in the soil deserves to be addressed.

4. Conclusions

Tylosin A with an average concentration of 1.6 mg/L in swine waste was degraded rapidly when fed to a high-rate anaerobic digester (ASBR), resulting in Tylosin A levels below detection in digester effluent. Batch studies with ASBR biomass showed a Tylosin A half-life of ~2.5 h.

A substantial increase of MLS_B resistance in ASBR biomass was shown at the initial stages of the operating period. Subsequently, during a 7-month operating period, the MLS_B resistance levels in ASBR biomass averaged 44.9 ± 8.7% ($n = 9$), which was substantially higher than the MLS_B resistance levels in the swine waste fed during this period (18.0 ± 5.0%). Levels of MLS_B-resistant bacteria in biomass samples from bioreactors with limited exposure to antimicrobials averaged 10.1 ± 3.3%.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.watres.2008.01.005

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