Changes in Performance and Active Microbial Communities Due to Single and Multiple Effects of Mixing and Solid Content in Anaerobic Digestion Process of OTC Medicated Cattle Manure

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Abstract- Oxytetracycline (OTC) is used in animal husbandry which is poorly metabolized and excreted in manure. In this study, effect of two operational parameters (mixing rate and solid content) and presence of OTC on dynamics of active microbial populations were investigated along with inhibition on biogas production and elimination of OTC. Anaerobic digester studies were carried out batch-wise in 1000 ml digesters with active volume of 600 ml for 30 days. Two different sets were conducted by diluting blank and medicated manures to 5-6% and 8-9% Volatile solid (VS) content. Inoculum seed from a manure digester was added at a ratio of 1:10. Manure slurries were digested then at mixing rates of 90 rpm and 120 rpm at 37^{o}C. In all digesters, VS content decreased by 30%-50% after 20 days as biogas yields of medicated manure digesters decreased by 14%-24% in respect to control digesters after 30 days. Decrease in biogas yield was lower (14%-17%) in high VS content digesters compared to decrease in low VS content digesters (20%-24%). Methane content of biogas was determined as 62±3% in all digesters. OTC concentration decreased by 55-70% indicating a half life of 22-27 days during anaerobic digestion. FISH analyses showed an increase in activity with time and mixing rate. Activity of microbial cells were decreased with increased VS content and presence of OTC. Digesters were dominated by hydrogenotrophic methanogens where acetoclastic methanogens were represented by *Methanosarcina* spp.

Keywords- Oxytetracycline, FISH, biogas, mixing rate, solid content

1. Introduction

Antibiotics were discovered in 1900s and since then they are used extensively on treatment of both humans and animals. Most of antibiotics leave animal body without or slightly changed (60-90%) in form of urine and manure [1]. Oxytetracycline (OTC) is a widely used antibiotic in animal husbandry which is favored by its low cost, low side effects and broad range of target microorganisms. OTC is a protein synthesis inhibitor effective on both gram positive and negative bacteria

Anaerobic digestion of biomass is a well established technology and an efficient renewable energy source. Animal manure is considered as main feedstock for digesters especially in rural areas. Extensive use of antibiotics for therapeutic and prophylactic purposes is the main reason of their presence in manure digesting systems. There are many studies giving valuable information on inhibitory effect of antibiotics on system performance, however, studies focusing antibiotics and its effect on microbial communities

in anaerobic manure digesters are rather scarce [2.] Also no studies showing relation of OTC inhibition and operational parameters has been seen in the literature.

The objective of this study is, therefore, to monitor dynamics of active microbial populations in OTC medicated manure and relation of inhibition with operational parameters like mixing rate and volatile solid content.

2. Materials and Methods

2.1. Animal Medication and Manure Sampling

Manure samples of Holstein race (2,5-3,5 years old, 400-500 kg body mass) dairy cows (n:8) which were kept in a barn belonged to Faculty of Veterinary Medicine, Istanbul University, were used as substrate for digester experiments. Manures in rectum were collected and stored at 4 °C until future use as control manure. The cows were then medicated once with Oxytetracycline injection solution (20mg OTC/kg bodyweight) according to the standard dosage in veterinary practice. Equal doses were injected to right and left body sides between *musculus semitendinosus and musculus semimembranosus* muscles. Manures were collected from rectum every 24 hours for 5

days and mixed together until homogenous state has been established. This mixed manure was then labeled as "medicated manure" and used throughout the experiments. Collected manure samples were stored in 1 L containers and brought to laboratory in cold chain. Until the experiments, samples are kept in $+4^{o}$C.

2.2. Chemicals and OTC detection

Oxytetracycline was purchased from Acros Organics N.V (NJ, USA). HPLC grade chemicals were obtained from Merck (NJ, USA). Extraction of antibiotic was performed as described elsewhere [3] with slight modifications. The HPLC instrument (Schimadzu LC-10 AD) was equipped with an UV detector; (UV VIS Detector, SPD 10-A), a degasser (DGU-14A) and a system controller

Table 1. Operational parameters changing in digester setups

Digesters	Temperature	Mixing rate (rpm)	Volatile Solid content (%)
Set 1 S1 (Control)	37^{0}C	90	5-6%
Set 1 S3			
Set 1 S4 (Control)			8-9%
Set 1 S6			
Set 2 S1 (Control)	37^{0}C	120	5-6%
Set 2 S3			
Set 2 S4 (Control)			8-9%
Set 2 S6			

(SCL-10A). The column used in this study was Inertsil ODS-3 HPLC column, (25 cm x 4.6 mm). Degassing of the solvents was achieved by sonication in a transonic ultrasonic bath (ELMA D-78224, Singen/Htw) prior to use. The mobile phase consisted of 75% 0.1 M oxalic acid buffer and 25% Methanol:Acetonitrile (1:1.5) solution which was delivered isocratically at a flow rate of 1 ml/min. Total run time was 20 min. Wavelength for the detection of OTC was 357 nm, at which the retention time was 8.4±0.8 min. All results were analyzed by the system software, LC Solutions (Schimadzu Scientific Instruments Inc., MD, USA). In order to determine extraction efficiencies, triplicate samples of non-medicated manure were spiked with different OTC concentrations (5, 20, 200, 1000 mg/L) and incubated for 3 hours and extracted as described above. Recovery results were calculated as a means of triplicate samples at each concentration.

2.3. Anaerobic Digester Setups

Experiments were carried out batch-wise in 1000 ml digester for 30 days with active volume of 600 ml. Manure samples were diluted to a Volatile Solids (VS) concentration of 5-6% and 8-9%. Seed from a lab-scale manure digester was added at ratio of 1:10. Four digesters marked as Set1 and two control and two medicated manure (two having VS conc. of 5-6% and two having VS conc. of 8-9%) were digested at mixing rate of 90 rpm, other four digesters at 120 rpm (Table 1.). Biogas production was measured and samples for physical, chemical, analytical and molecular analysis were collected every 10 days.

2.4. Monitoring of Active Microbial Population Dynamics

Every ten days of operation, 5 ml sample from serum bottles were transferred into sterile containers, diluted with absolute ethanol (1:1, v/v) and fixed according to a protocol described previously [4]. Probes used, hybridization and visualization of samples were carried out according to a previous study [5], except that 50-75 times dilution of the fixed samples were spotted on teflon coated slides instead of gelatin coated ones.

2.5. Statistical Analysis

Statistical analyses were carried out with SPSS Inc. 11.5 for windows (Chicago, USA) to explain relations between microbial populations, OTC level and environmental parameters.

3. Results and Discussion

3.1. OTC Concentrations in Digesters

In this study, two sets of anaerobic digesters were prepared with OTC medicated cattle manure in two different VS concentrations and operated in two different mixing rates. Inhibitions caused by OTC concentration were monitored as decrease in biogas yield throughout the study.

HPLC analysis was used to determine OTC concentration during operation. OTC concentrations were measured in range of 1.1-3.4 mg/l at the start up. During operation OTC concentrations were gradually decreased and after 30 days of operation it has been determined a decrease in detected OTC level in range of 55-73% of the initial OTC concentration. Therefore it has been calculated a half life of 28 and 22 days for low mixing and high mixing rate digesters, respectively (Table 2.)

Concentration of tetracyclines in manure samples are affected by various factors like type of medication and antibiotic, species of the animal, diet and age of the animal etc.[6].Therefore they can be measure in a wide range of concentrations (0,1-173 mg/kg)[7]. Arikan and his co-workers [2] measured 3,1 mg/l OTC in manure slurries of calves which are fed orally with OTC. In another study 5,88 mg/kg OTC concentration has been monitored in pig manure samples collected from different farms[8].

Table 2. Concentrations, elimination and half-life of OTC throughout the study

Digester	OTC concentrations at operation day (mg/l)				Elimination (%)	Half life (day)
	0^{th} day	10^{th} day	20^{th} day	30^{th} day		
Set 1 S3	2,2	1,6	0,8	1,0	55	28
Set 1 S6	3,4	2,6	1,3	1,5	56	27
Set 2 S3	1,1	0,9	0,8	0,4	64	24
Set 2 S6	2,2	2	1,1	0,6	73	21

Digestion of animal manure before its disposal not only helps recovering energy but also promotes elimination of OTC [9]. In study of Arikan and coworkers [2] a half life of 56 days has been found in anaerobic digestion of manure slurry with VS content of 4%. In our study, lower half-lives have been observed as the VS content increased. Half-lives of OTC in anaerobic digestion are lower than the half life of OTC in soil where half life was found to be 105 days [10].

3.2. Effects of Mixing Rate, Solid Content and OTC Concentration on Biogas Production

Relation between selected operational parameters with biogas yield was investigated along with the inhibitory effect of OTC. Biogas and methane yield of digesters at two different operation points are shown in Table 3. The results indicate that mixing rate and solid content do not have a significant effect in control digesters. Mixing rate and solid content are negatively related each other since high solid content decreases mixing rate by increasing viscosity of the slurry. A recent study showed that effects of different mixing rates and types on biogas yield vary and was different among each other [11]. Although solid content do not affect biogas yield, it has a slight positive effect on methane yields. Methane yield of high VS digesters are slightly higher than low VS digesters due to higher methane content in produced biogas.

Table 3. Biogas yields (L/kgTVS) and inhibitions caused by OTC presence in digesters

Digester	20 days of operation		30 days of operation		Inhibition (%)
	Biogas Yield	Methane Yield	Biogas Yield	Methane Yield	
Set 1 S1 (Control)	109	53	121	75	
Set 1 S3	75	40	92	55	24
Set 1 S4 (Control)	100	60	119	76	
Set 1 S6	83	47	95	62	20
Set 2 S1 (Control)	92	39	118	70	
Set 2 S3	85	39	102	60	14
Set 2 S4 (Control)	90	41	118	73	
Set 2 S6	73	31	98	63	17

Statistical analysis show a negative relation between OTC and biogas yield ($r=-0,366$ $p<0,01$). Operational parameters have slight effect on OTC inhibition. Biogas yields were decreased by 20-24% in low mixing rate digesters and by 14-17% in high mixing rate digesters in respect to control digesters. This result may indicate mixing rate itself decreased OTC inhibition by 30-40%. Studies showing OTC inhibitions have similar results as 3.1 mg/L OTC in calf manure causing 27% inhibition in biogas yield in the study of Arikan and co-workers [2].

3.3. Effects of Mixing Rate, Solid Content and OTC Concentration on Process Stability

Process stability is an important aspect of an efficient biogas digester and can be monitored by different control parameters like pH, VFA concentration, biogas composition, ammonia and sulfide level etc. It is crucial to understand the relation between operational and control parameters.

Table 4. Values of TS, VS and decrease in VS

Digester	Operation time (Day)	Total Solid (%)	Volatile Solid (%)	Decrease in VS (%)
Manure		16 -18	13 -16	
Set 1 S1 Control	0	6,9	5,8	48
	20	4	3	
Set 1 S3	0	7,4	6,2	44
	20	4,5	3,5	
Set 1 S4 Control	0	10,8	8,9	39
	20	6,8	5,4	
Set 1 S6	0	11,1	9	34
	20	8,6	6,5	
Set 2 S1 Control	0	6,4	5,4	35
	20	4,7	3,8	
Set 2 S3	0	6,1	5	30
	20	4,7	3,8	
Set 2 S4 Control	0	9,3	7,9	33
	20	7	5,9	
Set 2 S6	0	9,5	7,9	29
	20	7,3	6	

In this study, VS elimination, biogas composition and VFA accumulation have been used as control parameters to monitor process stability. Biogas composition does not show a significant difference in digesters showing inhibition. Biogas produced in all digesters have 62±3% CH4 and 38±3% CO_2. Although accumulation of acetic and propionic acid has been monitored on low levels at start up period, those concentrations do not inhibit system [12]. No VFA accumulation has been observed after 10^{th} day of operation in all digesters. Decrease in VS content was slightly higher in low mixing digesters to high mixing digesters (34-48% and 29-34%, respectively) and it shows itself in biogas yield. Decrease in VS content and biogas yield seems not relate to each other since 14-24% decrease in biogas yield only reflected as 4-5% decrease in VS content.

3.4. Changes in Active Microbial Populations of Digesters

Effects of changing operational parameters and OTC presence on dynamics of active population were determined by FISH (Figure 1.). Use of FISH technique for identification of microorganisms in manure digesters is a proven technique [13, 14]. But these studies mainly focus on general digester populations rather changes in it.

In our study, groups of *Methanomicrobiales*, *Methanobacteriales and Methanosarcinales* were targeted as methanogens. *Methanosarcina* spp. was dominated group *Methanosarcinales* which was previously reported in a similar study [14]. In our study, we observed a dominance of hydrogenotrophic methanogens especially *Methanobacteriales* over acetoclastic ones. In study of Karakashev and co-workers [14], 9 manure digesters and 6 biosludge digesters were investigated by FISH technique for archaeal community structures. Study revealed two different structures for two type of feedstock. Archaeal diversity of manure digesters mainly composed of hydrogenotrophic methanogens and group *Methanosarcinaceae*. It has been found that acetate utilizing methanogens are more sensitive to ammonia concentrations, therefore they are abundantly present biosludge digesters rather than manure digesters.

Statistical analyses performed on output of FISH studies showed relation between activity of microbiota and different environmental factors. Analysis on activity and time showed that activity increased significantly after 20 days of operation ($p<0.05$ r=0,240). Analysis on OTC revealed that control digester harbored more active cells than OTC containing digesters. Microbial activity is higher in low VS digesters than high VS digesters. Mixing rate has also great positive impact on microbial activity. No significant relation has been found between OTC and activity of bacteria where a negative relation has been found between OTC and activity of methanogens. Group *Methanosarcinaceae* was negatively related to concentration of OTC ($p<0.05$ r=-0.405).

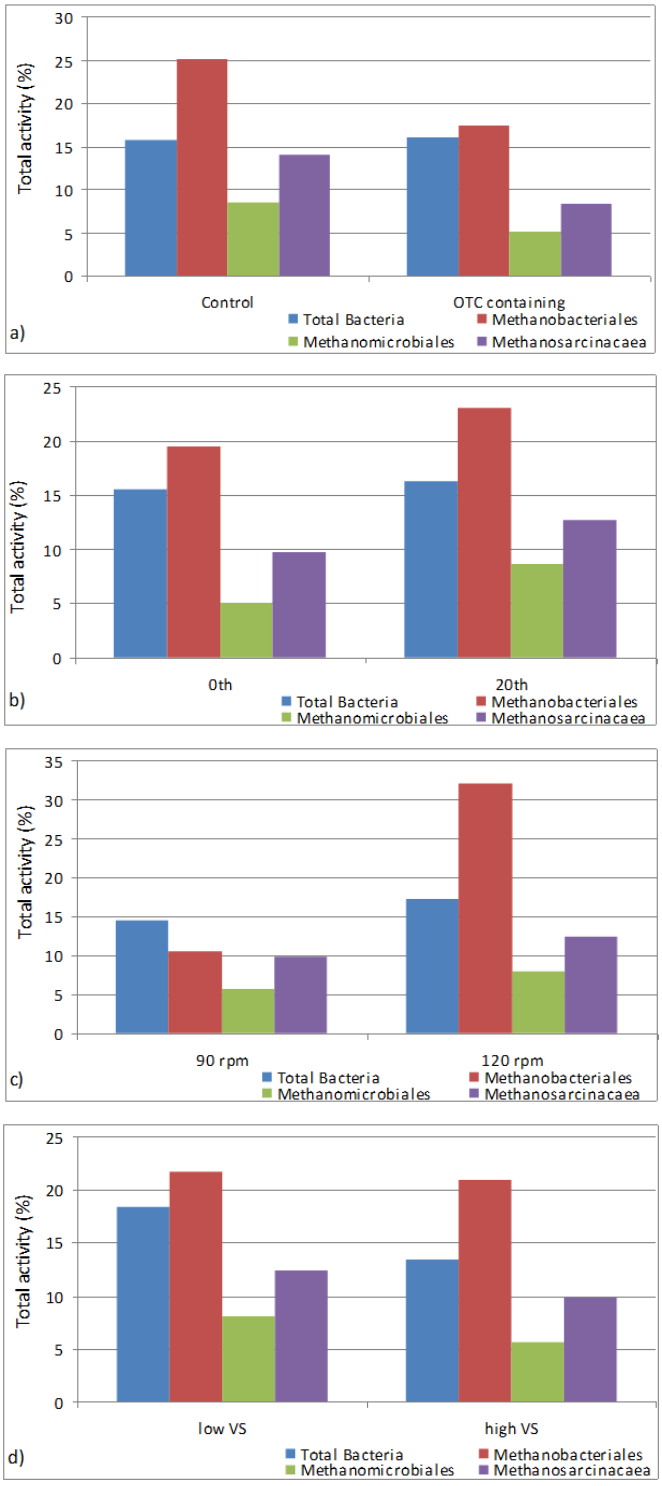

Fig. 1. Activity of digester microbial communities under various environmental parameters a) OTC, b) Time, c) Mixing rate, d) VS concentration

4. Conclusion

In this study, effects of a common veterinary antibiotic on biogas yield, system performance and active microbial populations were observed along with changing operational parameters. 118-121 L/kgTVS biogas yield was observed in control digesters. Results show a decrease of 14%-24% in

biogas yield in OTC containing digesters. A lower inhibition was observed at high VS concentrations. OTC was also eliminated from the system with time indicating a half life of 22-27 days in mesophilic anaerobic manure digesters. Presence of OTC do not affect system performance since no VFA accumulation and change in biogas composition was observed in OTC containing digesters. Changing operational parameters do not affect biogas production significantly. FISH analyses showed an increase in activity with time and mixing rate where presence of OTC and high VS concentrations affected activity negatively. Methanogens were dominated by hydrogenotrophic methanogens. To further analyze the system, a RNA based Q-PCR should be conducted along with community diversity analysis.

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