QUANTIFICATION OF MULTI-CLASS ANTIBIOTICS BY UHPLC-MS/MS IN ANIMAL MANURE AND FATE OF ANTIBIOTICS DURING RAPID COMPOSTING

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ABSTRACT

QUANTIFICATION OF MULTI-CLASS ANTIBIOTICS BY UHPLC-MS/MS IN ANIMAL MANURE AND THE FATE OF ANTIBIOTICS DURING RAPID COMPOSTING

Massive amounts of animal waste are generated from livestock raising and proper management of this waste is critical for the elimination of manure pollutants including various gaseous, nutrients, pathogens, and antibiotics. A comprehensive multiresidue method for the analysis of 33 antibiotics from 7 prevalent classes was comparably investigated for both dairy and poultry manure. Following salting-out-assisted extraction with Acetonitrile (MeCN), the antibiotics were quantified with Ultrahigh-Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MS/MS) without a clean-up step. Although the physicochemical properties of selected antibiotics were quite different, the apparent recovery from dairy and poultry manure samples were 86–121% and 89–113%, respectively. Rotary drum composting was performed in closed systems with controlled aeration for the reduction of detected antibiotics and volume of manure. Two identical reactors were operated up to 20 days. After the characterization of manure samples, proper compositions for rapid composting were prepared by blending of dairy (D), broiler (B), and layer-hen (L) manures. The performances of composting process for DB and DL manure mixtures as well as B and D manures were evaluated. Time to attain the peak composting temperature (68-73°C) was reduced and higher peak temperatures were achieved in mixed manures compared to the composting of D manure alone. While the composting with high aeration rate ($17\pm3 m^3.h^{-1}$) caused a decrease in composting temperature, the elevated temperature achieved with low aeration rate (3.6 ± 0.2 $m^3.h^{-1}$) did not improve antibiotic reduction, and high aeration reduced the half-lives of antibiotics regardless of manure blending indicating the importance of biotic processes.

ÖZET

ÇOKLU ANTİBİYOTİKLERİN UHPLC-MS/MS İLE HAYVAN GÜBRESİNDEKİ MİKTAR TAYİNLERİ VE ANTİBİYOTİKLERİN KOMPOSTLAŞTIRMA ESNASINDAKİ AKİBETLERİ

Çiftlik hayvanlarının yetiştirilmesinden kaynaklanan hayvan atıkları muazzam miktardadır ve bu atıkların uygun şekilde yönetilmesi içerisinde bulunan çeşitli gazlar, nütrientler, patojen mikrooganizmalar ve antibiyotikler gibi kirleticilerin giderilmesi için kritik derecede önemlidir. 7 geçerli gruptan toplam 33 antibiyotik tavuk ve inek gübrelerinde kapsamlı bir çoklu kalıntı analizi metodu için araştırılmıştır. Tuz yardımıyla MeCN çözeltisinde uzaklaştırma yöntemini izleyerek çok yüksek performanslı sıvı kromatografisi ardışık kütle spektrometrisi (UHPLC-MS/MS) ile antibiyotiklerin miktar tayini herhangi bir ekstrakt temizleme aşaması uygulanmadan yapılmıştır. Seçilen antibiyotiklerin fizikokimyasal özellikleri birbirinden çok farklı olsa da inek ve tavuk gübresinden geri kazanım sırasıyla %86–121ve %89–113'tür. Döner tambur kompostlaması kontrollü havalandırma yapılan kapalı bir sistemde tespit edilen antibiyotiklerin ve gübre hacminin azaltılması için uygulanmıştır. İki özdeş reaktöre 20 güne kadar işletilmiştir. Gübre numunelerinin karakterizasyonu sonrasında süt ineği, etlik tavuk ve yumurtalık tavuk gübreleri hızlı kompostlaştırma sağlanması için uygun kompozisyonda karıştırılarak hazırlanmıştır. Kompostlaştırma prosesinin performansı süt ineği ve etlik tavuk, süt ineği ve yumurtalık tavuk gübreleri karıştırılarak ve etlik tavuk, süt ineği gübrelerinin ayrıca kompostlaştırılması ile değerlendirilmiştir. Karışık gübreler ile tek başına süt ineği gübresiyle yapılan kompostlaştırmadan daha yüksek pik sıcaklıklar elde edilmiştir ve en yüksek kompostlaştırma sıcaklığına (68-73°C) ulaşma hızı azaltılmıştır. Yüksek havalandırma hızı ($17\pm3 m^3.h^{-1}$) kompostlaştırma sıcaklığını düşürmüş olsa da düşük havalandırma hızı ($3.6\pm0.2 m^3.h^{-1}$) ile elde edilen yüksek sıcaklık antibiyotik giderimini arttırmamıştır ve yüksek havalandırma hızı gübre karışımlarından bağımsız olarak antibiyotiklerin yarılanma ömrünü azaltmıştır ki biyolojik proseslerin önemine işaret etmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
°C	Degree Celsius	
$\mu g.kg^{-1}$	Microgram per Kilogram	
ATU	Allylthiourea	
C	Analyte Concentration at Time t	$\mu g.kg^{-1}$
C/N or OC/ON	Carbon to Nitrogen or Organic Carbon to Organic Nitrogen	
C0	Initial Antibiotic Concentration	$\mu g.kg^{-1}$
$C_2H_2O_4 \cdot 2H_2O$	Oxalic Acid Dihydrate	
$C_6H_8O_7$	Citric Acid	
CaCl	Calcium Chloride	
cfu	Colony Forming Unit	
d	Day	
eV	Electron Volt	
$FeCl_3$	Ferric Chloride	
$g.kg^{-1}$	Gram per Kilogram	
$g.mol^{-1}$	Gram per Mole	
h	Hour	
H_2O	Water	
HCl	Hydrogen Chloride	
HCOOH	Formic Acid	
$HCOONH_4$	Ammonium Formate	
HNO_3	Nitric Acid	
k	Removal Rate	day^{-1}
KCl	Potassium Chloride	
KH_2PO_4	Potassium Phosphate Monobasic	
KOH	Potassium Hydroxide	
L	Liter	
Log k_{ow}	Log Octanol Water Partition Coefficient	
Log p	Log Partition Coefficient	
m/z	Mass to Charge Ratio	
$m^3.h^{-1}$	Meter cube per hour	

mEq	Milli Equivalent
mg $O_2.L^{-1}$	Milligram Oxygen per Liter
$Mg(NO_3)_2$-$NH_3.H_2O$	Magnesium Nitrate Ammonium Water
$mg.kg^{-1}$	Milligram per Kilogram
$mg.L^{-1}$	Milligram per Liter
$mg.mL^{-1}$	Milligram per Milliliter
$mgO_2.gVS^{-1}.h^{-1}$	Milligram Oxygen Gram Volatile Solid per Hour
$MgSO_4$	Magnesium Sulfate
min	Minute
$Na_2EDTA \cdot 2H_2O$	EDTA Acid Dihydrate
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
$ng.mL^{-1}$	Nanogram per Milliliter
NH_4	Ammonia
nm	Nanometer
pKa	Acid Dissociation Constant
R^2	Coefficient of Determination
rpm	Rounds per Minute
t ½	Half-life day
V	Volt

Abbreviation	**Explanation**
APCI	Atmospheric Pressure Chemical Ionization
B	Broiler
BOD	Biological Oxygen Demand)
CAFOs	Combined Animal Feeding Operations
CEC	Cation Exchange Capacity
CHL	Chloramphenicol
Cİ	Concentration of Antibiotics in Raw Manure Samples
CIP	Ciprofloxacin
COD	Chemical Oxygen Demand
CT	Concentration of Antibiotics in the Composted Manure Samples
CTC	Chlortetracycline

D	Dairy
DAN	Danofloxacin
DAPs	Diaminopyrimidines
DB	Dairy and Broiler Manure
DEM	Demeclocycline
DIF	Difloxacin
DL	Dairy and Layer Hen Manure
DO	Dissolved Oxygen
DXC	Doxycycline
E.Coli	*Escherichia Coli*
EC	Electrical Conductivity
EC	European Commission
EDTA	Ethylenediaminetetraacetic Acid
EMV	Electron Multiplier Voltage
ENR	Enrofloxacin
EPA	Environmental Protection Agency
ERY	Erythromycin
ESI	Electrospray Ionization
ESVAC	European Surveillance of Veterinary Antibiotic Consumption
EU	European Union
FLF	Florfenicol
FLU	Flumequine
FQs	Fluoroquinolones
HLB	Hydrophilic Lipophilic Balance
ICP	Inductively Coupled Plasma
JOS	Josamycin
L	Layer-hen
LC–MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LINs	Lincosamides
LOD	Limit of Detection
LOQ	Limit of Quantification
MAR	Marbofloxacin
MC	Moisture Content
MC	Moisture Content
MCs	Macrolides

ME	Matrix Effect
MeCN	Acetonitrile
MeOH	Methanol
Na_2EDTA	Disodium Ethylenediaminetetraacetic Acid
NH_2	Amino
NOR D_5	Norfloxacin D_5
NOR	Norfloxacin
NORMAN	Network of Reference Laboratories, Research Centers and Related Organizations for Monitoring of Environmental Substances
ON	Organic Nitrogen
OTC	Oxytetracycline
OXO	Oxolinic Acid
P	Phosphorus
PLMs	Pleuromutilin
PMXs	Polymyxins
PR	Percent Reduction
PSA	Primary Secondary Amine
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RD	Rotary Drum
ROX	Roxithromycin
RSD	Relative Standard Deviation
RT	Retention Time
SAR	Sarafloxacin
SAs	Sulphonamides
SAX	Strong Anion Exchange
SCP	Sulfachloropyridazine
SD	Standard Deviation
SDM	Sulfadimethoxine
SDX	Sulfadoxine
SDZ	Sulfadiazine
SFX	Sulfisoxazole
SMH D_4	Sulfamethoxazole D_4
SMH	Sulfamethoxazole
SMT	Sulfamethizole
SMX	Sulfamethoxypyridazine

SMZ	Sulfamethazine
SOUR	Specific Oxygen Uptake Rate
SPE	Solid Phase Extraction
SPI	Spiramycin
SQX	Sulfaquinoxaline
ST	Static Compost
STZ	Sulfathiazole
TCA	Trichloroacetic acid
TCs	Tetracyclines
THI	Thiamphenicol
TIL	Tilmicosin
TKN	Total Kjeldahl Nitrogen
TOC	Total Organic Carbon
TP	Total phosphorus
TRI	Trimethoprim
TYL	Tylosin
UHPLC-MS/MS	Ultrahigh-performance liquid chromatography tandem mass spectrometry
USE	Ultrasonic extraction
WEN	Water Extractable Nitrogen
WEOC	Water Extractable Portions of Organic Carbon

1. BACKGORUND

1.1. Antibiotics and Animal Industry

Antibiotics have been used for infection therapy of human and animal since 1950s (Carvalho and Santos, 2016). In animal farming, antibiotics can be routinely used for two main purposes: to treat diseases and to promote growth; in other words, they can be used for therapeutic or sub-therapeutic purposes (Kumar et al., 2005). Sub-therapeutic use of antibiotics promotes the growth of animals and decreases the stress that makes the animal more prone to infection (Aust et al., 2008). Feeding antibiotics at therapeutic and sub-therapeutic doses to swine, cattle, poultry, and fish is an integral part of the farm animal/fish production.

Although the use of antibiotics for non-medicinal purposes has been phased out in animal husbandry in the European Union (EU) in 2006 they are used for therapeutic purposes and their eventual excretion causes their occurrence in manure. Kools et al., (2008) reported the consumption of 5,393 tons of veterinary antibiotics in 25 EU countries in 2004 before implementation of the regulation. In the Seventh ESVAC (The European Surveillance of Veterinary Antibiotic Consumption) Report (2017), the sales of veterinary antibiotics from 2010 to 2015 was estimated as 8,936 tons and the largest proportions were accounted for Tetracyclines (TCs) (33%), Penicillins (25%), Sulphonamides (SAs) (12%), Macrolides (MCs) (7%), Lincosamides (LINs)(3.5%), Fluoroquinolones (FQs) (2%) and Trimethoprim (TRI) (1.6%).

In the USA where the administration of antibiotics for food-producing animals is approved, approximately 13,990 tons of veterinary antibiotics were sold in a year, (US FDA, 2017). Like the results obtained for Europe the largest proportions were accounted for by TCs (42%). However, the second group of antibiotics was Ionophores (33%). On the other hand, there is no published information about the consumption rate of antibiotics for animal farming in Turkey. However, previous studies performed in Turkey shows us the presence of antibiotics in the manure of farm animals (Karcı and Balcıoğlu, 2009; Cengiz et al., 2010a; Cengiz et al., 2010b).

Due to heavy antibiotic treatment on animals in the farms, foodstuffs from animal origin included variety of antibiotic residues (Karcı and Balcıoğlu, 2009; Zhao et al., 2010). Therefore, European Commission established legislation about the acceptable residue limits of pharmacologically active

substances in food stuffs of animal origin (EC, 2007). Besides, analytical criteria and requirements for the determination of analytes were regulated by European Commission (EC, 2002) for food stuffs. There is not any regulation about the determination of analytes and their maximum residue limits in environmental samples although administered antibiotics to animals was discharged up to 90% into the environment via feces and urine (Kemper, 2008; Carvalho and Santos, 2016). Because of this antibiotic pollution threat of antibiotic resistant microorganisms (Martinez, 2009) and unintended exposure of antibiotics with the consumption of plants that already uptake these compounds (Tasho and Cho, 2016, Chowdhury et al., 2016) are also environmental burden that have to be considered by authorities.

Due to the constant input of the emerging contaminants to the environmental sources such as surface water and soil, special attention is being paid by network of reference laboratories, research centers and related organizations for monitoring of environmental substances (NORMAN). Norman identified a list of the most frequently discussed emerging substances and emerging pollutants in February 2016; however, the maximum residue limits of those compounds have not still been identified for environmental matrices.

1.2. Antibiotic Occurrence in Animal Manure and Environment

It is known that 250 different registered compounds are used as human and veterinarian antibiotics (Kumar et al., 2012). After taken of divergent antibiotics on animal body, they are generally poorly absorbed in the animal gut and do not accumulate in the animal tissue. As a result, large number of antibiotics given to animals is excreted in urine and feces. Excretion rates of Chlortetracycline (CTC), Sulfamethazine (SMZ), and Tylosin (TYL) were 65-75, 90, and 28-100 %, respectively (Kim et al., 2011). Worse than that antibiotic residues could be detected in soil due to the direct application of animal manure (Ho et al., 2012) and hence this pollution might lead to the occurrence of them in aquatic environments by runoff to the surface water and leaching to the ground water (Baran et al., 2011; Carvalho and Santos, 2016). Therefore, animal manure is an important source for the pollution with veterinary antibiotics since it is not generally processed as conventional wastewater treatment plants (Papageorgiou et al., 2016) before given to the environment.

Occurrence concentrations of antibiotics in dairy and poultry manure have been investigated in numerous studies and some of them listed in Table 1.1 below. In those studies, it was reported that mean antibiotic concentrations were varied in a broad range in dairy (0.001-59.5 $mg.kg^{-1}$) and poultry (0.001-1421 $mg.kg^{-1}$) manure. Poultry manure included several types of antibiotics at higher

concentrations than dairy manure. For instance, Enrofloxacin (ENR) was an antibiotic that detected at extremely high concentration (1421 $mg.kg^{-1}$) in poultry manure in China (Zhao et al., 2010).

Table 1.1. Occurrence of antibiotic in animal manure.

Group	Antib.	Manure	Conc. ($mg.kg^{-1}$)	Country	Reference
SAs	SMH	Dairy cow	0.22-1.02	China	Li et al., 2013
		Chicken	2.23-7.1		
	SCP	Dairy cow	0.15-0.26	Turkey	Karcı and Balcıoğlu, 2009
		Chicken	0.27-0.33		
		Poultry	35.53		
		Cow	0.36	China	Zhao et al., 2010
		Chicken	0.2-0.71		
	SDZ	Chicken	0.03-3.12	Austria	Martinez-Carballo et al., 2007
		Chicken, Turkey	51, 91		
		Cattle	0.001-0.081	The Netherlands	Berendsen et al., 2015
		Broiler	0.012-5.8	Malaysia	Ho et al., 2012
	SMZ	Poultry	3.76	Turkey	Karcı and Balcıoğlu , 2009
		Turkey litter	10.8	USA	Doliver et al., 2008
FQs	NOR	Dairy cow	0.43-1.76	China	Li et al., 2013
		Chicken	0.5-9.52		
		Cow	1.23-2.76	China	Zhao et al., 2010
		Chicken	0.85-225.45		
		Poultry litter	0.8-4.55	Brazil	Leal et al., 2012
	ENR	Dairy cow	0.46-4.17	China	Li et al., 2013
		Chicken	0.33-15.43		
		Cow	1.72-46.7	China	Zhao et al., 2010
		Chicken	0.33-1421		
		Poultry litter	0.39-30.97	Brazil	Leal et al., 2012
	CIP	Dairy cow	0.28-0.84	China	Li et al., 2013
		Chicken	0.33-2.94		
		Cow	0.49-29.59	China	Zhao et al., 2010
		Chicken	0.68-45.59		
		Poultry litter	0.65-2.13	Brazil	Leal et al., 2012
		Cattle	0.013	The Netherlands	Berendsen et al., 2015
	DAN	Cow	0.41-3.06	China	Zhao et al., 2010
		Chicken	0.08-2.48		
MCs	ERY	Broiler	0.027-0.032	Malaysia	Ho et al., 2012
	TYL	Dairy cow	0.22-0.28	China	Li et al., 2013
	TYL	Turkey litter	3.7	USA	Doliver et al., 2008

Table1.1. Continued

Group	Antib.	Manure	Conc. ($mg.kg^{-1}$)	Country	Reference
TCs	TC	Dairy cow	0.43-2.69	China	Li et al., 2013
		Chicken	0.54-4.57		
		Cattle	0.003-0.112	The Netherlands	Berendsen et al., 2015
	OTC	Dairy cow	0.21-10.37	China	Li et al., 2013
		Chicken	0.96-13.39		
		Cow	0.32-59.59	China	Zhao et al., 2010
		Chicken	0.27-10.56		
		Cattle, poultry	0.05-0.5	Turkey	Karcı and Balcıoğlu, 2009
		Broiler	0.004-21	The Netherlands	Berendsen et al., 2015
	CTC	Dairy cow	0.61-1.94	China	Li et al., 2013
		Chicken	0.57-3.11		
		Cow	0.24-27.59	China	Zhao et al., 2010
		Chicken	0.16-17.68		
		Cattle, poultry	0.25-0.35	Turkey	Karcı and Balcıoğlu, 2009
		Chicken, Turkey	1.7	Austria	Martinez-Carballo et al., 2007
		Turkey litter	1.5	USA	Doliver et al., 2008
	DXC	Cow	0.44-1.05	China	Zhao et al., 2010
		Chicken	0.92-10.91		
		Broiler	0.3-78.5	Malaysia	Ho et al., 2012
		Broiler	0.005-0.152	The Netherlands	Berendsen et al., 2015
Other	TRI	Poultry	0-17	Austria	Martinez-Carballo et al., 2007
		Broiler	0.009-3.4	Malaysia	Ho et al., 2012
	LIN	Broiler	0.001-0.017	The Netherlands	Berendsen et al., 2015

Antib.; Antibiotic, Conc.; Concentration, SMH (Sulfamethoxazole), SCP (Sulfachloropyridazine), SDZ (Sulfadiazine), NOR (Norfloxacin), CIP (Ciprofloxacin), DAN (Danofloxacin), ERY (Erythromycin), OTC (Oxytetracycline), DXC (Doxycycline)

As mentioned above, the occurrence of antibiotics in animal manure at high concentration poses a potential risk of contaminating adjacent agricultural soil. Detection of TCs, SAs, and MCs in fertilized soil was reported by Kim et al., (2011). In addition, detection of six different veterinary antibiotics in broiler manure and respective manure amended agricultural soil samples were revealed out in a recent study (Ho et al., 2012). In this study, detected DXC concentration in manure amended soil was found at nearly 0.207 $mg.kg^{-1}$ while same compound was detected at about 78.5 $mg.kg^{-1}$ in manure that was used as fertilizer. Detection of same compounds in soil and manure proves that manure could be main source for antibiotic pollution and similar fertilization activities might be a

potential threat for environment and human health.

1.3. Antibiotic Fate and Transformation Products

Since animal manure is applied to the agricultural field as a fertilizer, antibiotic laden manure might pollute the environmental sources such as fertilized soil with adjacent ground water and surface water by leaching and run off, respectively. Antibiotics are not only excreted as a parent material, but also high proportion of active and inactive metabolites are released to the environment, or metabolites might revert back to the parent compounds (Kim et al., 2011). Fate of some group of antibiotics and their degradation products were identified under environmentally relevant conditions. For instance, biotransformation of SAs is generally occurred as oxidation, acetylation or hydroxylation (Baran et al., 2011). It is known that, 50–70% of the orally administered dose of SAs is excreted in urine as N4-Acetyl SAs, and 15–20% as N^1-glucuronides (Baran et al., 2011). Although these acetic acid conjugates of SAs do not possess high biological activity as parent compounds, they may revert back to the parent compound during storage since the acetyl moiety is cleaved by bacteria (García-Galán et al., 2008). For instance, N-ac-SDZ (Zarfl et al., 2009) and N-ac-SMH (Höltge and Kreuzig, 2007), may potentially be transformed back to original antibiotic form after excretion.

Available degradation products of TCs in environment were listed by Halling-Sørensen et al., (2002). TCs could be transformed to 5,a 6-anhydrotetracycline hydrochloride, 4-epitetracycline hydrochloride, and 4- epianhydro tetracycline hydrochloride. CTC and its degradation products are 5a,6-anhydrochlorotetracycline hydrochloride, 4-epi-chlorotetracycline hydrochloride , 4-epi-anhydro-chlorotetracycline hydrochloride, and iso-chlorotetracyclines. OTC, and its degradation products are 4-epi-oxytetracycline, α-apo-oxytetracycline, β -apo-oxytetracycline, and terrinolide. Arıkan et al., (2009) studied the effect of composting on CTC residues that were ECTC and iso-CTC. More than 98% degradation achieved in 30 days with temperature dependent abiotic processes for both parent and degradation products by applying composting.

TYL is a MC group of antibiotics that metabolized to cysteinyl TYL (TYL A), desmycosin (TYL B), macrocin (TYL C) and relomycin (TYL D). TYL D is excreted in feces and TYL A is excreted in urine (Lewicki et al., 2009). In general, oral administration of TYL resulted in a maximum 67% of excretion via feces (Chee Sanford et al., 2009). But, there is limited information about the excretion routes of other group of antibiotics.

In addition, as a FQs antibiotic, the main metabolite of ENR is CIP and more potent than the parent compound (Trouchon and Lefebvre, 2016). It is known that CIP is not the only metabolites of ENR but the others do not have antibiotic effects (Trouchan and Lefebvre, 2016; Assis et al., 2016). ENR could not be metabolized by poultry as much as pig and cattle hence detected amount of ENR in poultry manure might close to the administered dose of antibiotic (Assis et al., 2016).

1.4. Antibiotic Pollution Control in Animal Waste

The intensive livestock production poses a potential pollution risk due to high livestock waste that includes not only excessive nitrogen, phosphorus, heavy metals and pathogens but also veterinary antibiotics. Administered antibiotics can nearly be extracted as non-metabolized (up to 90%) from animal organisms via feces and urine (Kemper, 2008). Therefore, proper management of these waste has prime importance before releasing to the soil since manure is traditionally used as fertilizer. Issues of antibiotic resistance microbial pollution (Martinez, 2009) and unintended exposure of antibiotics with the consumption of plants that already uptake these compounds from manure amended soil are resulted from application of antibiotic laden manure to the soil (Tasho and Cho, 2016, Chowdhury et al., 2016). Regarding these adverse effects, various treatment studies have been conducted to reduce antibiotic content before land application of manure.

Prior to manure disposal to environment (e.g. soil, surface water and groundwater), animal manure can be managed through a variety of manure-handling practices including composting, anaerobic digestion, pit storage, pile storage, and aerated lagoons. Among these, pit storage and aerated lagoons are only applied for the swine manure due to its slurry character and antibiotics could not be degraded efficiently. Kolz et al., (2005) reported TYL degradation in manure lagoon slurries in which 90% of it was degraded with aerobic and anaerobic conditions at 22 °C at long storage conditions; however, TYL residuals remained in slurry even after eight mounts of incubation.

Although anaerobic digestion is an alternative for the management of manure utilization and the production of a unique substrate biogas, it has been unpopular due to relatively low biodegradability and biogas yields (Mohring et al., 2009, Alvarez et al., 2010, Lateef et al., 2014). Furthermore; before land application digestate requires a final polishing step due to its high humidity and pathogen content, (Bustamante et al., 2013). Numerous studies revealed the inefficiency of anaerobic conditions for the removal of antibiotics from manure. Approximately 46% degradation of TCs were achieved in an anaerobic digestion batch test, (Çetecioğlu et al., 2014). On the other hand, Mohring et al., (2009) did not observe detectable degradation of SMZ and STZ (Sulfathiazole) in fermentation

tests. In another study, the presence of several antibiotics up to 9 $mg.kg^{-1}$ (201 $mg.kg^{-1}$ dry matter) were detected in 15 German biogas plants after the anaerobic digestion, (Spielmeyer et al., 2014). Moreover, above critical concentrations antibiotics can have potential to inhibit the anaerobic reactions and deteriorate the production of biogas/energy (Ray et al., 2017). Most studies have focused on the inhibition of anaerobic digestion at the high antibiotic concentrations typically found in manure, (İnce et al., 2013; Shi et al., 2011; Alvarez et al., 2010; Chelliapan et al., 2011; Gartiser et al., 2007; Lallai et al., 2002). For instance; OTC caused 50-60 % decrease in biogas production even at low (1-3.3 mg/L) concentrations, (İnce et al., 2013).

As it was mentioned above long storage periods of lagoons and sensitive microbial processes of anaerobic digestion prevent the common usage of those processes to treat manure (Ray et al., 2017). Additionally, it is known that antibiotics are generally disseminated in the order of composting>anaerobic digestion>manure storage>soil (Massé et al., 2014). Hence, composting process could be the best alternative to cope with antibiotic laden manure dependent pollution.

1.5. Quantification of Multi-class Antibiotics

Livestock is one of the fastest-growing agricultural sectors to meet the food demand of rising human population. Currently, most meat and dairy products are produced on large farms instead of small-diversified ones to improve efficiency. Massive amounts of animal waste are generated from these industrial farms and estimated annual production rate of animal manure is 7 billion tons, which is higher than that of other biosolids (Thangarajan et al., 2013). Proper management of this waste has prime importance to minimize adverse effects on the environment since manure is traditionally used as fertilizer.

However, mismanagement of animal waste can cause a risk especially on natural resources because of not only gaseous emissions, excessive nutrients, and pathogens but also emerging contaminants. In this context, the utilization of veterinary antibiotics as growth promoters for food-producing animals has been banned in European countries and the United States since 2006 and 2016, respectively (ESVAC, 2017, US FDA, 2017). However, the complete elimination of their therapeutic, prophylactic and metaphylactic use is not feasible, and high consumption of antibiotics is still an important issue in animal husbandry. Therefore, actions are urgently required at multiple levels to control antibiotic pollution, whereas new policies on livestock rearing practices have been implemented especially in confined animal feeding operations. In this regard, a reliable and sensitive

Table 1.2. Literature on the multi-class antibiotic analysis in manure with LC-MS/MS.

Compounds	Manure	Extraction	SPE	Recovery (%)	LOD ($\mu g.kg^{-1}$)	Reference
10 SAs, 4 TCs	Cattle and swine	(MeOH+ethanol +dichloromethane 1/3/1)+ (0.05 M EDTA +1 M citrate buffer, pH 4.7), 80/20; one cycle	-	73-126	10.0-80.0	Spielmeyer et al., 2014
3 SAs, 2 TCs, 3 FQs, 1 DAPs (TRI), 1 β-Lactams	Poultry	(MeCN) + (McIlvaine buffer pH 3.6) 75/25; one cycle with USE	-	89-108	1.8-9.2	Gorissen et al., 2015
1 SAs, 2 TCs, 1 MC, 1 DAPs (TRI), 1 β-Lactams, 2 PMXs	Swine	MeCN, 6% TCA, one cycle with USE	-	94-118	1.1-20.2	Meersche et al., 2016
1 SA, 1 TC, 3 FQs, 3 MCs, 1 DAPs (TRI)	Poultry	(MEOH, MeCN, 30/20)/(0.1 M EDTA + McIlvaine buffer, pH 4, 25/25) 50/50; three cycles with USE	HLB	63-113	1.0-5.0	Ho et al., 2012
15 SAs, 5 TCs, 11 FQs, 6 MCs, 2 APs, 1 DAPs (TRI)	Swine	(MeCN)/(0.2 g Na_2EDTA+Mcllvaine buffer, pH 3), 50/50; three cycles with USE	SAX+HLB	<10-259	0.4-5.2	Zhou et al., 2012
8 SAs, 4 TCs, 4 FQs, 1 MCs	Poultry	(MeCN)+ (0.1 M EDTA, sodium phosphate buffer, pH 4)/($Mg(NO_3)_2$-$NH_3.H_2O$) , 3/1; three cycles with USE	HLB	69-136	0.5-14.1	Huang et al., 2013
16 SAs, 4 TCs, 9 FQs, 9 MCs, 1 LINS, 8 β-Lactams	Swine	(MeCN) +(Lead acetate + 0.2 M EDTA + Mcllvaine buffer pH 4), 1/19; one cycle	Strata X	84-147	0.5-32.0	Berendsen et al., 2015
6 SAs, 4 TCs, 6 FQs, 6 MCs, 2 LINs, 2 PLMs	Swine	(MeOH + MeCN+ 0.1 M EDTA, Mcllvaine buffer pH 4) $MgSO_4$, NaCl, 12.5/37.5/50; two cycles with USE	PSA+C18	32-110	0.01-1.86	Guo et al., 2016

*DAPs (Diaminopyrimidines), PMXs (Polymyxins), PLMs (Pleuromutilin), MeOH (Methanol) , HLB (Hydrophilic Lipophilic Balance), SAX (Strong Anion Exchange), TCA (Trichloroacetic acid), USE (Ultrasonic extraction), LOD (Limit of Detection), SPE (Solid Phase Extraction), PSA (Primary Secondary Amine), Na_2EDTA (Disodium ethylenediaminetetraacetic acid)

multi-class analysis method for animal waste, in which antibiotics end up, is of great concern for the scientific community and regulatory authorities, as manure has agronomic applications.

Relative to the number of reports on other environmental matrices, there are only a few studies on the analysis of multiresidue antibiotics in manure by using liquid chromatography tandem mass spectrometry (LC–MS/MS) (Larivière et al., 2017) and these are presented in Table 1.2. The success of multi-class antibiotic analysis is expected to be highly dependent upon the extraction process. This extraction process is more challenging for a solid manure sample or a solid fraction of liquid manure that mainly harbors the antibiotics (Wallace and Aga, 2016; Pan et al., 2011) than for liquid manure (Jacobsen and Halling-Sørensen, 2006; Moral et al., 2005). In most of the studies listed in Table 1.2, extraction is based on the method of Blackwell et al., (2004) for which a mixed solvent combined with aqueous and non-aqueous solvents is applied to the solid and liquid manure matrices that may be rich in organics and metals.

The fact that many of the antibiotics in the manure are retained can be ascribed to their interaction with the matrix components through complexation and ion exchange, in addition to hydrophobic interactions resulting from the ionic properties of the antibiotics themselves (Tolls, 2001). Therefore, the addition of Ethylenediaminetetraacetic Acid (EDTA) and/or citrate as a chelating agent(s) to the extraction solvent together with MeOH can facilitate the release of the antibiotics into the extraction solvent (Ho et al., 2012; Berendsen et al., 2014; Gorissen et al., 2015; Huang et al., 2013; Zhou et al., 2012). However, Meersche et al., (2016) achieved a high recovery of selected SAs, TCs and TRI from liquid manure by using acidified MeCN instead of a solvent mixture containing a chelating agent. On the other hand, the extraction of the same antibiotics from a solid manure sample was performed by Martínez-Carballo et al. (2007) by using only an aqueous solution of EDTA and McIlvaine buffer.

Although various investigations have been performed by changing the type of non-aqueous solvent (Meersche et al., 2016; Pan et al., 2011; Huang et al., 2013; Janusch et al., 2014), MeCN is commonly used as the sole organic solvent or in combination with MeOH for a wide range of antibiotics from various classes. Furthermore, a suggested benefit of extraction with the use of a MeCN/solvent mixture is that the mixture provides cleaner extract result of the precipitation of the proteins found in manure (Martínez-Carballo et al., 2007).

Besides the composition of the extraction solvent, the pH of the extraction buffer is another parameter that can affect the performance of extraction by influencing the interactions between the antibiotics sorbed on the manure and the extraction solvent. However, during multi-class antibiotic

analysis in manure, assessment of the pH effect is not straightforward, as each antibiotic has different physicochemical properties and the interaction of the extraction solvent with various matrix components can be complex. Although in various studies, the extraction of antibiotics from manure was performed with acidic solvents (Table 1.2), the extraction of the TC, SA and FQ classes of antibiotics individually with a neutral or alkaline extraction buffer was more effective in terms of recovery (Janusch et al., 2014; Li et al., 2015; Haller et al., 2002). Zhou et al. (2012) did not achieve a consistent effect for a wide range of antibiotics by using a buffer with a pH value between 3 and 7.

During extraction, the influence of the solvent can be enhanced by ultrasonication, by application of high pressure or by microwave irradiation of solid manure (Díaz-Cruz and Barceló, 2007); yet, these extraction techniques can cause the co-extraction of natural organic matter (Aga et al., 2016), and this may require an additional clean-up step of the extract prior to LC–MS/MS analysis. However, in various studies, the extracts obtained from manure with and without the use of these extraction techniques were subjected to a clean-up step, and this mainly involved SPE with the use of different SPE materials.

Multi-class antibiotic analysis was previously performed in manure extracts with a HLB cartridge alone (Ho et al., 2012; Pan et al., 2011; Huang et al., 2013) and in tandem with a SAX cartridge (Zhou et al., 2012) or NH_2 (Amino) cartridge (Wallace and Aga, 2016) and by using PSA and C18 as dispersive SPEs (Guo et al., 2016). A recent comprehensive review demonstrated the non-selectivity of HLB cartridges by highlighting their application to various pollutants, including pesticides, polycyclic aromatic hydrocarbons, emerging contaminants and others (Andrade-Eiroa et al., 2016). Furthermore, the possible loss of TRI and SDZ antibiotics through the application of SPE was reported (Meersche et al, 2016), yet, the influence of an appropriate SPE protocol on the loss in recovery cannot be ignored.

Although the analysis of multi-class antibiotics in liquid and solid manure without using SPE was previously performed in three different recent studies, the lowest detection limits of the antibiotics were relatively high (Meersche et al., 2016; Gorissen et al., 2015; Spielmeyer et al., 2014). Whereas the recovery of four different TCs (i.e. CTC, DXC, OTC and TC) and one of SAs (sulfaguanidine) was not achieved at the lowest concentration of the investigated spiking range (150-2500 $\mu g.kg^{-1}$) in both cattle feces and swine liquid manure (Spielmeyer et al., 2014) without the application of SPE, a high recovery rate of a few TCs (i.e. CTC, DXC) at a spiking concentration of 50 $\mu g.kg^{-1}$ was obtained in poultry manure (Gorissen et al., 2015). This difference in the results is probably related to the compositions of the manure samples, which were not characterized in the

investigations mentioned above, as it is known that different extraction methods may be necessary to achieve high performance for different manure samples (Martínez-Carballo et al., 2007; Larivière et al., 2017). Indeed, the ability to extract TCs was selected as a criterion by Spielmeyer et al., (2014) for multiresidue analysis owing to their strong interaction with the matrix components of the manure in the extraction step and low signal response in the subsequent quantification step (Huang et al., 2013). For the extraction of some TCs from manure, remarkably poor results were achieved in recent studies (Berendsen et al., 2015; Zhou et al., 2012).

Considering the disadvantages of SPE versus liquid-liquid extraction, Guo et al. (2016) applied a so-called quick, easy, cheap, effective, rugged and safe (QuEChERS) method for the analysis of 26 antibiotics in liquid manure through some modifications. The extraction solvent used in the study was composed of 0.1 M EDTA/McIlvaine buffer and a mixture of organic solvents (MeOH and MeCN), as in case of previous studies (Zhou et al., 2012; Ho et al., 2012). The target antibiotics were concentrated in the organic phase, which was separated from the aqueous phase by salting out. As opposed to the original QuEChERS method developed for pesticides, two cycles of extraction were required to extract the antibiotics from the liquid manure and, furthermore, the extract required concentration by evaporation. Consequently, a few antibiotics (i.e. DXC, SQX, enoxacin, NOR, and valnemulin) were recovered at a level of <70%. The performance of this method together with the others is shown in Table 1.2 in terms of LOD. However, these results cannot be directly compared, as it is unclear whether the dry or wet weight of manure was used in calculating the performance of the method.

1.6. Fate of Antibiotics in Composting Process

Proper management of animal manure has prime importance before releasing to the soil since it is traditionally used as a fertilizer. It is highly criticized that mismanagement of manure can cause substantial environmental impact due to not only its pathogen and excessive nutrient content but also its emerging contaminants such as antibiotics. Antibiotics have been commonly used drugs in livestock industries that are the fastest growing agricultural sectors. To provide increased food demand resulted from rising population, antibiotics have been administered to animals for growth promotion and/or disease treatment, (Kumar et al., 2005). Issues of antibiotic resistance microbial pollution and unintended exposure of antibiotics with the consumption of plants that already uptake these compounds from manure amended soil are resulted from application of antibiotic laden manure to the soil. Therefore, various treatment studies have been conducted to reduce antibiotic content before land application of manure. For this purpose, storage of manure in lagoons (Chee-sanford et

al., 2009) and anaerobic digestion (Beneregama et al., 2009) were applied besides the composting. However, long storage periods for lagoons and sensitive microbial process for anaerobic digestion prevent the common usage of those processes to treat manure. Recent investigations clearly introduced the composting as an antibiotic reduction process with reducing density and volume of manure and decreasing noxious odors and toxins. As opposed to composting, waste lagoons, which are used to treat animal waste, are not effective for the reduction of antibiotic resistance pollution (Wang et al., 2012). It was suggested that the presence of compost particles enhanced the degradation of antibiotics (Mitchell et al., 2015).

The effectiveness of composting on the degradation of various veterinary antibiotics from four main groups in manure has been investigated in recent literature (Table 1.3). Although the type of manure, the conditions of composting process, initial concentrations of antibiotics in manure and the analytical techniques for the determination of antibiotics were remarkably different, generally composting process exhibited high antibiotic reduction potential (Cessna et al., 2011; Ho et al., 2013; Mitchell et al., 2015; Amarakoon et al., 2016) with few exceptions (Dolliver et al., 2008; Bao et al., 2009). The reasons of relatively lower degradation rates of antibiotics in some studies are not easily explained; however, low moisture content can be an important factor that prevents oxygen transformation to maintain microbial activity and elevates composting temperatures resulted in low antibiotic degradation rates (Zhang et al., 2019). Dolliver et al. (2008) performed the composting of manure with 37-44%, moisture content that led to lower percent reduction of antibiotics (Table 1.3 and 1.4).

The importance of abiotic reactions during the composting process cannot be ignored. However, the possible effects of abiotic factors on the reduction of antibiotics have been only studied in the research of Arıkan et al. (2009). It has been shown that elevated temperature to 55ºC was responsible for the reduction of CTC and its transformation products in sterilized manure (Arikan et al., 2009). Although thermal instability of TCs antibiotics has been known (Ingerslev et al., 2001) there is not such information for the other groups of antibiotics in manure.

Table 1.3 Literature about the fate of antibiotics during manure composting.

Antibiotics	Manure	Moisture (%)	Antibiotic		Fortification	Peak Comp. Temp. (°C)	Comp. Period (d)	Reference
			Conc. (mg kg^{-1})	Degradation (%)				
				SAs				
SCP, SQX	Swine, Chicken	65	10,200	62.23-100	YES	64	35	Qui et al., (2012)
SDZ	Broiler	55	30,640	>99.99	YES	45.7	40	Ho et al., (2013)
SMZ	Turkey litter	37-44	10,800	no change	YES	54-64	22, 35	Dolliver et al.,(2008)
SMZ	Beef feedlot	54.1	150	40, 93.2	NO	60	126	Cessna et al., (2011)
SMZ, SDM	Dairy	73	400, 2,200	98-97	YES	66-73	21	Mitchell et al., (2015)
SMZ	Cattle	48-68	360, 780	93, 99	YES	67-69	30	Amarakoon et al., (2016)
SMZ	Dairy	55-65	992	98	NO	>55	42	Ray et al., (2017)
				FQs				
ENR, NOR, FLU	Broiler	55	5,090- 42.030	>99.81	YES	45.7	40	Ho et al., (2013)
				MCs				
TIL, TYL, ERY	Broiler	55	5,240-62,570	>99.27	YES	45.7	40	Ho et al., (2013)
TYL	Beef feedlot	54.1	41	93.16	NO	60	126	Cessna et al., (2011)
TYL	Turkey litter	37-44	3,700	76	YES	54-64	22, 35	Dolliver et al.,(2008)
TYL	Dairy	73	2,800	98	YES	66-73	21	Mitchell et al., (2015)
TYL	Cattle	48-68	100, 4,500	85, 99	YES	67-69	30	Amarakoon et al., (2016)
TYL	Dairy	55-65	161	86	NO	>55	42	Ray et al., (2017)
				TCs				
DXC	Broiler	55	13,640	99.8	YES	45.7	40	Ho et al.,(2013)
OTC, CTC	Beef calves	68.3, 74.3	Max 115,000	≥99.8	NO	65	35	Arıkan et al.,(2007, 2009)
CTC	Swine	50-60	Max 879,600	89-27	NO	50	42	Bao et al.,(2009)
CTC	Layer-Hen	50-60	Max 150,000	94.51-100	YES	50	42	Bao et al.,(2009)
CTC	Turkey litter	37-44	1,500	>99	YES	54-64	22-35	Dolliver et al.,(2008)
CTC, TC, OTC	Hen, swine	65	Max 60,000	93.8-98.5	YES	55, 62	45	Hu et al.,(2011)
CTC	Cattle	48-68	240, 310	97, 99	YES	67-69	30	Amarakoon et al., (2016)
CTC	Dairy	55-65	675	84	NO	>55	42	Ray et al., (2017)
TC	Dairy	55-65	81,600	72	NO	>55	42	Ray et al., (2017)

Conc.; Concentration, Comp.; Composting, Temp; Temperature, Max; Maximum, TIL; Tilmicosin, SDM ; Sulfadimethoxine

Table 1.4 Half-life and initial concentrations of antibiotics studied in literature.

	Initial Conc. ($\mu g.kg^{-1}$)	Half-life (d)	References
SMZ	150	26.8-237	Cessna et al., 2011
SDZ	30,640	1.4	Ho et al., 2013
SMZ	360-780	9	Amarakoon et al., 2016
SMZ	992	2.03-2.78	Ray et al., 2017
ENR	36,770	2.8	Ho et al., 2013
TYL	41	20.3-43.5	Cessna et al., 2011
ERY	15,580	1.4	Ho et al., 2013
TYL	3,700	19	Dolliver et al., 2008
TYL	1,000-4,500	12	Amarakoon et al., 2016
TYL	161	18	Ray et al., 2017
ISO-CTC	173-783	13.5-26.5	Cessna et al., 2011
CTC	1,500	1	Dolliver et al., 2008
CTC	240-310	1.9	Amarakoon et al., 2016
CTC (Broiler)	94,710	12	Bao et al., 2009
CTC	74,300	4-5	Arıkan et al., 2009
CTC	675	6.1-8.7	Ray et al., 2017
TRI	21,980	3.7	Ho et al., 2013

As it is well known the peak temperature achieved in composting process is an indicator for efficient composting process and Mitchell et al., (2015) suggested that higher composting temperatures enhanced the antibiotic degradation rate. However, even at relatively high peak temperature (60º C) of the composting longer half-lives of 27 and 43.5 d were reported (Table 1.4) (Cessna et al., 2011). On the contrary, very high reduction rates of antibiotics with relatively short half-lives (1.3-3.8 d) were achieved at 46ºC (Ho et al., 2013). These contradictions in the results of the studies can be related with the quantification of antibiotics. The abatement in extractable antibiotic concentration during composting can be attributed to higher sorption tendency of antibiotics (Kim et al., 2012) due to the presence of organic bulking agent in manure and/or the formation of humic substances in mature compost. Furthermore, it was suggested that composting results in higher sorption sites for organics and inorganics (Hartlieb et al., 2003). Strong sorption of antibiotics on solid matrices can adversely affect extraction efficiency for quantification. It can be concluded that temperature effect on degradation of antibiotics is suspicious and not only temperature but also other abiotic factors (sorption, aeration, etc.) and biotic factors might reveal synergetic effect during composting.

One other point is that the manure fortified with antibiotics have been generally composted to investigate the fate of antibiotics. However, strong sorption tendencies of antibiotics (especially MCs and TCs) prevent the degradation process when aged manure composted, (Bao et al., 2009; Amarakoon et al., 2016). Compare to the fortified samples, lower degradation rates of detected

antibiotics after digestion in animal manure could prove the high sorption capacities of antibiotics by metabolizing those of compounds in animal manure. 90% versus 27% reduction of CTC in hog manure (Bao et al., 2009), and 99% versus 85% reduction of TYL in cattle manure (Amarakoon et al., 2016). Therefore, degradation of fortified antibiotics during composting investigations could not reflect the real degradation pathways.

Another important concern for composting process is the static versus turned composting (Ray et al., 2017). Turning helps the necessary contact time of air and homogenous moisture content dissociation in manure pile. Eventually it provides homogeneous humus material at the end of the composting period. This homogeneity might accelerate the proliferation of microorganisms and microbial activity that leads to probable enriched biodegradation of antibiotics (Ray et al., 2017; Zhang et al., 2019).

Balanced Carbon to Nitrogen (C/N) ratio is a controversial issue that leads to bedding and bulking agent addition to the manure (Arıkan et al., 2009; Hu et al., 2011) hence it is resulted in a substantial increase about the amount of waste. Not only for balanced C/N ratio, but also for obtaining suitable moisture content, bulking agent has been extensively added to the manure before composting. Recommended initial C/N ratio is in the range of 20:1-40:1; however as low as 14:1 C/N ratio could be effective for animal manure composting (Herbert et al., 2010). Whereas the moisture content is recommended to be in the range of 40 to 65% (Herbert et al., 2010). However, farmers avoid using even bedding material in concentrated feeding operations to cut down the costs of transport and disposal of manure. Hence, blending bulking agents to manure increases the amount of waste and another method must be discussed to provide optimum nutrient and moisture content.

Long composting times are important concern but there is not necessary attention in the several studies to reduce the time of waste treatment. Composting takes a very long time up to 126 days (Cessna et al., 2011) in those studies. However, rapid composting methods are necessary to handle huge amounts of waste produced from modern animal husbandry facilities.

2. PURPOSE

Livestock is one of the fastest-growing agricultural sectors to meet the food demand of rising human population. Massive amounts of animal waste are generated from large industrial farms and estimated annual production rate of animal manure is 7 billion tons, which is higher than that of other biosolids (Thangarajan et al., 2013). Proper management of manure is a vital concern to minimize lethal adverse effects on the environment since manure is traditionally used as a fertilizer. However, mismanagement of animal waste can cause a risk especially on natural resources because of not only gaseous emissions, excessive nutrients, and pathogens but also emerging contaminants. As an emerging contaminant group, veterinary antibiotics which are used for growth promotion and disease treatment or prevention is of great concern since their occurrence can result in antibacterial resistance microorganism pollution.

Several multi-residue analytical methods have been developed for antibiotics on environmental matrices. Multi-residue analytical methods are still studied to reduce time and cost of already present analysis. They are generally performed by coupling of liquid chromatography (LC) with tandem mass spectrometry (LC-MS/MS) to provide high sensitivity and selectivity. Since various antibiotics displayed very different physical and chemical properties, they may exhibit different sorption tendencies on solid matrices hence non-selective sample preparation procedures can be applied to obtain considerable recovery range for different target antibiotic groups.

In accordance with perspectives given above, our investigations were focused on the following objectives:

- Considering the significance of antibiotic contamination, the first purpose of this thesis is that developing an advanced multi-residue analytical method to analyze target antibiotics in manure. Additionally, method was developed for two different matrices which were dairy and poultry manure.
- Different extraction solutions and equipment were tested to determine optimum conditions for detection of selected target antibiotics from 7 different groups.
- To demonstrate the applicability of the developed method, three different types of manure samples, broiler, layer hen and dairy, were collected from CAFOs (Combined Animal Feeding Operations) monitored in the Marmara region of Turkey.

- After the development and application of analytical method to antibiotics, the fate of excreted antibiotics in raw dairy and poultry manure was investigated during composting process. Since rapid composting methods are necessary to handle huge amount of waste in large farms as opposed to methods applied for small to medium-sized farms composting was performed by rotary drum technique to reduce process time.
- Considering necessary initial nutrient balance and moisture content, studies were performed by blending dairy manure with poultry waste to elevate peak-composting temperature in a short time period. Effect of temperature on antibiotic degradation and compost quality was determined by comparing different compost samples.
- Besides the antibiotic degradation, variation of physical-chemical characteristics of compost samples that might control the degradation kinetics of antibiotics and composting efficiency were simultaneously evaluated during composting process.

3. QUANTIFICATION OF MULTI-CLASS ANTIBIOTICS BY UHPLC-MS/MS ANALYSIS: COMPARATIVE EVALUATION OF DAIRY AND POULTRY MANURE

A part of this chapter was published in 'International Journal of Environmental Analytical Chemistry' journal entitled 'Quantification of Multi-class Antibiotics by UHPLC-MS/MS Analysis Combined with Salt-Assisted Acetonitrile Extraction: Comparative Evaluation of Dairy and Poultry Manure'. This chapter describes the development of antibiotic analysis method in manure samples.

3.1. Introduction

The release of antibiotics into the environment is a consequence of their use not only for human medicine but also for veterinary medicine (Kümmerer, 2009). Worldwide actions have already been taken to curb the extensive consumption of antibiotics in the agricultural sector, as these antibiotics can significantly contribute to the emergence and prevalence of antibiotic resistance, which threatens the health of both humans and animals by reducing the effectiveness of antibiotics for disease treatment. Many antibiotics fallen into the major classes are regarded as contaminants of emerging concern (Barbosa et al., 2016) due to their potential adverse effects (Aga et al., 2016; Knapp et al., 2010; Boxall et al., 2003). Therefore, actions are urgently required at multiple levels to control antibiotic pollution. In this regard, a reliable, quick, easy and sensitive multi-class analysis method for poultry and dairy waste, in which antibiotics end up, is of great concern for the scientific community and regulatory authorities, as manure has agronomic applications.

Since antibiotics represent very different physical and chemical properties, they exhibit different sorption tendency on manure matrices (Tolls, 2001) hence non-selective sample preparation procedures can be applied to obtain successful detection concerning recovery. For manure, the extraction methods of antibiotics are based on liquid portioning assisted by use of Ultrasound, Mechanical Shaking or advanced techniques such as Pressurized Liquid Extraction and Microwave Extraction. Due to non-selective extraction some of these methods have been followed by a clean-up step based on SPE by using HLB, SAX and mixed mode cation exchange cartridge to eliminate matrix interferences. However, clean-up steps are not applied in some generic extraction procedures to minimize analyte losses, solvent consumption and sample preparation time, (Spielmeyer et al., 2014; Janusch et al., 2014; Gorissen et al., 2015; Meersche et al., 2016).

The selection of appropriate organic solvent influences the performance of analytical method for simultaneous analysis of multiple classes of veterinary antibiotics. Although MeCN, MeOH, ethanol and dichloromethane were commonly selected solvents in literature (Table 1.2.). MeCN has substantial ability related to precipitate proteins, to denaturate enzymes, and to minimize the co-extraction of lipids (Kaufmann et al., 2008). However, the extraction efficiency of MeCN for polar analytes like TCs and Penicillines (β-lactams) is not sufficiently high, (Ho et al., 2012; Dasenaki and Thomaidis, 2015). On the other hand, high extraction efficiency of MeOH results in co-extraction of many matrix components and produced a very colored sample, causing the complication of following clean-up step, (Blackwell et al., 2004; Dasenaki and Thomaidis, 2015).

In addition, extraction with aqueous buffers (e.g. Mcllvaine Buffer) reduces co-extraction of non-polar matrix components, (Dasenaki and Thomaidis, 2015). To adjust the pH, Mcllvaine Buffer has been commonly used because of its wide range of pH values (2 to 8) (Dorival-Garcia et al., 2013). Regarding of those properties of extraction solvents, to enhance the extraction efficiency, bipolar extraction can be applied combining MeCN and a buffer containing complexing agents. EDTA is frequently added as a complexing agent which is necessary for FQs, TCs, (Tolls, 2001) and MCs (Wang et al., 2015) since these antibiotics have a strong tendency to form chelates with divalent metallic cations. As a purifying agent, NaCl was added in extraction solvent to determine its salting out effect for MeCN which is miscible with water. The addition of salt reduced this miscibility and lead to phase separation of MeCN from aqueous phase. As a result, mainly water-soluble matrix components could be separated from target analytes that migrate to organic phase (Dorival-Garcia et al., 2015).

In this part of this study development of a reliable, sensitive, and selective multi-residue method to quantify widespread used 7 different groups of 33 veterinary antibiotics in dairy and poultry manure at low concentrations ($\mu g.kg^{-1}$) using UHPLC-MS/MS are explained. For this purpose, chromatography, mass spectrometry and extraction conditions were optimized for selected conditions. While developing the method, purification with SPE was not examined due to its expensive, time consuming and labor sensitive aspects. Instead of using cartridge NaCl was used to separate water and organic phase that carries target compounds to following steps. Few multi-analyte methods (Pan et al., 2011; Ho et al., 2012; Zhou et al., 2012; Meersche et al., 2016) examined the matrix effect of method in manure. However, matrix effects were calculated for each method to explain recovery results in our study.

There are two options that was used as ionization source to produce ions for mass condition which are Electrospray Ionization (ESI) or Atmospheric Pressure Chemical Ionization (APCI) sources. However, ESI was generally preferred for polar analytes that more liable to matrix effect than APCI source, (Souverain et al., 2004) and our equipment includes ESI source. To enhance the ionization by improving peak shape and sensitivity of TCs, Oxalic Acid was commonly used that was known as chelating agent and has ability to prevent the clogging of the interface of MS instrument (Konak et al., 2016; Chico et al., 2008; Gajda and Posyniak, 2015). This addition was also suggested by the U.S. Environmental Protection Agency, (2007). Hence, the addition of Oxalic Acid to the aqueous part of mobile phase was assessed by developing the method ionization procedure in this study. Additionally, attention was given to maximize the signal intensities of the antibiotics, especially those of TCs, to increase their quantification sensitivity.

3.2. Materials and Methods

3.2.1. Chemicals and Antibiotic Standards

A total of 33 target veterinary antibiotics with different physicochemical properties (Appendix A) were used in this study. The analytical standards, including APs (Amphenicols); CHL (Chloramphenicol), FLF (Florfenicol), THI (Thiamphenicol), DAPs; TRI LINs; LIN, MCs; ERY, JOS (Josamycin), SPI (Spiramycin), TIL, TYL, FQs; CIP, DAN, DIF (Difloxacin), ENR, FLU (Flumequine), OXO (Oxolinic Acid), SAR (Sarafloxacin), TCs; CTC, DXC, OTC, SAs; SCP, SDZ, SDX (Sulfadoxine), SMZ, SMT (Sulfamethizole), SMH, SMX (Sulfamethoxypyridazine), SQX, SFX (Sulfisoxazole) and STZ, were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Two other FQ standards, NOR and MAR (Marbofloxacin), were supplied by Fluka (Steinheim, Germany). The internal standard DEM (Demeclocycline) and SMH D_4 (Sulfamethoxazole D_4) were obtained from Dr. Ehrenstorfer GmbH (Germany), whereas NOR D_5 (Norfloxacin D_5) and ROX (Roxithromycin) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Fluka (Steinheim, Germany), respectively. All the antibiotic standards were of the highest available purity (≥95%).

The stock antibiotic standard solutions (1 $mg.mL^{-1}$) and corresponding internal standards (2 $mg.mL^{-1}$) were individually dissolved in an appropriate solvent, which was usually MeOH, whereas OXO and NOR were dissolved in 0.1 M NaOH (Sodium Hydroxide), NOR D_5 was dissolved in Chloroform, and CIP and FLU were dissolved in MeOH including 2% (v/v) Formic Acid (HCOOH), and all of the stock solutions were stored in amber glass bottles at −30 ± 2°C. A multicomponent

working standard solution (10 mg.L^{-1} per analyte) and a mixed internal (2 mg.L^{-1} each) standard solution were prepared by appropriate dilution of the stock solution with MeOH.

HPLC-grade MeCN, (≥99.9%) and MeOH, (≥99.9%) were purchased from Merck, and Chloroform (≥99.9%) was obtained from Sigma-Aldrich. Solid reagents used in the extraction of the antibiotics from manure and in quantification with a LC–MS/MS system were all analytical grade: HCOOH, (98–100%), NaOH (99.5%), NaCl, (99.5%) and Oxalic Acid Dihydrate ($C_2H_2O_4 \cdot 2H_2O$, ≥99.5) were purchased from Merck; disodium EDTA acid dihydrate ($Na_2EDTA \cdot 2H_2O$, 99%), Ammonium Formate ($HCOONH_4$, ≥99.9 %) and Citric Acid ($C_6H_8O_7$, ≥99.5) were obtained from Sigma-Aldrich (St. Louis, MO, USA); and Sodium Phosphate dibasic (Na_2HPO_4, 98–100.5%) was purchased from BDH (GPR, UK).

3.2.2. LC-MS/MS Analysis

Analysis of the manure extracts was carried out using an LC (Agilent 1260) coupled to a triple quadrupole mass spectrometer equipped with an electrospray ionization interface (ESI-MS/MS, Agilent Technologies 6460 Triple Quadrupole). Chromatographic separation was performed by using a C18 Acquity UHPLC BEH column (1.7 μm, 100 mm × 2.1 mm) at 40°C with a VanGuard precolumn of the same packing (130 Å, 1.7 μm, 2.1 mm × 5 mm). A binary mobile phase consisting of eluant A (0.2% HCOOH in water + 1mM $HCOONH_4$ [Aqua I]) and eluant B (0.1% HCOOH + 1mM $HCOONH_4$ in MeCN) was used with a gradient elution program, which was t = 0–5 min, 10–15% B; t = 5–7 min, 15–20% B; t = 7–11 min, 20–40% B; t = 11–15 min, 40–60% B; t = 15–16 min, 60–10% B; and t=16–20 min, 10%B. In a separate run, 1mM Oxalic Acid was added to mobile phase A (Aqua II) to evaluate the effect of the composition of the mobile phase on analysis performance. The injection volume was 5 μL, running at a flow rate of 0.2 mL.min^{-1}.

Detection of ions was achieved using Jet Stream ESI source in positive and negative ion modes. Instrumental parameters were studied with several trials for selecting the optimum conditions. As a result, the MS/MS system was operated at the following working conditions; Ionspray Voltage ±3.5 kV, Nozzle Voltage 0 and 1.5 kV at positive and negative ion modes; Electron Multiplier Voltage (EMV) +115 and -600 V; Source and Sheath gas temperature 300 and 325° C; Source and Sheath gas (nitrogen) flow rate 6 and 11 L min^{-1}, respectively. Nebulizer gas (nitrogen) pressure was 45 psi. Data acquisition for quantification and confirmation was applied in the Dynamic Multiple Reaction Monitoring Mode (dMRM). All the acquisitions were controlled by Mass Hunter Software.

3.2.3. Manure

Method optimization was performed both for dairy and poultry blank manure matrices, which were free of the target antibiotics. To evaluate the differences in analytical method performance of matrices the manure samples were characterized by physicochemical properties, which are listed in Table 3.1. Additionally, manure characteristics are elaborately analyzed and described in 5th. Chapter. Total nitrogen content of manure samples was determined by the Kjeldahl method and crude protein content was calculated as 6.25 times of Total Kjeldahl Nitrogen (TKN) content on dry matter basis (Wilkie and Mulbry, 2002). Inductively Coupled Plasma (ICP) was used for the metal concentrations of manures. Muffle furnace was used for the estimation of total organic carbon content by multiplying total organic matter content with the conventional "Van Bemmelem factor" of 1.724 in five hours (Tiquia, 2005). Cation Exchange Capacity (CEC) of the manure samples was determined according to Method 9081 of Environmental Protection Agency (EPA).

Table 3.1. Physicochemical properties of manure samples.

Manure Characteristics	Dairy manure	Poultry manure
pH	8.7	8.4
CEC (mEq 100 g^{-1})	372	353
Total Organic Carbon (g kg^{-1})	527	370
TKN (g kg^{-1})	14	33
Crude protein (g kg^{-1})	88	206
Metals (mg kg^{-1})		
Fe	1,760	5,448
Al	622	14,145
Cu	161	117
Zn	218	790
Mn	240	1,451
Ca	24,071	148,750
Mg	4,903	7,982

Before spiking, blank manure samples were first dried, ground and then passed through 2 mm mesh sieve. The optimized method was applied to broiler, layer hen and dairy manures collected from different CAFOs in the Marmara region of Turkey. Manure samples transported to the laboratory were freeze dried and stored at -20 C° at most one week before analysis. The evaporation of water from collected samples were performed by lyophilization to prevent degradation of analytes.

3.2.4. Extraction of Antibiotics

After comparative evaluation of reference concentrations of antibiotics in environmental samples (e.g Karcı and Balcıoğlu, 2009; Zhao et al., 2010) spiking was performed at four concentration levels (25, 50, 100, 200 µg.kg^{-1}) since the maximum residual limit for antibiotics in manure is not recommended. After thorough mixing, the fortified manure samples with mixed antibiotic standard and corresponding internal standards were kept in the dark under the hood for 2 h at room temperature for equilibration and evaporation of solvent before extraction. Solid-liquid extraction technique to perform antibiotic analysis by using different methodologies as summarized in Figure 3.1.

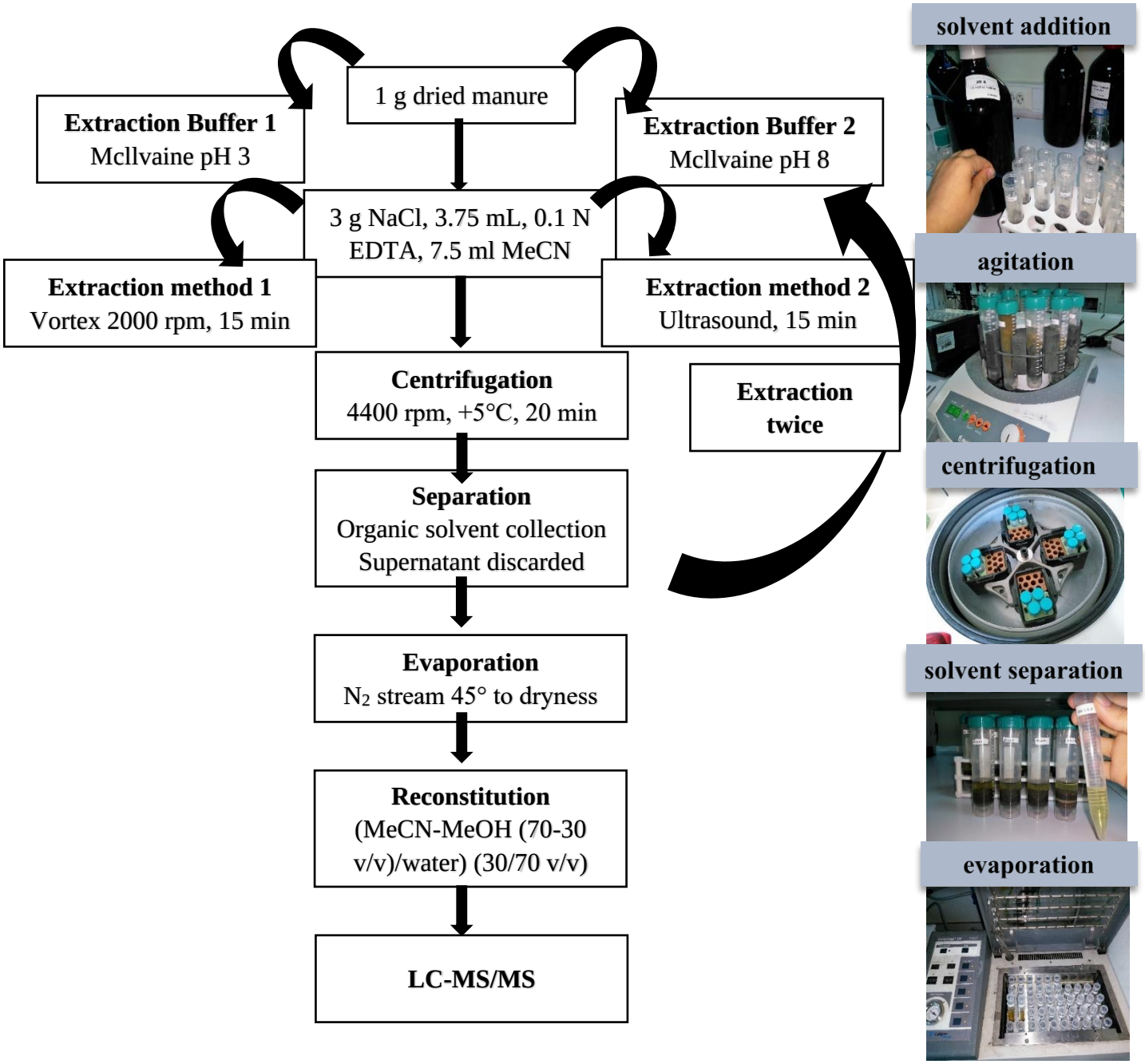


Figure 3.1. Schematic diagram of the sample extraction method.

An aliquot of 1 g spiked dairy or poultry manure placed into a 50 ml polypropylene centrifuge tube was extracted with a mixture of MeCN as organic solvent and aqueous buffer. An aqueous buffer was used at two different pH values (pH 3 and 8) in extraction: 3.75 mL Mcllvaine buffer =100 mM Citric Acid + 100 mM Na_2HPO_4. As chelating agent, 0.1 M EDTA was added in each case. Efficient contact of manure samples with extraction solvent was accomplished by using either mechanical shaker (Heidolph, Germany) or ultrasonic bath under predefined conditions (Figure 3.1). After centrifugation (Heidolph, Germany), salt-induced organic phase separation was carried out and the supernatant was transferred to a 15 ml tube. The manure was extracted once more and combined supernatants were subjected to evaporation to dryness under a gentle stream of nitrogen at 45°C with automated evaporation system (Caliper, Germany). Obtained residue was reconstituted with 50 µL of MeCN+MeOH (70-30 v/v)): H_2O (30/70 v/v). Without application of additional cleanup procedure samples were filtered through 0.2 µm (PTFE-membrane, Sartorius) filter before injection to LC-MS/MS.

3.2.5. Method Performance

It is known that there is not any document established for performance criteria of analytical method specifically for antibiotics in manure. Although European Commission Decision 657/EC (2002), which is for the residues in the products of animal origin, was extended to environmental samples in some studies (e.g. Guo et al., 2016; Gorissen et al., 2015; Meersche et al., 2016), the complex nature of manure could limit its strict application (Berendsen et al., 2015; Spielmeyer et al., 2014). In this thesis, the performance of applied analysis method was evaluated as a set of experimental tests which determines specificity, linearity, recovery, repeatability, LOD, limit of quantification (LOQ), and matrix effect based on relevant publications (EC 2002, Burns et al., 2002; Matuszewski et al., 2003; Chandran and Singh, 2007). In table 3.2, the calculation of these parameters is summarized.

The linearity and recovery tests consisted of non-fortified and fortified manure samples at four different concentration levels (25-200 $\mu g.kg^{-1}$) of target antibiotics and fixed concentration (400 $\mu g.kg^{-1}$) of internal standards. Linearity of calibration curves was obtained by least-squares linear regression analysis of analyte/internal standard the response ratio versus concentration of analyte/internal standard ratio. The results of non-fortified manure samples were also used to evaluate specificity of the applied method. Repeatability was evaluated as Relative Standard Deviation (RSD) at each concentration of each matrix for each method. The calculations for the LOD and LOQ were based on the residual standard deviation of the linear regression (SD) and the

slope (b) of the method linearity regression line. Additionally, matrix effect (ME) was evaluated by performing separate experiments in which the calibration curve of antibiotic standards in solvent and in matrix extract were established.

Confirmation of the identity of selected antibiotics in manure samples was performed by the evaluation of precursor ion, product ions and retention time. The relative retention time of each antibiotic corresponded to that of the standard solution at a tolerance of 2.5% as confirmation criteria (EC, 2002). The extracts of manure samples with antibiotic concentrations out of the range of calibration curve (25–200 $\mu g.kg^{-1}$) were estimated by extrapolation.

Table 3.2. Method performance characteristics for the determination of antibiotics in manure samples.

Method Performance Characteristics	Calculation	Comment
Apparent recovery	$\text{Recovery (\%)} = \frac{\text{Cm}}{\text{Cs}}$ x100	Cm=Measured concentration Cs=Spiked concentration
Repeatability	$\text{RSD (\%)} = \frac{(100 \text{ x SD})}{\text{Mean concentration}}$	SD= Standard deviation
Limits of detection and quantification	$\text{LOD} = \frac{3\text{xSD}}{\text{b}}$ $\text{LOQ} = \frac{10\text{xSD}}{\text{b}}$	b=Slope of matrix spiked calibration curve
Matrix effect	$\text{ME (\%)} = (\frac{\text{E}}{\text{S}} - 1) \times 100$	E=Slope of analyte calibration curve prepared in matrix extract S=Slope of analyte calibration curve prepared in solvent

3.3. Results and Discussion

3.3.1. Optimization of Mass Spectrometry Parameters

As part of the method development, the first step was to optimize the mass spectrometry parameters by infusing each of the 33 antibiotic standard solutions at a concentration of 10 $ng.mL^{-1}$

in MeOH with a mobile phase flow rate of 0.2 $mL.min^{-1}$. Following the isolation of the most-abundant parent ion, two characteristics of MRM transitions for each antibiotic were acquired by collision-induced dissociation of the precursor ion, whereas for the internal standards, one qualifier was monitored. Considering its higher sensitivity, the positive-ion mode was selected for all antibiotics, except the APs. The results of the precursor ions, product ions and MS/MS parameters under the optimized conditions for the instrument, are presented in Table 3.3.

Table 3.3. MRM Parameters for antibiotic standards.

Compound	Precursor Ion (m/z)	Product Ion 1 (m/z)	Collision Energy (Ev)	Product Ion 2 (m/z)	Collision Energy (Ev)	Fragmentor (V)
SCP	285.0	156.0	14	91.8	28	100
SDZ	251.0	185.0	15	156.0	10	120
SDX	311.1	156.0	12	92.0	30	140
SMZ	279.1	186.0	12	92.1	28	90
SMT	271.0	156.1	8	92.0	26	90
SMH	254.1	156.0	10	92.0	26	100
SMX	281.1	156.0	12	92.1	28	100
STZ	256.0	156.0	10	92.0	24	90
SQX	301.0	155.8	18	91.8	30	170
SFX	268.0	155.8	13	91.8	28	120
CIP	332.1	314.0	17	288.0	13	120
DAN	358.0	314.0	26	96.0	36	120
DIF	400.2	356.0	14	299.0	26	130
ENR	360.1	342.3	17	316.4	15	120
FLU	262.1	202.1	32	174.0	35	100
MAR	363.0	320.0	18	72.0	20	120
NOR	320.1	276.1	12	233.1	20	120
OXO	262.0	244.0	12	216.0	28	100
SAR	386.1	368.1	18	299.0	27	140
ERY	734.4	576.3	14	158.2	30	180
JOS	828.5	174.0	30	109.0	46	150
SPI	843.4	539.8	35	174.1	40	270
TIL	869.5	696.4	44	174.1	49	320
TYL	916.4	772.4	30	174.2	40	280
CTC	479.0	444.0	20	153.9	28	120
DXC	445.2	410.1	20	428.1	30	110
OTC	461.3	443.1	9	426.3	15	120
TC	445.0	410.0	20	153.9	25	120
CHL	321.0	257.0	2	152.0	8	120
FLF	356.0	336.0	4	185.0	12	120
THI	354.0	290.0	2	185.0	8	110
LIN	407.2	359.0	15	126.0	30	150
TRI	291.1	230.1	22	123.1	22	120
SMH D_4	258.1	160.0	10			100
NOR D_5	325.1	307.1	16			130

Table 3.3. Continued

Compound	Precursor Ion (m/z)	Product Ion 1 (m/z)	Collision Energy (Ev)	Product Ion 2 (m/z)	Collision Energy (Ev)	Fragmentor (V)
ROX	837.4	158.1	34			190
DEM	465.0	448.0	12			120

3.3.2. Preliminary Studies

For the preparation of method development, detailed preliminary studies were carried out to make method applicable for 33 antibiotics from 7 different groups. Results of these preliminary studies are summarized in this part. Several preliminary experiments were performed by using different mobile phases consisting of MeOH or MeCN and water with different concentrations of formic acid and acetic acid (0.01–0.5%) for simultaneous analysis of the target antibiotics from seven different classes, as it is known that the composition of the mobile phase can have an influence on both chromatographic separation and the signal intensity of the various analytes observed by LC–MS analysis (Kostiainen and Kauppila, 2009). Although the combination of formic acid and ammonium format in both water (Aqua I) and MeCN resulted in high responses of the antibiotics, as the results reported in previous studies (e.g. Refs. (Ho et al., 2012; Berendsen et al., 2015)), the addition of Oxalic Acid to the aqueous phase of the binary mobile phase (Aqua II) resulted in a pronounced improvement in the simultaneous detection of target antibiotics in a mixed standard solution in terms of peak intensity and peak shape. Notably, variation of the composition of the mobile phase led to a shift in the retention time, which was considered during analysis of the manure extracts.

As a first concern, gradient total run time of chromatographic separation was determined by applying it from total 10 to 20 min options. Other LC-MS/MS conditions were kept constant while gradient run of chromatographic separation was determined. Although apparent recoveries of most of antibiotics were more successful when 10 min gradient run (t=0-0.5 min, 5-25% B; t=0.5-5.5 min, 25-90% B; 5.5-7 min, 90-5% B; and 7-10 min, 5% B), 20 min alternative of the gradient run was applied (t = 0-5 min, 10-15% B; t = 5-7 min, 15-20% B; t = 7-11 min, 20–40% B; t = 11-15 min, 40-60% B; t = 15-16 min, 60-10% B; and t=16-20 min, 10%B). Indeed, repeatability of 10 min gradient run method could not be as successful as 20 min run due to peak shape disturbances and poor peak sensitivity resulted from characteristics of multi-residue method. Hence, chromatographic separation was performed with total 20 min gradient run for following method development studies. Acquired amount of manure, extraction solvent, buffer and salt for prosperous extraction were optimized by changing the concentrations in preliminary studies.

After evaluating the method performance, the parameters were optimized by using Aqua II as the aqueous mobile phase, MeCN/EDTA/McIlvaine buffer at pH 3 and pH 8 as the extraction solvent and a mechanical shaker to assist extraction of the dairy manure matrices (3.4 and 3.5) for total 10 min gradient run of chromatographic separation. Method performance for the quantification was in the concentration range of 25-200 $\mu g.kg^{-1}$. To evaluate the extraction performance signal responses were demonstrated at logarithmic scale (Figure 3.2) at 200 $\mu g.kg^{-1}$. pH 8 buffer improved signal responses of SAs and FQs whereas pH 3 accelerated MCs. Signal responses of other antibiotics were nearly identical for both pH conditions. Resembling the similar results as signal responses of antibiotics validation results of extraction buffers demonstrated nearly identical performance. The obtained results show that 30 of 33 antibiotics could be detected with acidic buffer and 28 of 33 antibiotics with alkaline buffer. Linearity of some investigated antibiotics had a lack of performance which must be with a correlation coefficient of at least 0.90. Due to low signal intensity and lack of repeatability of investigated methods linearity of correlation coefficient was poor hence SCP, SQX, OTC at pH3 and SQX, SAR, CIP, DAN, LIN at pH 8 buffer could not be detected in dairy manure. All in all, gradient run for chromatographic separation was increased to 20 min in following sections to improve the method.

Table 3.4. Method performance parameters for dairy manure with pH 3 extraction buffer in antibiotic concentration range of 25-200 $\mu g.kg^{-1}$.

	Mean recovery (%) (intra-day RSD%) n=2				RT	Linearity
	25 ($\mu g.kg^{-1}$)	50 ($\mu g.kg^{-1}$)	100 ($\mu g.kg^{-1}$)	200 ($\mu g.kg^{-1}$)	(min)	(R^2)
SDZ	112.8 (13.0)	93.7 (8.6)	101.0 (8.4)	102.3 (0.3)	2.9	0.998
STZ	156.8 (44.4)	99.0 (11.1)	98.3 (2.0)	100.0 (1.9)	3.7	0.996
SMZ	118.0 (18.7)	95.8 (5.6)	108.3 (15.4)	105.0 (4.7)	4.8	0.998
SMT	185.0 (63.4)	114.2 (5.9)	107.5 (32.2)	130.3 (26.3)	5.2	0.975
SMX	109.8 (15.2)	109.1 (6.4)	109.0 (14.3)	103.7 (4.4)	5.2	0.999
SDX	117.2 (13.0)	92.2 (0.3)	120.6 (59.3)	91.2 (15.3)	6.4	0.996
SMH	96.4 (17.0)	138.7 (15.0)	109.8 (25.5)	105.7 (6.5)	6.4	0.973
SFX	111.2 (26.5)	101.3 (15.5)	104.4 (8.3)	102.3 (4.5)	6.8	0.993
DIF	109.0 (14.3)	104.6 (5.9)	92.2 (16.7)	99.0 (0.5)	6.3	0.999
MAR	107.4 (16.6)	108.5 (2.0)	96.7 (3.4)	93.2 (9.5)	5.0	0.997
OXO	91.2 (1.2)	105.3 (7.7)	86.6 (22.1)	92.0 (9.9)	7.1	0.994
FLU	99.2 (1.1)	102.7 (4.8)	87.5 (21.8)	97.6 (3.0)	7.1	0.999
SAR	99.0 (7.1)	95.8 (17.7)	88.8 (15.9)	95.7 (5.5)	6.2	0.997
NOR	94.2 (0.9)	117.3 (15.3)	78.5 (42.3)	96.7 (2.6)	5.2	0.998
ENR	100.6 (9.3)	100.0 (11.3)	76.1 (42.0)	95.9 (5.2)	5.7	0.997
DAN	104.8 (2.7)	94.6 (3.6)	86.0 (22.4)	98.7 (3.6)	5.6	0.997
CIP	118.6 (43.6)	98.8 (7.4)	70.0 (80.8)	93.8 (4.5)	5.3	0.985
SPI	127.8 (25.9)	104.0 (9.2)	94.9 (2.5)	101.7 (0.3)	6.9	0.998
ERY	131.4 (32.9)	130.5 (34.4)	98.7 (2.7)	104.8 (6.6)	7.6	0.999
TYL	111.4 (15.0)	105.5 (4.4)	95.3 (3.1)	101.2 (0.6)	7.7	0.999
JOS	102.2 (8.0)	98.5 (5.0)	99.4 (5.5)	103.9 (7.7)	8.2	0.998
TIL	98.0 (12.1)	98.7 (19.1)	106.0 (8.1)	103.3 (6.8)	7.4	0.993
TC	142.2 (47.5)	110.7 (3.4)	96.3 (2.5)	94.8 (7.6)	5.6	0.997
DXC	90.2 (9.1)	93.7 (17.7)	102.9 (5.6)	96.3 (5.0)	7.1	0.998
CTC	100.2 (12.1)	99.1 (6.4)	102.2 (12.8)	110.8 (8.6)	6.8	0.993
THI	84.8 (11.3)	102.4 (8.8)	115.8 (16.9)	109.0 (14.3)	5.0	0.998
FLF	89.4 (6.6)	97.4 (12.5)	107.5 (6.6)	102.6 (6.1)	6.5	0.997
CHL	102.8 (13.2)	99.9 (10.1)	105.0 (5.4)	101.7 (4.3)	7.1	0.997
TRI	103.4 (11.8)	101.7 (5.1)	105.0 (5.4)	102.4 (4.9)	5.0	0.998
LIN	112.6 (19.3)	89.6 (24.3)	105.5 (10.8)	110.8 (13.2)	4.5	0.998

*RT (Retention Time), R^2 (Coefficient of Determination)

Table 3.5. Method performance parameters for dairy manure with pH8 extraction buffer in antibiotic concentration range of 25-200 $\mu g.kg^{-1}$.

	Mean recovery (%) (intra-day RSD%) n=6				RT (min)	Linearity (R^2)
	25 ($\mu g.kg^{-1}$)	50 ($\mu g.kg^{-1}$)	100 ($\mu g.kg^{-1}$)	200 ($\mu g.kg^{-1}$)		
SDZ	99.4 (3.4)	95.4 (1.3)	101.0 (3.8)	100.3 (1.0)	3.6	0.999
STZ	108.5 (5.1)	100.5 (4.0)	94.1 (7.0)	97.5 (5.4)	3.9	0.999
SMZ	93.2 (9.4)	99.5 (3.1)	98.7 (3.5)	100.7 (2.1)	4.4	0.995
SMT	96.7 (4.0)	94.7 (3.1)	103.6 (0.1)	100.5 (1.9)	4.5	0.994
SMX	97.2 (5.3)	89.7 (1.4)	93.9 (3.1)	99.4 (1.1)	4.8	0.996
SCP	106.0 (0.8)	99.1 (6.9)	95.3 (0.3)	99.9 (0.3)	4.9	0.999
SDX	102.4 (2.4)	96.5 (2.0)	100.2 (1.6)	99.4 (3.8)	5.0	0.997
SMH	101.1 (3.3)	99.4 (3.4)	103.6 (0.4)	101.2 (1.4)	5.0	0.998
SQX	101.6 (2.5)	98.8 (0.6)	102.1 (0.5)	100.5 (0.2)	5.4	0.998
DIF	97.1 (4.6)	97.9 (0.9)	101.2 (4.8)	102.4 (4.1)	4.9	0.998
MAR	100.5 (2.8)	99.1 (2.7)	100.7 (1.8)	100.7 (0.6)	4.3	0.998
OXO	100.9 (6.1)	96.9 (0.9)	102.0 (2.9)	104.9 (0.8)	5.9	0.948
FLU	100.9 (4.5)	102.0 (1.6)	101.4 (0.1)	102.3 (0.7)	5.8	0.996
NOR	150.3 (8.8)	96.0 (9.8)	85.7 (3.9)	98.0 (7.8)	4.5	0.973
ENR	81.6 (118.7)	81.7 (6.9)	93.5 (7.9)	101.1 (0.3)	4.7	0.981
SPI	100.5 (5.7)	100.7 (1.6)	102.0 (0.7)	99.8 (1.1)	5.4	0.997
ERY	101.6 (1.5)	102.9 (1.0)	99.2 (1.2)	101.0 (0.9)	5.6	0.997
TYL	98.7 (3.9)	100.9 (3.5)	99.8 (1.8)	99.2 (0.0)	5.7	0.998
JOS	101.0 (6.0)	101.5 (1.7)	100.5 (1.3)	100.2 (0.7)	6.1	0.997
TIL	105.5 (2.1)	100.2 (1.1)	109.6 (0.7)	101.6 (0.2)	6.4	0.991
OTC	95.4 (9.1)	96.8 (9.1)	98.7 (1.3)	100.3 (1.1)	4.4	0.998
TC	100.7 (4.7)	102.3 (3.8)	103.0 (0.1)	99.5 (0.5)	4.6	0.998
DXC	104.2 (10.6)	100.7 (5.6)	100.2 (0.8)	100.4 (2.4)	4.6	0.997
CTC	103.0 (4.1)	101.4 (1.1)	100.3 (1.8)	99.1(0.3)	5.1	0.998
THI	103.7 (6.1)	79.1 (37.2)	102.5 (3.0)	99.4 (0.7)	4.4	0.999
FLF	102.7 (3.6)	98.4 (6.1)	101.1 (2.3)	100.5 (1.5)	5.0	0.997
CHL	99.5 (4.0)	97.5 (3.1)	96.8 (0.6)	100.9 (0.6)	5.3	0.995
TRI	101.4 (16.1)	93.1 (11.1)	99.3 (2.2)	96.5 (6.6)	4.4	0.997

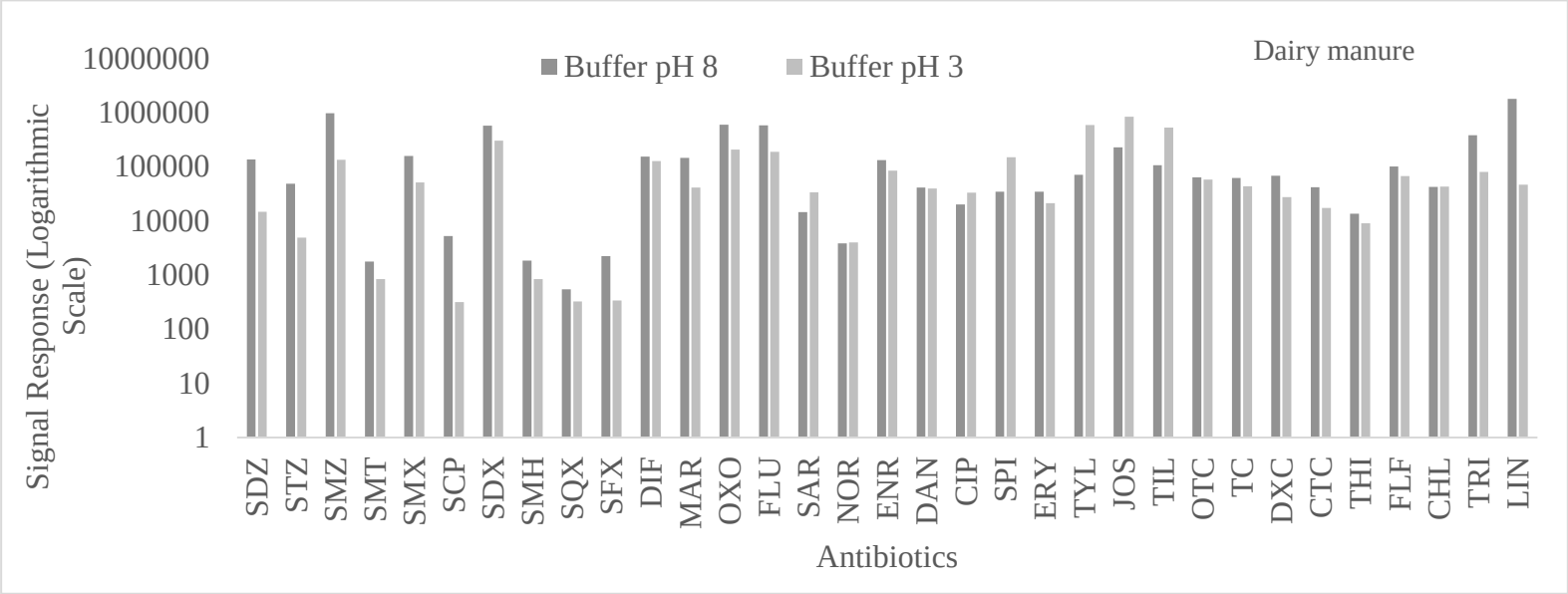


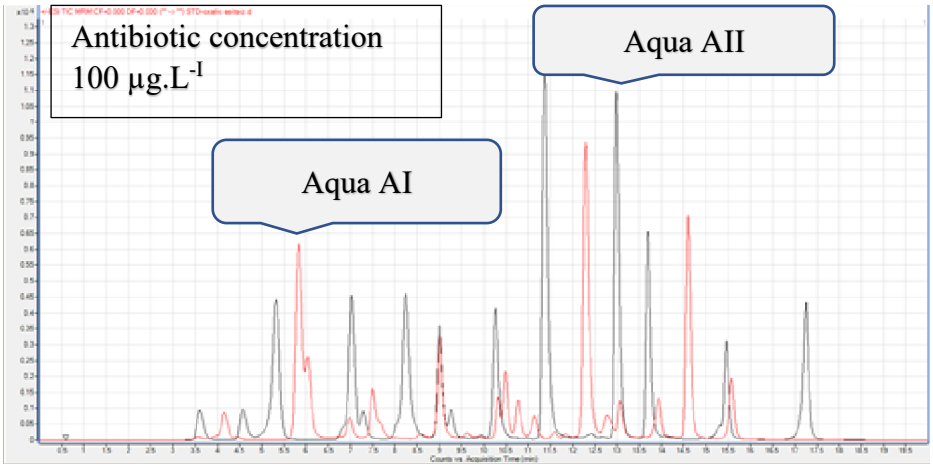
Figure 3.2. Effect of aqueous buffer pH on the signal responses of antibiotics (200 μg.kg^{-1}) in dairy manure samples.

3.3.3. Development of a Sensitive LC-MS/MS Method

Although the combination of Formic Acid and Ammonium Format in both water and MeCN resulted in relatively higher responses of antibiotics the addition of Oxalic Acid to the aqueous phase of binary mobile phase (Aqua II = 0.2% HCOOH, 1 mM $HCOONH_4$ and 1 mM Oxalic Acid) provided pronounced improvement in the simultaneous detection of some antibiotics extracted from poultry and dairy manure matrices by using a solution of MeCN/EDTA/Mcllvaine acidic buffer and these increases are shown in Figure 3.4 for each antibiotics with signal enhancement ratios (signal response with Aqua II/Aqua I). As shown in total ion chromatograms of mixed antibiotic standard (Figure 3.3) achieved with two different aqueous mobile phases (Aqua I and II), the addition of Oxalic Acid to the mobile phase (AII) increased the signal intensity of some antibiotics together with a shift in their retention time (Figure 3.4) and these time shifts were considered during the analysis of manure extracts. Except for Sulfamonomethoxine and Sulfadimethoxine, retention times of other 33 antibiotics could be distinguished hence it led to the separation of their signal responses.

The positive influence of adding Oxalic Acid to the aqueous mobile phase was previously described by several authors for TCs (Pena et al., 2007; Chico et al., 2008; Zhu et al., 2001) and this addition was also suggested by the EPA, (2007). However, the results of this study indicate that the beneficial effect is not limited to TCs, the addition of 1mM Oxalic Acid to the aqueous mobile phase (Aqua II) also increased the signal responses of 94% and 70% of the investigated antibiotics extracted from dairy and poultry manure samples, respectively.

As shown in Figure 3.4 in dairy manure, only SPI exhibited lower responses with Aqua II compare to those obtained with Aqua I whereas in poultry manure 10 antibiotics showed similar behavior and among these antibiotics, FLF, THI and CHL have noticeably low responses with Aqua II in this manure. The responses of antibiotics in poultry manure were generally lower than those in dairy manure (except for ERY, TYL, TC, and CTC), since poultry manure with higher protein and metal contents has more complex characteristics than dairy manure, as shown in Table 3.1. However, the antibiotics exhibited generally similar amount of response in both manure samples and the enhancements in the signal responses for OTC, DXC, and CHL were remarkable in dairy manure and OTC, DXC in poultry manure with Aqua II (Figure 3.4). Considering the increased signal intensity in most antibiotics, subsequent studies were performed with Aqua II.



Antibiotics	Aqua AI RT (min)	Aqua AII RT (min)
SDZ	3.6	3.6
STZ	4.5	4.6
SMZ	10.8	11.4
SMT	7.6	8.1
Sulfamonomethoxine	9.6	6.8
SMX	9.6	6.8
SCP	9.9	10.4
Sulfadimethoxine	10.8	11.4
SDX	10.8	11.4
SMH	11.1	11.5
SQX	13.2	7.9
SFX	11.8	10.9
DIF	10.5	11.4
MAR	6.0	7.3
OXO	14.6	15.5
FLU	14.6	15.5
SAR	10.3	11.3
NOR	7.0	7.3
ENR	9.0	10.3
DAN	8.6	6.0
CIP	7.5	7.5
SPI	11.6	12.7
ERY	17.5	14.2
TYL	13.9	14.0
JOS	15.6	15.8
TIL	12.8	13.0
OTC	6.7	7.4
TC	6.2	7.3
DXC	6.2	7.3
CTC	11.5	11.4
THI	6.8	6.3
FLF	11.2	11.7
CHL	12.4	13.1
TRI	5.8	7.0
LIN	4.2	5.3

Figure 3.3. Ion chromatograms of mixed antibiotic standards for Aqua I-II mobile phase.

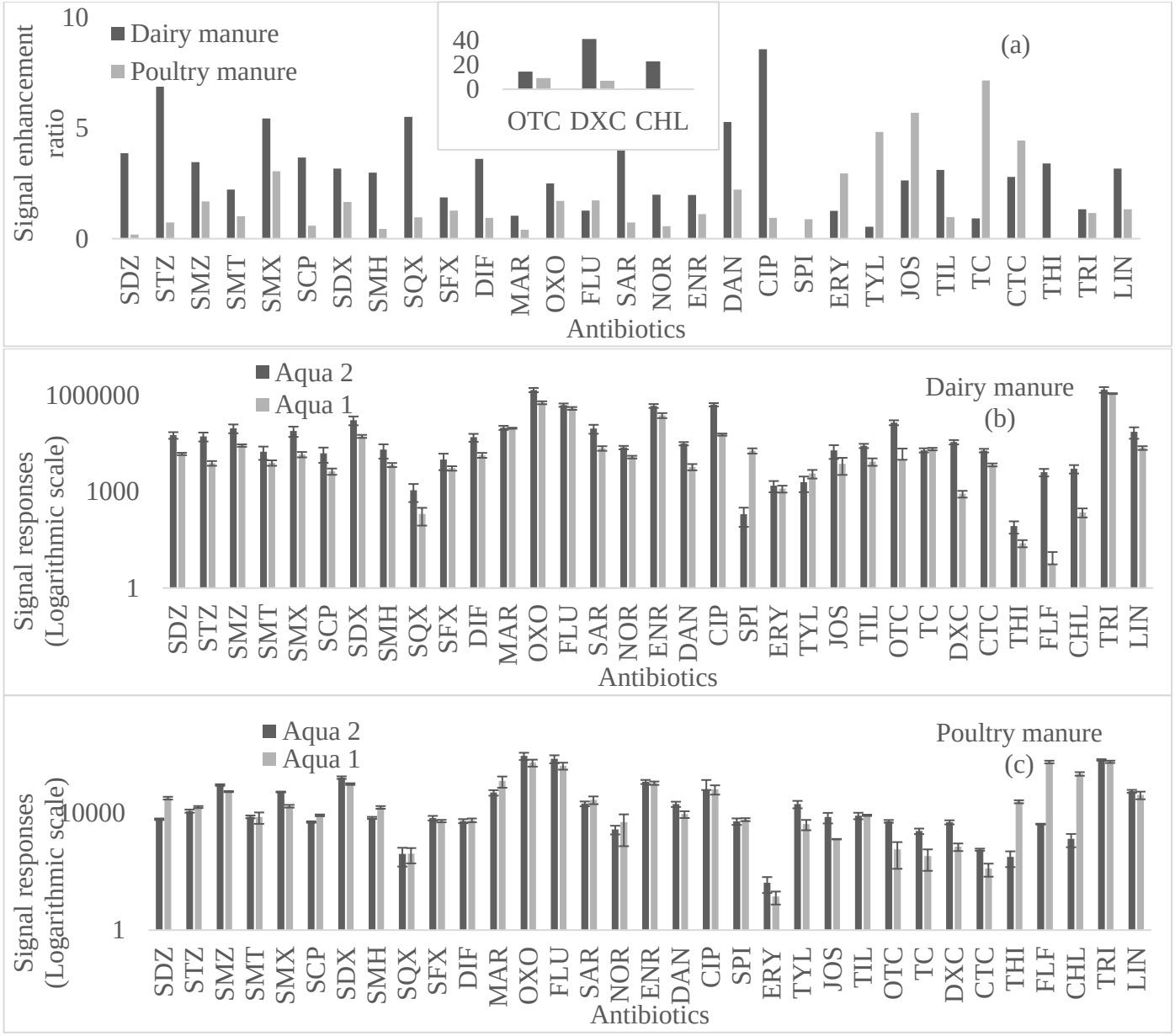


Figure 3.4. Signal responses (b, c) and enhancement ratios (a) of antibiotics (each 100 μg.kg^{-1}) with Aqua I-II in dairy and poultry manure samples.

The effects of the composition of the mobile phase on overall analysis performance were compared by the parameters given in Table 3.2 for both manure matrices. By using Aqua II as the mobile concentration range of 25–200 μg.kg^{-1}, apparent recovery and RSD percentages obtained at a concentration of 100 μg.kg^{-1} are shown as an example in Figure 3.5. This figure is constituted as comprising the results of antibiotics that were quantitatively analyzed for the selected concentration range. Although a noticeable signal response enhancement of antibiotics could positively influence the performance of analysis, the matrix components of manure samples could represent a limitation in quantitative analysis. For instance, the signal response of THI in dairy manure samples was increased at a pronounced ratio by using Aqua II mobile phase (Figure 3.4) but this was not enabled for quantification of THI in poultry manure due to poor analytical performance (e.g. apparent recovery >150).

As deduced from Figure 3.5, the recovery and repeatability percentages of all the investigated compounds achieved with this method by using Aqua II as the mobile phase were quite satisfactory in dairy manure, whereas there were a few exceptions in poultry manure. Drastic differences were not observed in the recovery values of both manure matrices, although the signal intensities of the antibiotics were dependent upon the type of manure matrix. This result can be clearly attributed to the addition of internal standards to each manure matrix to compensate for matrix effects and possible losses during extraction.

The positive influence of the Aqua II mobile phase on the matrix effect was also assessed in Figure 3.6. A very high matrix effect in extracts from both types of manure was reduced, especially for LIN and TCs, by the addition of Oxalic Acid to the aqueous mobile phase. Even if the various manure samples were extensively purified prior to tandem MS (LC–MS/MS) analysis, co-eluted components of the matrices could result in an increase or decrease in the intensity of the signals of the antibiotics (Ho et al., 2012; Zhou et al., 2012; Salvia et al., 2012). Therefore, matrix-matched calibration with suitable internal standards is a prerequisite to increase the accuracy of the analysis.

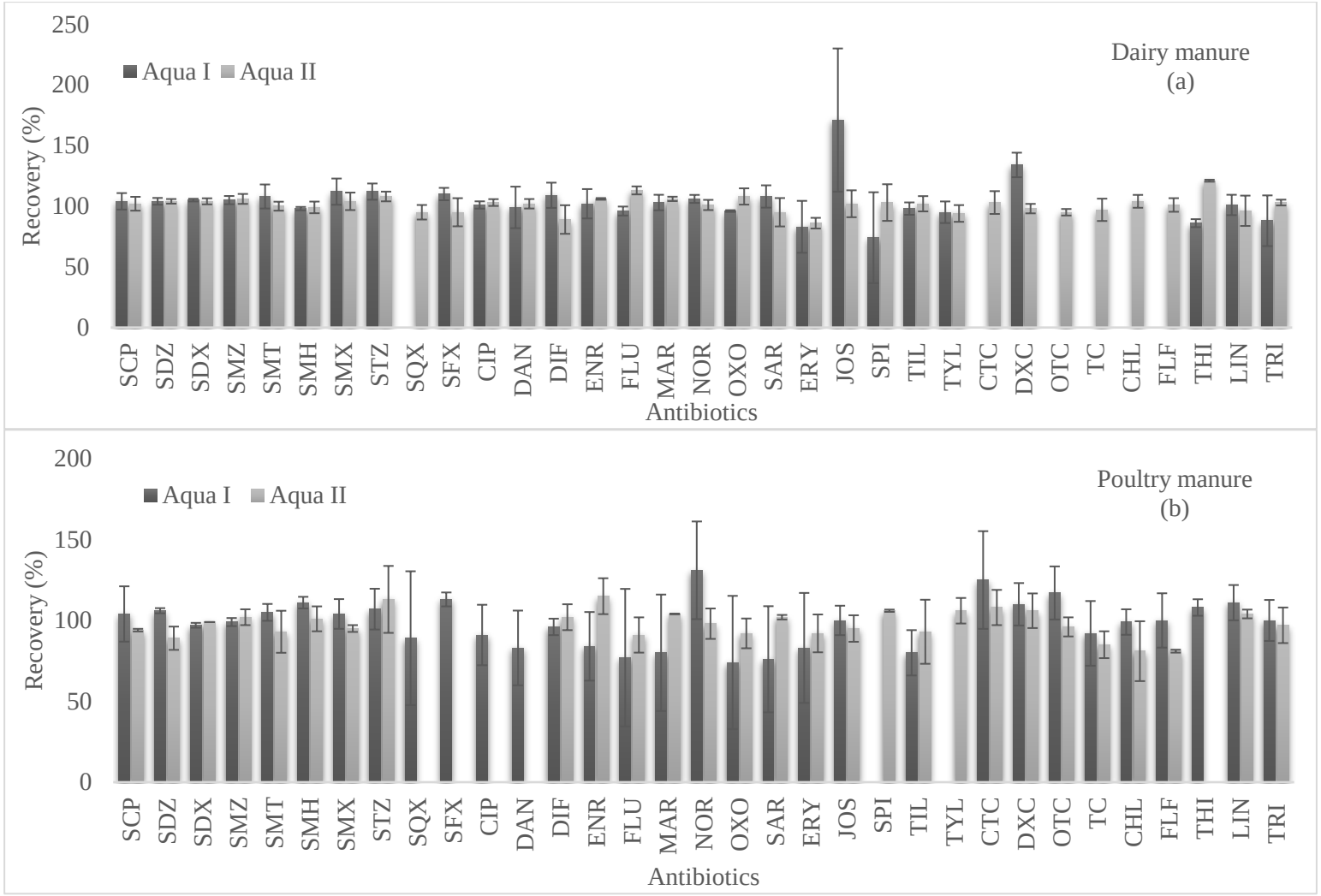


Figure 3.5. Effect of aqueous mobile phase on the recovery and RSDs percentages of antibiotics (each 100 µg kg−1) in dairy (a) and poultry (b) manure samples. Mobile phase Aqua I = 0.2% HCOOH,1 mM $HCOONH_4$ in water; Aqua II = 0.2% HCOOH, 1 mM $HCOONH_4$ and 1 mM Oxalic Acid in water (n = 3).

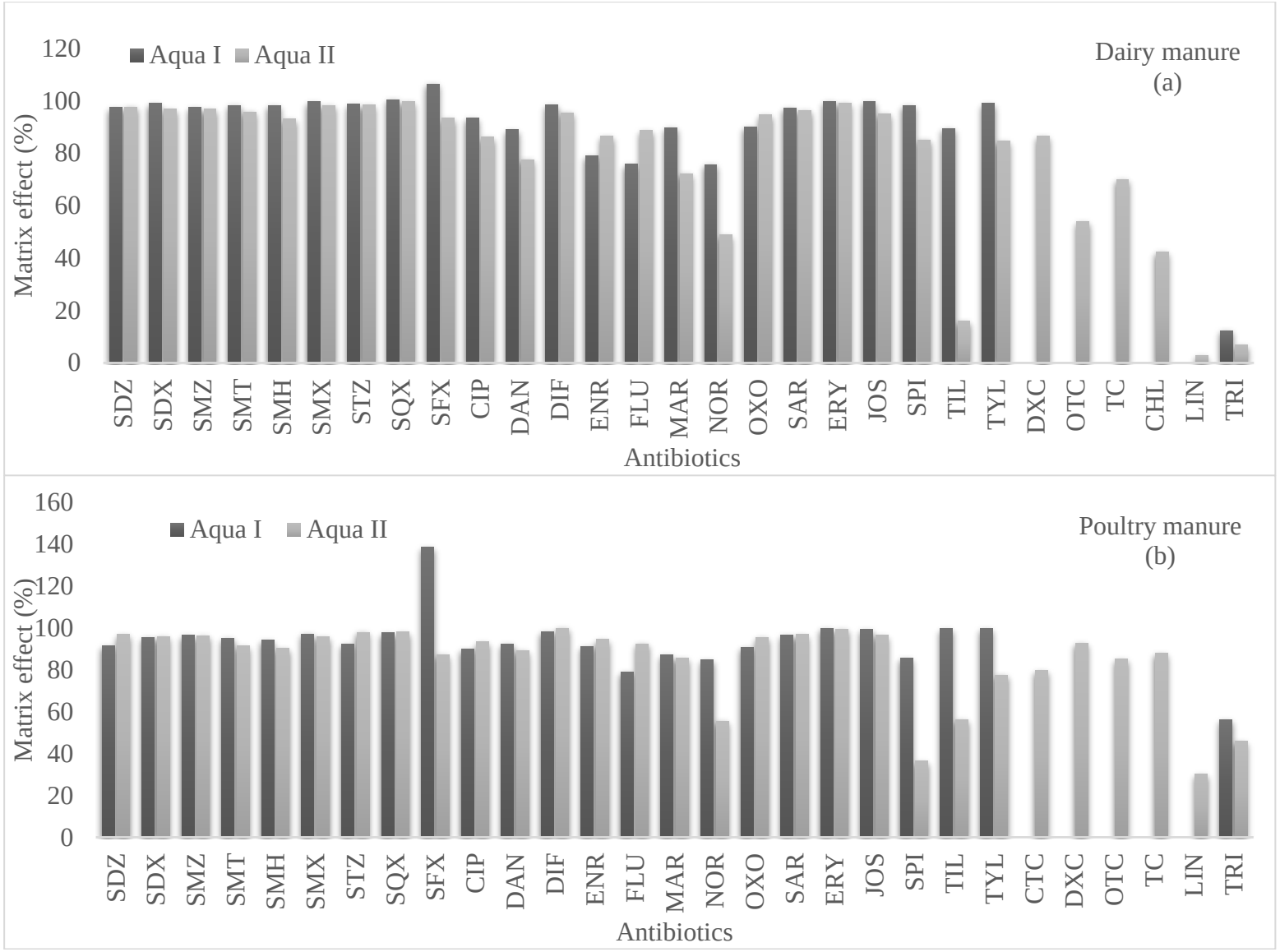


Figure 3.6. Matrix effect in determination of antibiotics (n=3, 25-100 µg/kg) in dairy manure (a) and poultry manure (b) by using two different mobile phases in LC-MS/MS analysis. The antibiotics with ME>150 are not shown.

3.3.4. Development of a Sample Extraction Method

To achieve simultaneous analysis of multi-class antibiotics in both dairy and poultry manure samples, a sample preparation method was developed with an extraction solvent composed of MeCN and EDTA/McIlvaine buffer at a ratio of 50:50 (v/v) at two different pH values of the McIlvaine buffer and with the assistance of mechanical or ultrasonic extraction without a clean-up step. The results obtained for the targeted antibiotics at four different concentration levels (25, 50, 100 and 200 $\mu g.kg^{-1}$ each) were compared in terms of antibiotic apparent recovery constituted with matrix matched calibration and linearity of these calibration curves. Although the combination of MeCN and MeOH was suggested for several multiresidue antibiotic analysis in manure (Ho et al., 2012; Guo et al., 2016), MeCN was the common organic solvent utilized in the extraction because of its easy separation of organics from the aqueous phase by salting out. This type of extraction method based on salt-induced phase separation of MeCN was already used to develop for high-throughput analysis of pesticides in food matrices (Anastassiades et al., 2003), and then the QuEChERS method was extended to the analysis of antibiotics in various matrices; however, this solvent composition for the extraction of pesticides is not suitable for analysis of the TC and FQ antibiotics, which requires the addition of a chelating agent, Na_2EDTA, as shown in matrices other than manure (Bourdat-Deschamps et al., 2014; Frenich et al., 2010). In addition, Meersche et al., (2016) applied the original QuEChERS method to liquid manure, but a 10-fold decrease in the recovery of TCs was observed that might result from the lack of Na_2EDTA in extraction solvent. Considering the results of studies performed with and without QuEChERS, 0.1 M EDTA with McIlvaine buffer in a 50:50 (v/v) ratio was used as the aqueous extraction solvent to achieve not only SA and MC antibiotics but also a high recovery rate for the TC and FQs.

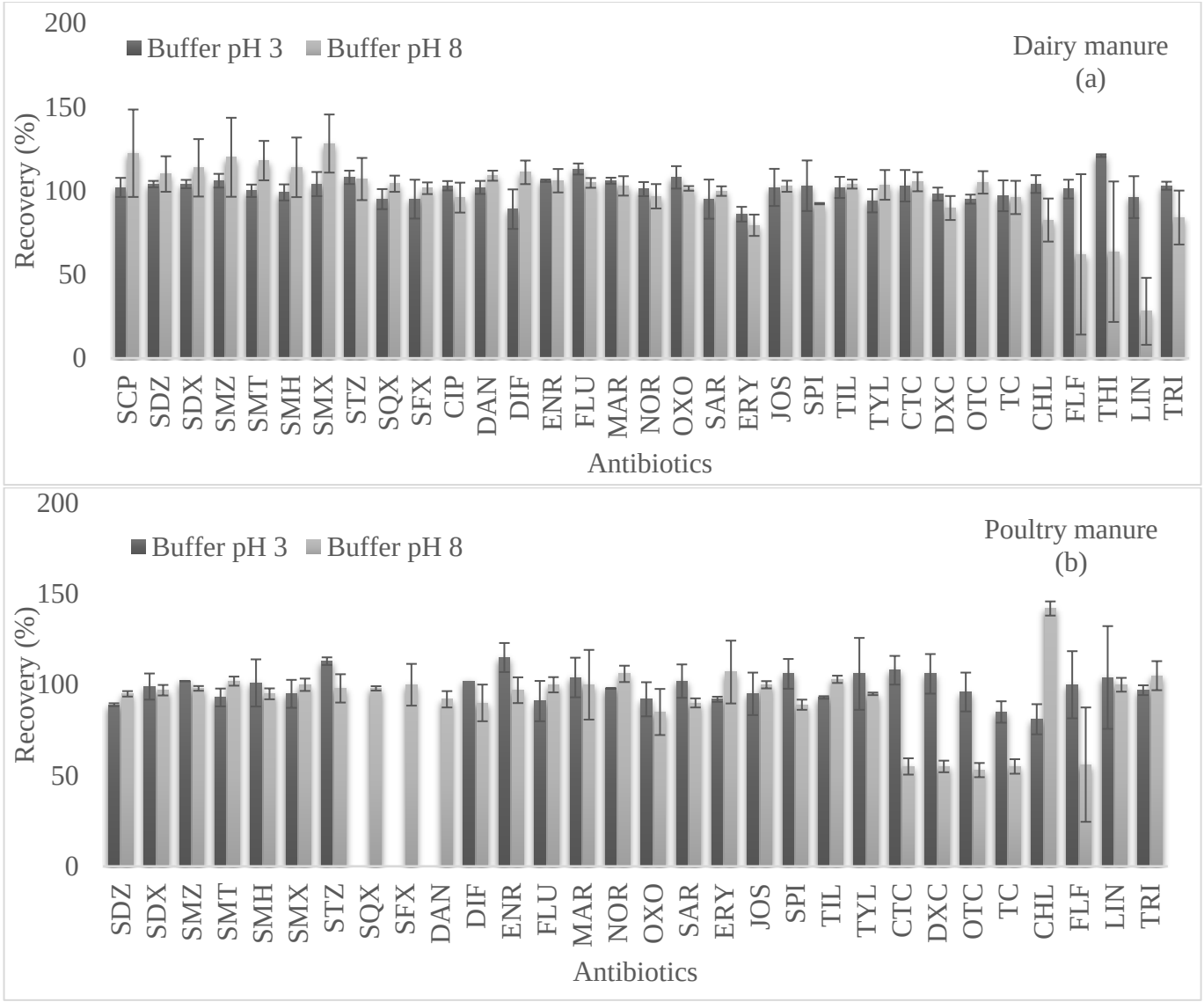
It must be pointed out that a lower dose of EDTA in the extraction solvent led to lower recoveries, especially from poultry manure owing to its relatively high metal content, as shown in Table 3.1. To induce the salting-out effect to promote the partition of water from the organic phase in which the antibiotics were concentrated, only NaCl was applied, as unsatisfactory results were achieved for TCs and FQs in the presence of $MgSO_4$; this contrasts QuEChERS methodology, which suggests the use of both $MgSO_4$ and NaCl. Although Uslu and Balcıoğlu (2009) applied magnesium nitrate to cow manure to solubilize OTC and although Huang et al. (2013) utilized an extraction solvent containing a magnesium salt for the multiresidue analysis of antibiotics in poultry manure, $MgSO_4$ as a dehydrating agent in salting-out extraction reduces the recovery of TCs and FQs probably because of the formation of insoluble complexes during hydration of this salt (Tolls, 2001). A negative effect of magnesium salts on the recovery of TCs and FQs was observed in some other studies performed on

various matrices (Chung et al., 2017; Dorival Garcia et al., 2015) Furthermore, in the study of Peysson and Vulliet (2013), the inability to extract TC and FQ compounds was attributed to the use of $MgSO_4$ as a drying agent. However, Guo et al. (2016) did not mention such an adverse effect for liquid manure.

Bearing in mind that the ratio of solvent volume to manure amount could influence the release of antibiotics into the extraction solvent and their partitioning between the phases of the ternary complexes, in this study, antibiotics were extracted from dairy and poultry manure samples at two cycles by providing fresh solution to enhance effective partitioning of the antibiotics into the MeCN phase. Following the extraction step, further clean-up of the extract was not performed; however, further enrichment of the antibiotics by evaporation of MeCN was necessary to increase the analytical sensitivity, as the signal responses of the antibiotics were much lower than those of some other compounds such as pesticides. In Figure 3.7, apparent recovery values of the antibiotics that exhibited linearity with pH 3 and 8 buffer are compared for dairy and poultry manure samples.

The partitioning of un-ionized antibiotics into the organic phase could be expected during salting-out-induced extraction. Although the pKa values of the target antibiotics vary over a wide range (Appendix A), the extraction efficiencies of most of the antibiotics, except for the APs and TCs, were quite high regardless of the buffer pH (Figure 3.7). However, it should be taken into consideration that the proportion of citrate in the extraction solvent is different at pH 3 and 8. Whereas the recovery of the APs depended upon the pH of the extraction buffer in both manure matrices, the recovery rate of the TCs varied with the type of manure. The performance of the method for TCs in poultry manure was lower at pH 8 than at pH 3, and this can be ascribed to the ternary complexes (Mackay and Canterbury, 2005) formed between the antibiotics. The organic content and the metal content of poultry manure resulted in complexed structure of this manure because of its high metal content (Table 3.1).

Moreover, CIP could not be detected in poultry manure, regardless of the buffer type. Changing the pH of the aqueous buffer from 3 to 8 allowed the determination of some antibiotics (e.g., SQX, SFX and DAN in poultry manure), but recovery of some other antibiotics (e.g., TCs and FLF in poultry manure and APs and LIN in dairy manure) decreased remarkably at the alkaline pH. Furthermore, recoveries of CHL for poultry and SMX for dairy manure increases extremely to 142 and 128 respectively at alkaline pH that lead to weaken the method performance. Consequently, the obtained results show that all the investigated antibiotics in dairy manure and 28 of 33 antibiotics in poultry manure could be detected with acidic buffer.



% Recovery range		
	pH3	pH8
SAs	95-108	102-128
FQs	89-113	96-111
MCs	86-103	79-104
TCs	98-103	90-105
APs	101-121	64-83
	% Mean recovery	
SAs	102	114
FQs	103	103
MCs	97	96
TCs	98	99
APs	109	69

% Recovery range		
	pH3	pH8
SAs	89-113	95-102
FQs	98-107	85-106
MCs	91-102	89-107
TCs	99-104	53-55
APs	94-98	56-142
	% Mean recovery	
SAs	98	98
FQs	101	95
MCs	98	99
TCs	99	55
APs	91	99

Figure 3.7. Effect of aqueous buffer pH on the analysis performance of antibiotics (each 100 μg.kg^{-1}) in dairy (a) and poultry (b) manure samples (n = 3).

To increase the number of antibiotics detected in poultry manure, acidic buffer extraction was performed with ultrasound treatment besides mechanical shaking. In order to determine the pH effect, the comparison of ultrasound assisted extraction with mechanical shaking was performed at four different antibiotic concentration levels to evaluate linearity (Figure 3.8). Whereas ultrasound-assisted extraction provided the analysis of CIP in poultry manure, this positive effect was not achieved for all antibiotics, and it even caused a decrease in the extraction performance for some of antibiotics (i.e. APs, CTC) with lower signal intensity in dairy manure compare to the mechanical agitation performance. It was also reported in a recent study that ultrasonic extraction did not improve the extraction efficiency of various antibiotics (Meng et al., 2017) it was attributed to the small amount of sieved solid sample usage in this analysis and the high diffusion ability of water-miscible MeCN.

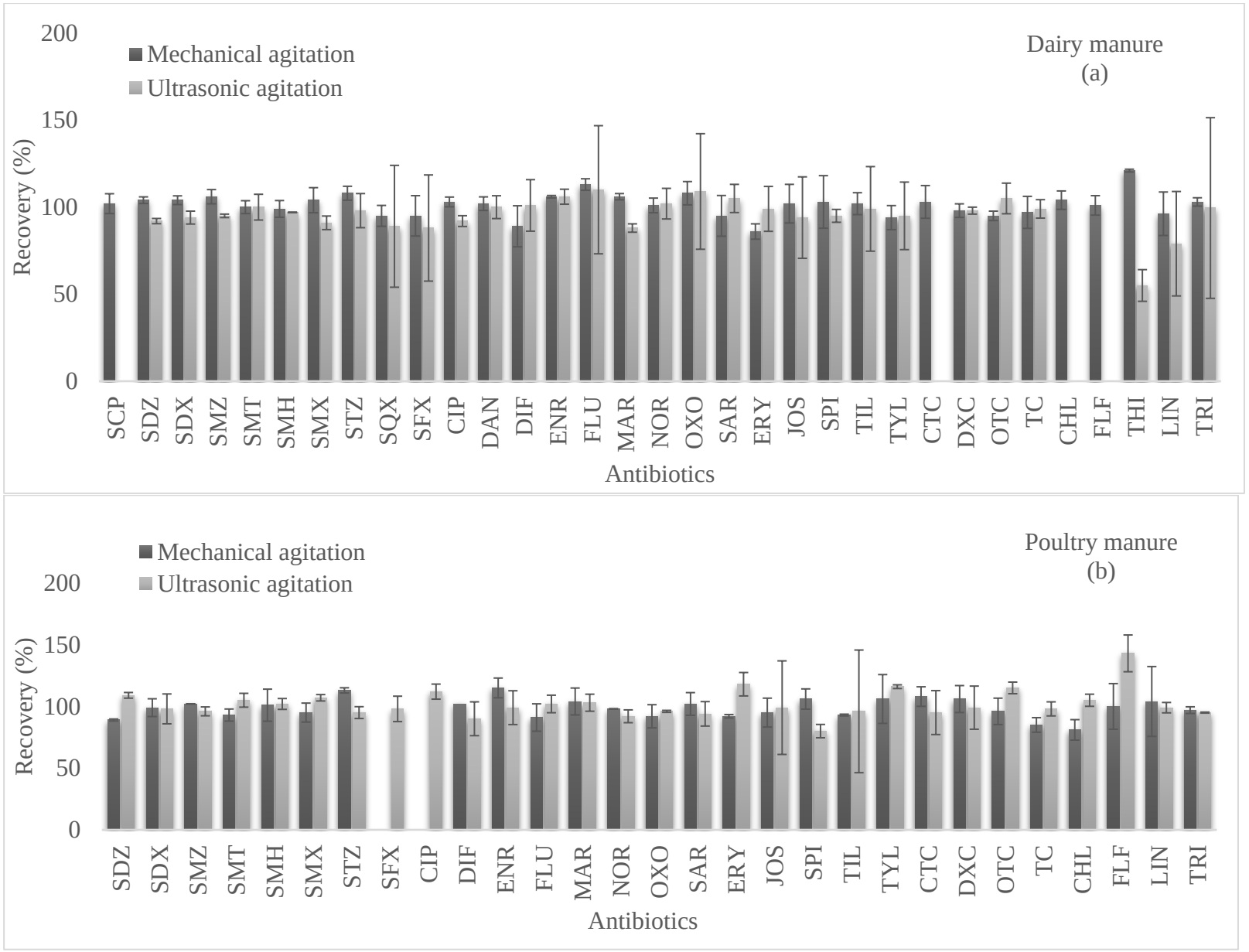


Figure 3.8. Effect of agitation type on the extraction performance of antibiotics (each 100 µg/kg) in dairy (a) and poultry (b) manure samples (n=3).

3.3.5. Method Performance

To evaluate the method performance, the specificity of the method used in this study was confirmed by observing the absence of interfering signals around the retention time of the targeted antibiotics during LC–MS/MS analysis of various blank manure samples at first. Then, the parameters were evaluated by using Aqua II as the aqueous mobile phase A, and MeCN/EDTA/McIlvaine buffer pH 3 as the extraction solvent with mechanical agitation to assist extraction of the dairy and poultry manure matrices (Tables 3.6 and 3.7). Linearity of almost all the investigated antibiotics with a correlation coefficient of at least ≥0.99 was achieved in dairy manure (Table 3.6), even for APs, which exhibited low signal intensity, whereas there were a few exceptions (i.e. SQX, SFX, DAN, CIP and THI) in poultry manure for which there was a lack of method performance for quantification in the concentration range of 25–200 $\mu g.kg^{-1}$ (Table 3.7). Excessively high matrix effect (>200%) of poultry manure could be the reason of this result achieved for these antibiotics. It was also observed that the recovery did not depend upon the spiking concentration of the antibiotics. Although the RSD percentages of the investigated antibiotics are generally reasonable (<20%) for most of the antibiotics, the values for MC antibiotics are quite high for both manures, especially at the lowest antibiotic spiking level (Tables 3.6 and 3.7).

LODs are between 0.1 and 10.3 $\mu g.kg^{-1}$ for dairy manure and between 0.1 and 5.9 $\mu g.kg^{-1}$ for poultry manure regardless of the antibiotic class. Even in the presence of a high matrix effect, these LOD values are much lower than those reported in the literature for manure without application of SPE clean up (Meersche et al., 2016; Spielmeyer et al., 2014). It is proposed that clean up may be essential to reduce the matrix effect (Pan et al., 2011; Janusch et al., 2014). Nevertheless, SPE can be insufficient to reduce the effect of the matrix components, as it was detected in amounts up to 364% for CTC and 174% for TYL in manure extracts in studies performed by Zhou et al., (2012) and Ho et al., (2012), respectively. It should be considered that the performance of the analytical method depends upon various factors, which is not only matrix effect, including type of matrix, spiking levels of the analytes and calculation of the recoveries or LODs. Eventually, the performance of the applied method herein clearly reveals the possibility for the simultaneous analysis of antibiotics in both manure samples, even though the target antibiotics have diverse physicochemical characteristics.

For the comparative evaluation of method performances apparent recovery, RSD percentages and matrix effects of mentioned unsatisfied method conditions, which were explained in 3.3.3 and 3.3.4 sections, such as usages of Mcllvaine buffer at pH8, ultrasonic agitation and Aqua I application

Table 3.6. Method performance parameters for dairy manure in antibiotic concentration range of 25-200 μg.kg^{-1}.

	Mean recovery (%) (intra-day RSD%) n=6				(inter-day RSD%) n=9	LOD	LOQ	Linearity	ME (%)
	25 (μg.kg^{-1})	50 (μg.kg^{-1})	100 (μg.kg^{-1})	200 (μg.kg^{-1})	100 (μg.kg^{-1})	(μg.kg^{-1})	(μg.kg^{-1})	(R^2)	
SCP	98 (9.2)	93 (5.7)	102 (5.7)	106 (4.5)	101 (5.8)	1.2	4.1	0.999	98.4
SDZ	98 (13.8)	108 (4.0)	104 (1.9)	101 (1.6)	105 (11.2)	0.1	0.1	0.998	97.6
SDX	83 (0.7)	100 (6.1)	104 (2.5)	102 (6.0)	98 (3.6)	0.1	0.1	0.998	96.8
SMZ	81 (0.1)	99 (7.1)	106 (4.1)	99 (0.1)	102 (6.1)	0.9	2.9	0.997	96.8
SMT	117 (0.2)	93 (6.8)	100 (3.7)	106 (10.5)	96 (7.1)	2.3	7.8	0.997	95.6
SMH	95 (9.9)	96 (11.6)	99 (4.8)	102 (2.0)	100 (5.6)	2.1	6.9	0.998	93.0
SMX	104 (18.5)	106 (9.6)	104 (7.2)	100 (0.1)	102 (8.7)	1.2	3.8	0.998	98.2
STZ	103 (14.9)	106 (7.5)	108 (4.0)	105 (5.5)	104 (9.4)	1.0	3.5	0.992	98.5
SQX	130 (6.5)	102 (2.8)	95 (6.0)	102 (1.4)	112 (5.7)	7.1	23.7	0.989	99.7
SFX	109 (12.2)	97 (10.5)	95 (11.6)	101 (1.9)	96 (13.2)	6.6	22.1	0.999	93.4
CIP	110 (10.1)	97 (3.3)	103 (2.8)	96 (4.0)	101 (5.1)	2.2	7.2	0.998	86.1
DAN	95 (2.1)	101 (1.4)	102 (3.9)	97 (2.1)	108 (3.8)	1.7	5.7	0.916	77.6
DIF	108 (20.3)	99 (11.9)	89 (11.8)	103 (1.7)	105 (14.2)	0.7	2.3	0.995	95.4
ENR	92 (2.3)	99 (5.9)	106 (0.7)	100 (1.5)	101 (4.8)	1.6	5.2	0.997	86.5
FLU	85 (23.2)	99 (2.8)	113 (3.3)	107 (7.4)	98 (5.2)	0.6	1.8	0.991	88.7
MAR	103 (1.0)	96 (2.4)	106 (1.8)	98 (1.2)	96 (2.7)	0.5	1.8	0.998	72.2
NOR	94 (0.6)	96 (2.6)	101 (4.2)	99 (2.3)	99 (4.5)	2.8	9.5	0.998	48.8
OXO	89 (0.1)	97 (8.9)	108 (6.7)	103 (3.5)	102 (7.0)	2.2	7.3	0.997	94.5
SAR	110 (13.4)	98 (8.8)	95 (11.7)	102 (7.6)	104 (15.3)	4.8	15.8	0.996	96.3
ERY	103 (6.8)	93 (12.5)	86 (4.4)	103 (0.8)	95 (6.7)	4.3	14.4	0.989	99.1
JOS	90 (9.4)	93.8 (21.4)	102 (11.1)	102 (6.7)	105 (17.1)	3.2	10.5	0.997	95.1
SPI	91 (5.0)	113 (3.5)	103 (15.1)	101 (0.0)	102 (14.5)	0.7	2.5	0.992	85.0
TIL	120 (23.6)	104 (1.9)	102 (6.3)	109 (10.4)	100 (10.2)	4.2	14.1	0.999	16.0
TYL	118 (2.4)	105 (1.2)	94 (6.9)	99 (3.9)	97 (7.2)	2.2	7.4	0.991	84.6
CTC	99 (0.9)	105 (4.4)	103 (9.4)	101 (0.3)	103 (8.3)	1.6	5.3	0.997	>150
DXC	96 (3.8)	104 (10.2)	98 (3.9)	102 (5.0)	97 (5.8)	1.3	4.2	0.997	86.5

Table 3.6. Continued

	Mean recovery (%) (intra-day RSD%) n=6				(inter-day RSD%) n=9	LOD	LOQ	Linearity	ME
	25 ($\mu g.kg^{-1}$)	50 ($\mu g.kg^{-1}$)	100 ($\mu g.kg^{-1}$)	200 ($\mu g.kg^{-1}$)	100 ($\mu g.kg^{-1}$)	($\mu g.kg^{-1}$)	($\mu g.kg^{-1}$)	(R^2)	(%)
OTC	106 (9.0)	97 (1.3)	95 (2.7)	96 (4.5)	93 (6.2)	0.6	2.1	0.998	53.9
TC	82 (7.0)	96 (5.8)	97 (9.2)	97 (4.1)	95 (11.0)	0.4	1.3	0.994	70.0
CHL	98 (16.8)	104 (2.9)	104 (5.3)	112 (14.0)	106 (12.5)	10.3	30.9	0.995	42.4
FLF	101 (5.4)	108 (4.7)	101 (5.6)	100 (2.1)	110 (6.7)	4.5	13.4	0.989	>150
THI	144 (9.8)	105 (5.1)	121 (0.8)	74 (0.5)	112 (5.9)	2.5	7.4	0.902	>150
LIN	110 (11.4)	99 (8.8)	96.2 (12.5)	105 (7.3)	108 (15.4)	2.8	9.1	0.998	2.7
TRI	111 (8.6)	101 (11.4)	103 (2.4)	105 (5.1)	98 (10.7)	0.1	0.1	0.998	6.8

Table 3.7. Method performance parameters for poultry manure in antibiotic concentration range of 25-200 $\mu g.kg^{-1}$.

	Mean recovery (%) (intra-day RSD%) n=3				(inter-day RSD%) n=6	LOD	LOQ	Linearity	ME (%)
	25 ($\mu g.kg^{-1}$)	50 ($\mu g.kg^{-1}$)	100 ($\mu g.kg^{-1}$)	200 ($\mu g.kg^{-1}$)	100 ($\mu g.kg^{-1}$)	($\mu g.kg^{-1}$)	($\mu g.kg^{-1}$)	(R^2)	
SCP	102 (3.3)	99 (12.9)	94 (0.8)	107 (8.0)	105 (3.1)	4.8	16.1	0.997	97.6
SDZ	108 (12.9)	89 (8.0)	89 (7.2)	103 (2.8)	112 (15.1)	2.2	7.3	0.961	96.9
SDX	105 (4.0)	93 (4.6)	99 (0.1)	101 (0.8)	102 (4.1)	0.2	0.7	0.999	95.8
SMZ	97 (1.5)	96 (14.7)	102 (4.9)	102 (1.7)	102 (6.1)	0.4	1.2	0.998	96.3
SMT	96 (17.7)	104 (5.1)	93 (13.0)	107 (4.6)	98 (14.1)	1.9	6.5	0.979	91.4
SMH	108 (7.8)	91 (10.9)	101 (7.7)	106 (6.0)	103 (7.8)	0.6	2.0	0.997	90.4
SMX	111 (4.4)	101 (3.0)	95 (2.1)	102 (1.4)	99 (5.1)	0.6	1.9	0.998	95.6
STZ	101 (11.4)	108 (6.3)	113 (20.7)	108 (9.5)	112 (12.4)	0.9	2.9	0.999	97.9
DIF	90 (3.1)	97 (7.3)	102 (8.0)	107 (10.2)	108 (10.1)	1.6	5.4	0.999	99.9
ENR	76 (0.7)	106 (0.4)	115 (11.1)	104 (5.8)	105 (3.2)	0.9	3.0	0.995	94.6
FLU	112 (0.1)	90 (10.1)	91 (10.9)	98 (4.4)	96 (7.2)	0.1	0.1	0.998	92.3
MAR	87 (4.6)	98 (9.1)	104 (0.1)	100 (0.7)	102 (1.6)	1.4	4.7	0.997	85.6
NOR	96 (5.9)	99 (0.4)	98 (9.4)	104 (7.1)	100 (8.1)	3.8	12.4	0.998	55.5
OXO	110 (2.1)	89 (11.1)	92 (9.2)	98 (2.6)	99 (8.4)	0.1	0.4	0.999	95.6
SAR	106 (5.9)	101 (13.0)	102 (1.4)	101 (1.1)	101 (5.6)	4.5	15.1	0.998	96.9
ERY	86 (3.3)	132 (4.3)	92 (11.7)	91 (17.5)	98 (13.4)	0.7	2.4	0.556	99.5
JOS	104 (21.8)	123 (12.7)	95 (8.2)	102 (2.8)	103 (15.2)	3.0	9.9	0.976	96.6
SPI	102 (2.8)	86 (6.6)	106 (0.7)	96 (4.4)	105 (5.4)	1.8	6.0	0.921	36.4
TIL	103 (12.5)	99 (11.6)	93 (19.8)	100 (1.4)	95 (18.1)	1.5	5.1	0.994	56.3
TYL	84 (20.2)	110 (28.3)	106 (7.9)	100 (2.5)	102 (8.4)	5.0	16.7	0.991	77.5
CTC	112 (9.9)	95 (8.0)	108 (10.9)	99 (2.9)	105 (10.2)	5.9	19.6	0.997	79.7
DXC	94 (3.0)	107 (1.3)	106 (10.7)	100 (0.7)	101 (9.4)	0.4	1.5	0.999	92.7
OTC	102 (2.8)	109 (12.2)	96 (5.9)	100 (0.4)	98 (7.1)	3.8	12.7	0.999	85.1
TC	116 (14.6)	86 (16.4)	85 (8.3)	104 (2.7)	101 (14.2)	0.2	0.6	0.994	87.8
CHL	194 (19.0)	100 (5.7)	81 (18.5)	98 (13.4)	97 (9.6)	0.1	0.2	0.922	306.5
FLF	90 (3.1)	82 (3.5)	81 (0.9)	94 (3.8)	81 (0.9)	1.0	3.2	0.97	>150
LIN	112 (5.5)	89 (1.6)	104 (2.7)	97 (3.3)	89 (9.6)	0.1	0.5	0.892	30.1
TRI	99 (8.9)	104 (12.6)	97 (11.0)	101 (2.5)	101 (12.1)	0.1	0.2	0.988	45.8

as mobile phase A were also investigated and demonstrated in Appendix B-G. Method performances of these alternative analyses shows unsuccessful results for especially at low concentrations (25-50 $\mu g.kg^{-1}$). However, the condition of Aqua I application as mobile phase A for poultry manure; for instance, gave inefficient method performance results for not only low concentrations of antibiotic but also at the highest concentration (200 $\mu g.kg^{-1}$). Poor method performances of these methods resulted from lower signal responses, unaccomplished repeatability, and particularly matrix effects of components that were incredibly high (>500) such as TCs and APs by using method with Aqua I and ultrasonic agitation for poultry manure.

3.3.6. Application of Method to CAFO Manure Samples

To demonstrate the applicability of the developed method, three different types of manure samples, broiler, layer hen and dairy, were collected from CAFOs analyzed in the Marmara region of Turkey. Whereas nearly total targeted antibiotics were analyzed in each manure sample, a limited number were identified, especially in layer-hen manure (Table 3.8), as expected due to the legal constraints on the use of antibiotics. The literature data for the maximum concentrations of antibiotics detected in cattle and poultry manure samples, mainly compiled from recent publications, are presented in Table 3.9. Different types of antibiotics in both dairy and layer-hen manure samples (three samples for each manure) were identified, but their concentrations were not as high as those found in broiler manure.

The concentrations of ENR (444,000 $\mu g.kg^{-1}$) and its metabolite CIP (29,000 $\mu g.kg^{-1}$) in broiler manure ranged up to $mg.kg^{-1}$ level. Similarly, the analysis of remarkably high concentrations of FQ antibiotics was previously reported by Zhao et al., (2010). The detection of ENR and CIP at concentrations of 1,421,000 and 46,000 $\mu g.kg^{-1}$ in poultry manure was presented. In addition, OTC is another antibiotic that is frequently detected, and this was reported at high concentrations up to 59,000 and 60,000 $\mu g.kg^{-1}$ in cattle and swine manure samples, respectively (Zhao et al., 2010). In this study, TC antibiotics were found only in dairy manure (120 $\mu g.kg^{-1}$), and SA antibiotics (SDZ and SMH) were detected in broiler manure at lower concentrations than FQs and MLs. There is no doubt that both the types and quantities of antibiotics are related to farming practices of feeding animals; however, SAs as well as other classes of antibiotics could be detected by applying the method in different manure samples (Table 3.8). High amounts of antibiotics in manure can obviously be a source for environmental contamination.

Table 3.8. Antibiotic concentrations detected in manure samples in this study.

Antibiotic concentrations in manure samples (μg.kg^{-1})			
	Broiler	Layer Hen	Dairy
CIP	28,997 (403)	≤LOD	52 (2)
DAN	≤LOD	≤LOD	608 (119)
DIF	≤LOD	≤LOD	≤LOD
ENR	444,134 (359)	≤LOD	134 (23)
FLU	≤LOD	≤LOD	≤LOD
MAR	≤LOD	≤LOD	≤LOD
NOR	≤LOD	≤LOD	≤LOD
OXO	146 (11)	≤LOD	≤LOD
SAR	≤LOD	≤LOD	≤LOD
SCP	≤LOD	≤LOD	≤LOD
SDZ	74 (11)	351 (6)	22 (45)
SDX	≤LOD	≤LOD	≤LOD
SMT	≤LOD	≤LOD	≤LOD
SMH	695 (6)	≤LOD	≤LOD
SMX	≤LOD	≤LOD	≤LOD
SQX	≤LOD	≤LOD	≤LOD
SFX	≤LOD	≤LOD	≤LOD
STZ	≤LOD	≤LOD	≤LOD
CTC	≤LOD	≤LOD	≤LOD
DXC	≤LOD	≤LOD	≤LOD
OTC	≤LOD	≤LOD	116 (22)
TC	≤LOD	≤LOD	≤LOD
JOS	≤LOD	≤LOD	≤LOD
SPI	≤LOD	≤LOD	≤LOD
TRI	584 (40)	≤LOD	≤LOD
CHL	≤LOD	≤LOD	≤LOD
FLF	≤LOD	≤LOD	≤LOD
LIN	119 (6)	196 (79)	40 (5)
TIL	≤LOD	≤LOD	≤LOD
ERY	≤LOD	≤LOD	45 (9)
TYL	6,660 (412)	≤LOD	≤LOD

Parenthesis; (±standard deviation); ≤LOD=25 μg.kg^{-1}

Table 3.9. Literature values of maximum antibiotic concentrations detected in cattle and poultry manure.

	Cattle manure		Poultry manure	
	(Conc. $\mu g.kg^{-1}$)	Reference	(Conc. $\mu g.kg^{-1}$)	Reference
TYL	280	Zhao et al., 2010	13,740	Zhao et al., 2010
CHL	12,900	Zhao et al., 2010	4,830	Zhao et al., 2010
TC	12,010	Zhao et al., 2010	4,576	Zhao et al., 2010
OTC	59,590	Zhao et al., 2010	21,960	Zhao et al., 2010
Methacycline	960	Zhao et al., 2010	5,860	Martínez-Carballo et al., 2007
DXC	1,050	Ho et al., 2012	78,516	Zhao et al., 2010
CTC	27,590	Zhao et al., 2010	17,680	Zhao et al., 2010
Sulfamonomethoxine	300	Zhao et al., 2010	960	Zhao et al., 2010
Sulfamerazine	110	Li et al., 2013	890	Zhao et al., 2010
Sulfaguanidine	300	Ji et al., 2012	570	Li et al., 2013
Sulfadimidine	180	Aust et al., 2008	6,040	Ji et al., 2012
SDZ	4,570	Hu et al., 2010	91,000	Li et al., 2013
SCP	3,660	Ji et al., 2012	710	Zhao et al., 2010
SMZ	9,990	Zhao et al., 2010	1,630	Martínez-Carballo et al., 2007
SMH	9,360	Li et al., 2013	7,110	Zhao et al., 2010
Sulfanilamide	80	Li et al., 2013	1,590	Zhao et al., 2010
SDX	810	Li et al., 2013		
NOR	2,760	Zhao et al., 2010	225,450	Ho et al., 2012
Lomefloxacin	5,530	Zhao et al., 2010	7,030	Zhao et al., 2010
Fleroxacin	2,220	Zhao et al., 2010	99,430	Zhao et al., 2010
ENR	46,700	Zhao et al., 2010	1,420,760	Ji et al., 2012
DIF	2,630	Ji et al., 2012	12,380	Ho et al., 2012
DAN	3,060	Ji et al., 2012	2,480	Ho et al., 2012
CIP	29,560	Li et al., 2013	45,590	Ho et al., 2012
ERY			32	Zhao et al., 2010
TIL			85	Zhao et al., 2010
TRI			3,412	Zhao et al., 2010

3.4. Conclusions

Continuous monitoring of veterinary antibiotics in animal manure to control this substantial pollution at the source is necessary to mitigate its environmental burden. In this respect, there is an urgent demand for the development of a generic and simple analytical method for the identification and quantification of multi-class antibiotic residuals in manure samples. In this study, the developed method allowed the analysis of 33 antibiotics belonging to seven different classes in both poultry and dairy manure samples. Modification of the composition of the mobile phase used for LC–MS/MS increased the number of the antibiotics that could be simultaneously analyzed. Salting-out-induced extraction of antibiotics from manure samples with a MeCN/EDTA/McIlvaine buffer solvent mixture enabled successful application of the method without the use of SPE. Although the matrix effects of dairy and poultry manure samples were quite high, and the signal responses of these matrices were different, the use of internal standards made the method feasible for both manure samples (poultry and dairy manure). By applying the developed method to manure samples collected from dairy and poultry CAFOs, the detection of various antibiotics with high concentrations confirmed that manure is an important source of antibiotic pollution.

4. FATE OF ANTIBIOTICS DURING RAPID COMPOSTING OF ANIMAL WASTE

Fate of antibiotics during rotary drum composting process are mainly explained in this section. Physicochemical parameters of manure could have been associated with composting and antibiotic degradation directly related with the fate of antibiotics.

4.1. Introduction

Rapid composting methods are necessary to handle huge amounts of waste produced from modern animal husbandry facilities. Turned composting enhanced the antibiotic degradation rate and it was probably resulted from the improvement of oxygen availability and diffusion in manure pile for biologic activity, (Ray et al., 2017). In addition, turning provides enough contact time between air and manure samples and homogeneity of air lead to more uniform moisture content in composted manure. Hence, composting was performed by using rotary drum in this study. To understand the influence of turning, static composting was performed as a control in composting bin through the same composting condition.

Adequate oxygen supply is primary concern for biological processes, if necessary amount of oxygen is not present in composting samples, anaerobic microorganisms proliferates, composting slows down, and odorous materials exists (Herbert et al., 2010). Therefore, two different active aeration rates (3.6±0.2 and 17±3 $m^3.h^{-1}$) were performed to aerate manure samples since required air determination is necessary for evaluation of composting performance. Co-composting of dairy and poultry manure could provide uniform moisture content and balanced C/N ratio without using bulking agent that considerably increased the amount of waste. Therefore, in this study, efficiency of antibiotic degradation was compared between individual (dairy or poultry) and blended manure (dairy and poultry) composting studies. Several physicochemical parameters (e.g. nutrient and metal content) were analyzed to clear relation between antibiotic reduction and these parameters. Additionally, biological proliferation and decline was found out by SOUR (Specific Oxygen Uptake Rate) tests through composting process; hence, composting performance could be cleared out in biological perspective.

A broad profile of veterinary antibiotic was detected in dairy and poultry manure samples in section 3.3.6 by using multi residue analysis method. In this section, those excreted antibiotics in

manure samples, which were detected in the beginning of composting, were analyzed to determine the reduction of these compounds by using developed analysis method in section 3.

4.2. Materials and Methods

4.2.1. Sample Collection

Broiler, layer hen and dairy manure collected from three different CAFOs were subjected to composting in this study. Dairy manure was obtained from an industrial farm (1,000 dairy cow) in which urine and feces were collected by using scraper system to a solid floor. Collected manure was separated by using mechanical separator to liquid and solid phase. Broiler manure was collected from a large-scale barn (100,000-200,000 broiler) after broiler harvesting (30-40 days) and this waste contained approximately 10% of rice husk used as bedding material. Layer hen manure was collected from another large-scale intensive farm (300,000-400,000 layer hens) and this waste was solid and contained feather, broken shell and spilled feed.

4.2.2. Composting Design

Efficient composting of manure depends upon uniform moisture content and C/N ratio. Considering this fact dairy manure was blended with poultry waste. The moisture content of collected dairy manure was in the range of approximately 80% while the moisture content of broiler litter and layer hen manure were of 30% and 70%, respectively. Initial optimum moisture content of rotary drum was determined in preliminary studies and approximately 60% initial moisture content of composting samples accelerated the time to reach maximum peak temperature and increased the highest temperature since microbial activity sped up in this condition.

Therefore, blending of layer hen (DL) and broiler manure samples (DB) with dairy manure at certain ratio (3/1) can provide uniform moisture content. To decrease dairy manure initial moisture content (80%) to 60%, layer hen manure (69%) was dried to 30% under the hood and dairy/layer manure was blended at about 3/1 ratio. It was not necessary to dry broiler manure before blending with dairy manure (DB) due to its suitable initial moisture content (30%). Like DL case, dairy/broiler manure was again blended in the range of 3/1 ratio. Additionally, individual dairy (D) and broiler manure (B) samples were composted as two other cases to understand differences between composting approach (blending or not blending). For the preparation of individual manure samples for composting, 3/1 ratio of D was dried under the hood (30%) and blended with 80% initial moisture

content of D (2/3) to attain initial 60% moisture content for D compost whereas B was watered to elevate moisture content to 60%.

Manure composting experiments was carried out in two identical 30 L capacity laboratory-scale rotary drums, 400 mm high and 400 mm, in diameter. Each drum was made up of a 4-mm thick stainless-steel sheet and 40 mm thick layer of polyurethane foam insulation, which minimized the conductive heat loss along the reactor wall. The drums were mounted horizontally on four rubber rollers attached to a metal stand (4.1). This composting system was placed in a temperature-controlled room (30°C).

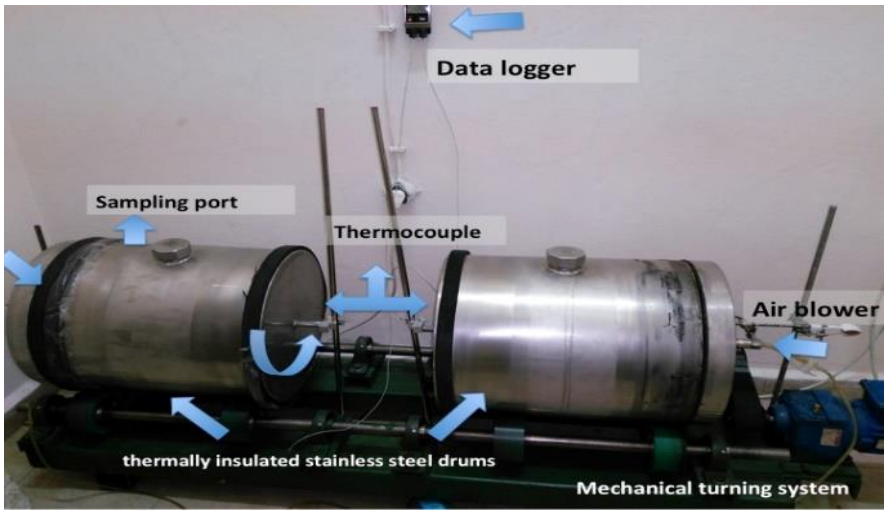


Figure 4.1. Laboratory scale rotary drum composter.

A 0.18 kW motor with gear reducer was used to turn the drum at a variable speed (0.1-15 rpm) and rotation of the drum provided the mixing and aeration of manure. Hence moisture content, temperature, and oxygen levels can be controlled during composting. Rotary drums had two main openings at both ends for continuous air blowing and escaping water vapor and gases generated during composting, respectively. During the composting, temperature was measured with thermocouple probe inserted to the core of the drum and recorded on a data logger (Testo 175-T3) continuously. Compost samples was taken from a screw cap sampling port at specified time intervals (1-2 days).

Air blowing was a variable parameter to investigate the fate of antibiotic. In this study, high (13-20 $m^3.h^{-1}$) and low air volumes (3.6 $m^3.h^{-1}$) were given to the reactors. Rotation speed of rotary drum composters were optimized during preliminary studies (0.1 rpm). Not only rotary drum (turning) but also static composting (control) was performed in the laboratory. Identical composting conditions were ensured for static and turning compost to compare the antibiotic reduction during these two-

different processes. Static compost was performed in the same temperature-controlled room with turning compost reactors. Manure was stirred manually in two days intervals for static compost.

Two groups of rotary drum composting experiments were investigated for D, DB, DL as a first group and D, DB, B as a second group of manure compost samples for 12 and 20 days intervals, respectively. For first group, aquarium pump (3.6 $m^3.h^{-1}$) was used as aeration source whereas air compressor was regulated for aeration of second group (13-20 $m^3.h^{-1}$). Static compost was investigated with the second group of compost samples and DB case from first group concurrently.

4.2.3. Evaluation of Composting Efficiency with the Fate of Antibiotics

Composting efficiency was evaluated considering the reduction of antibiotics. Antibiotics detected in manure and compost samples were determined with previously validated multi-residue analysis method, which included extraction and quantification steps (section 3). This analytical method provided rapid quantification of 33 different antibiotics in manure samples. In this study, samples taken from composting reactors were lyophilized and stored at -20º C prior to analysis.

Reduction of antibiotics were determined by calculating percent reduction (PR) (%) and half-life (day) of analytes. PR of antibiotics were calculated as;

$$PR(\%)=100 \times (C\dot{I} - CT)/C\dot{I}$$

Where Cİ means the concentration of calculated antibiotic in the initial compost, CT means the concentration of antibiotic in samples on the final day of compost samples.

The removal rate of antibiotics was expressed as first order kinetics in variety of studies, (Ho et al., 2012; Amarakoon et al., 2016; Cessna et al., 2011; Bao et al., 2009) like the most of analyte results in our study. Removal rate (k, day^{-1}) and half-life (t ½, day) of target antibiotics were calculated as;

$$C = C0 \times e^{kt},\ t\frac{1}{2} = -ln\frac{2}{k}$$

Where C0 means the initial antibiotic concentration ($\mu g.kg^{-1}$), C means the analyte concentration ($\mu g.kg^{-1}$) at time t (day).

4.2.4. *E.Coli* (*Escherichia Coli*) Determination

E.coli was used as an indicator for the pathogens. For this test after the extraction of manure or compost TBX agar was used to enumerate microorganisms as β-glucuronidase-positive *Escherichia coli*, (ISO 16649-2, 2001).

4.2.4.1. Extraction Manure samples were diluted in the range of 1:10 (w/v) with sterile dilution solution. Serial dilutions were performed from that solution if necessary.

4.2.4.2. Pour-plate method For enumeration, 1 ml of the homogenized sample was spiked onto a petri dish and 15 ml of the TBX Agar (cooled to 45-50 °C) poured onto sample. Petri dish was mixed gently and allowed to solidify. Inoculated dishes were inverted and placed in an incubator set at 44±1°C for 18-24 hours.

4.2.4.3. Counting After the specified period of incubation CFU of β-glucuronidase-positive *E. coli* in each dish were counted as blue dots. Number of dots in each dish was accepted when it was between 20-150.

4.2.5. Specific Oxygen Uptake Rate (SOUR)

A respirometric technique for the assessment of compost stability was applied by utilizing a dissolved oxygen probe to measure the variation of the oxygen concentration in the aqueous manure during composting. Optimum microbial activity and maximum reaction rates were obtained to measure specific oxygen uptake rate (SOUR), (Lasaridi and Stentiford, 1998). Test equipment is shown in figure (4.2) below.

Manure or compost was weighed about 5 g to 500 mL deionized water. 15 mL Phosphate Buffer, 5 mL Calcium Chloride ($CaCl_2$), 5 mL Ferric Chloride ($FeCl_3$), 5 mL Magnesium Sulfate ($MgSO_4$) and 1.1 mL (2 $mL.L^{-1}$) ATU (allylthiourea) were prepared according to BOD (Biological Oxygen Demand) method (APHA, 1995) and added with deionized water. The water temperature was kept close to 30 °C. DO (Dissolved Oxygen) Probe Sensor was at a depth of 5-7 cm. The suspension was continuously stirred by means of a magnetic stirrer. 20 min aeration and 15 min measurement periods were ensured. Readings were taken every 3 minutes and stored to the file. Any readings below 1 mg $O_2.L^{-1}$ were discarded. When oxygen demand was reached a maximum value, DO was reduced to zero and this maximum amount gave us SOUR value.

Figure 4.2. SOUR test equipment.

4.2.6. Characterization of Manure Samples

A complete description of the chemical composition of raw manure and compost samples were performed by using parameters listed in Table 4.1.

Table 4.1. Methods to measure manure characteristics.

Characteristics	Methods
pH	Potentiometric
Moisture	Gravimetric
Electrical Conductivity (EC)	Potentiometric
Organic Carbon (OC)	Gravimetric
Total Nitrogen	Kjeldahl digestion and Nessler
Ammonia	Nessler
Total Phosphorus	Digestion and Ascorbic Acid
Metal	MW (Microwave) digestion+ICP

Moisture content of the manure was determined by gravimetric method using automated moisture scale (Kern dbs) and after drying at 105 °C. pH and EC of the manure or compost were measured in deionized water at 1:10 w/v solid to liquid ratio with a pH probe (WTW pH 330 pH meter) and EC meter (WTW LF 320). Organic matter was determined by the loss of mass on ignition at 550 °C in a muffle furnace for five hours and total organic carbon content of manure was calculated from the organic matter content using the conventional “Van Bemmelem factor” of 1.724 (Tiquia, 2005). TKN-N of manure was determined after digestion (Digesdahl, Hach) with Sulphuric Acid and Hydrogen Peroxide. Ammonia was extracted from manure with 2 M KCl (1:10 w/v) and quantified by Nessler method (APHA, 2005). Total metals were determined by ICP atomic emission spectrometry (ICP-AES Perkin-Elmer Optima 2100 DV) after MW-assisted acid digestion of dry manure sample (US-EPA, 1996).

In addition to listed parameters in Table 4.1., Water Extractable Portions of Organic Carbon (WEOC), nutrients (Water Extractable Organic Nitrogen, WEON) and metals were analyzed. Water

extracts of manure and compost samples will be prepared from with deionized water at 1:10 (w/v) compost:water ratio and they were mechanically shaken for 30 min (Said-Pullicino et al., 2007). After centrifugation at 10,000 rpm for 30 min, extracts will be filtered through a 0.45 μm membrane filter (Sartorious, cellulose acetate). Sequential extraction of phosphorus was practiced with not only water but also $NaHCO_3$, NaOH and HCl and determination of phosphorus in extraction portions were carried out by ascorbic acid method. Mentioned method procedures are written in detail in following steps:

4.2.6.1. pH and EC pH and EC of manure or compost were measured in deionized water after 30 min waiting for settlement at 1:10 w/v solid to liquid ratio with a pH probe (WTW pH 330 pH meter) and EC meter (WTW LF 320).

4.2.6.2. Moisture Content (MC) (%) MC of manure was determined by gravimetric method using automated moisture scale (Kern dbs) and after drying at 105 °C.

4.2.6.3. Total Organic Carbon (TOC) Organic matter was determined by the loss of mass on ignition at 550 °C in a muffle furnace for five hours and total TOC content of manure was calculated from the organic matter content using the conventional "Van Bemmelem factor" of 1.724 (Tiquia, 2005). At first, manure sample was dried at 103 °C for 24 h. Crucibles were dried at 103 °C for 24 h and taken 1 h in muffle furnace at 550 °C and desiccated to reach constant weight. 5-10 g dried manure sample was weighed and taken to the muffle furnace at 5 hours.

Calculation

Ash content ($g.kg^{-1}$)= $\frac{Ash\ weight\ of\ compost\ (g)}{Dry\ weight\ of\ compost\ (kg)}$

Organic Matter Content ($g.kg^{-1}$)= 1000-Ash content ($g.kg^{-1}$)

TOC=0.58 x Organic Matter Content ($g.kg^{-1}$)

4.2.6.4. Ammonia (NH_4^+-N) KCl method Total Ammonia was extracted from manure with 2 N KCl (1:10 w/v) and quantified by Nessler method (APHA, 2005). 0.5-1 g (wet weight) manure samples were weighed into 50 mL centrifuge tubes. Blank sample was also carried through test in centrifuge tube. 30 mL 2 N KCl was added to centrifuge tubes and shaken 40 min with horizontal shaker. 1-5 mL supernatant was taken to 25 mL volumetric flask from clear upper part of the pellet. Then, 3 drop mineral stabilizer and polyvinyl alcohol were added to volumetric flask. Sample solution was diluted to 25 mL. Spectrometer was adjusted to 425 nm (program no: 380). 1 mL of Nessler reagent was

added on top and wait for 2 minutes. Samples were transferred to 25 mL quartz cuvettes and read after zeroing blank.

4.2.6.5. TKN (Total Kjeldahl Nitrogen) TKN-N of manure was determined after digestion (Digesdahl, Hach) with sulfuric acid and hydrogen peroxide. 0.15 g (wet weight) manure sample was weighed into 100 mL digesdahl digestion flask. Blank sample was also carried through test in digesdahl digestion flask. Heater was set at 440 °C. 6 mL sulfuric acid was added to the digestion flask. After 4 min boiling 12 mL ~50% hydrogen peroxide was added to the charred sample via the funnel on the fractioning column. After the addition of hydrogen peroxide was complete, hot flask was taken off the heater and the flask allowed to air cool. Digestion was diluted to 100 mL after cooling with deionized water.

pH adjustment for TKN 3 mL cooling sample was pipetted into a 25 mL graduated mixing cylinder. 1 drop of TKN indicator was added to the cylinder. 8 N KOH was added one drop at a time by swirling until the first pale blue color appears (pH 3). 3 drop mineral stabilizer and 3 drop polyvinyl alcohol were added each graduated cylinder and sample was diluted to 25 mL. Spectrometer was adjusted to 460 nm (program no: 399). 1 mL of Nessler reagent was added on top and wait for 2 minutes. Samples were transferred to 25 mL quartz cuvettes and read after zeroing blank.

Calculation

TKN % = $\frac{75 \times A}{B \times C}$

A= Absorbance value at 460 nm; $mg.L^{-1}$

B= Weight of dry manure taken in digestion flask; g

C=Volume of digested sample solution; mL

4.2.6.6. WEON (Water Extractable Organic Nitrogen) Water extracts of manure and compost samples were prepared with deionized water at 1:10 (w/v) compost: water ratio (5 g manure and 50 ml water) and they were mechanically shaken for 30 min (Said-Pullicino et al., 2007). After centrifugation at 10,000 rpm for 30 min, extracts will be filtered through a 0.45 μm membrane filter (Sartorious, cellulose acetate). After extraction of manure or compost, supernatant was subjected to Hach procedure (4.2.6.5). To calculate the Organic portion of nitrogen, amount of total ammonia was subtracted.

4.2.6.7. WEOC (Water Extractable Organic Carbon) Manure or compost was extracted as explained WEN procedure (4.2.6.6). After extraction of manure or compost, supernatant was subjected to COD

(Chemical Oxygen Demand) procedure according to APHA, (1995). Centrifuged sample was transferred to COD tube and necessary dilution was prepared before digestion. 1.5 mL digestion solution and 3.5 mL sulfuric acid reagent were added into diluted sample. Tubes were placed in block digester and preheated to 150 °C and refluxed for 2 h. Then, it was cooled to room temperature. After cooling, absorbance of each samples was measured at 600 nm after zeroing blank by spectrometer.

4.2.6.8. Sequential and total Phosphorus Phosphorus was fractioned with sequential extraction method (Takahashi, 2013). After sequentially extracted, phosphorus was determined with the digestion (Digesdahl, Hach) of extractants and measured with ascorbic acid method like total phosphorus analysis.

Sequential extraction 0.3 g (wet weight) manure was weighed. Manure was sequentially extracted with 30 ml deionized water, 0.5 M $NaHCO_3$, 0.1 M NaOH and 1 M HCl. (After NaOH extraction, pellet was extracted with deionized water once more before HCl extraction and supernatant discarded). For each solvent extraction, samples were shaken by horizontal shaker and centrifuged at 30 min (10000 rpm). After each sample extraction supernatant was taken and filtered using 0.45 μm cellulose membrane. Collected supernatants from each extraction step were implied further ascorbic acid method (APHA, 1995) directly to detect inorganic phosphorus. In addition, appropriate volume of supernatant was subjected to Hach digestion procedure to detect total phosphorus of each sequential extractant. Hence, organic phosphorus was determined by calculating total minus inorganic phosphorus results of each extractants.

Total phosphorus (TP) 0.15 g manure was weighed and Hach digestion procedure applied to determine TP (4.2.6.5). After digestion of manure, digestate was subjected to ascorbic acid method (APHA, 1995).

Ascorbic acid method 50 mL sample (or dilution was carried out, if necessary) was pipetted into flask after extraction. 1 drop phenolphthalein indicator was added to the flask. If a red color developed 5N sulfuric acid solution was added dropwise to discharge the color. 8 mL combined reagent was added to the flask and waited for 10-30 min. Absorbance of each sample was measured at 800 nm by spectrometer.

Combined reagent 100 mL combined reagent was prepared with 50 mL 5N sulfuric acid solution, 5 mL potassium antimonyl tartrate solution, 15 mL potassium ammonium molybdate solution and 30 mL potassium ascorbic acid solution. The reagent was stable for 4h.

4.2.6.9. Total and soluble metal analysis Total metal analysis was accomplished according to EPA, 3052, (1996). 0.2 g manure was dried at 103 °C. 9 mL HNO_3 and 2 mL concentrated HCl were added subsequently to MW reaction vessels. MW digester was adjusted to required program which MW temperature was reached at 180 ± 5 °C in approximately less than 5 min and maintained this temperature for 9.5 minutes. After cooling of digester, digestates were diluted to 25 mL. Samples were filtered through 0.45 µm filter paper and transferred to glass tubes. Total metals were determined by ICP atomic emission spectrometry (ICP-AES Perkin-Elmer Optima 2100 DV).

Soluble metal analysis After the extraction procedure that was explained in WEN procedure (4.2.6.6), extractants were subjected to metal analysis by inductively coupled plasma atomic emission spectrometry (ICP-AES Perkin-Elmer Optima 2100 DV).

4.3. Results and Discussion

4.3.1. Preliminary Studies

Composting experiments were initially tested in incubator to understand the optimum amount of manure for composting in rotary drum reactors. Especially, temperature of manure samples increased when minimum 6 kg of manure was composted. Therefore, volume of reactors was determined as minimum capacity could be filled in 6 kg manure sample.

Following preliminary rotary drum experiments, initial moisture content of manure, manure loading, aeration, turning frequency and room temperature were optimized to provide the necessary composting performance. There are some significant preliminary study results that guide following composting experiments of this study listed in below and shown in Table 4.2;

- Increasing of manure weight led to elevation of maximum composting temperature. (6.5 kg)
- Drying manure was carried out under the hood to decrease initial moisture content of manure to approximately 60% of MC, which led the highest maximum temperature.
- Aeration rate was enough when 3.6 $m^3.h^{-1}$ air volume was given to the reactors to reach more than 65 °C maximum temperature; however, air flow could be increased to decrease final moisture content.
- In view of maximum temperature static and rotary drum composting had minor difference; however, TKN, ammonia, bulking density were low during composting for rotary drum compare to static process.

- Water addition was necessary when MC of manure was lower than 55% through composting.
- Manure holding time before composting adversely affected composting temperature and performance parameters. Therefore, manure drying time was decreased to 1-2 days under the hood.

Table 4.2. Rotary drum optimization conditions (a) and physicochemical characteristics (b) for preliminary studies.

Sample (a)	Manure holding time (day)	Manure weight (kg)	Initial MC (%)	Final MC(%)	Max temperature (°C)	Time to reach max temp. (hour)	Turning frequency	Composting Time (day)
1.1	4	6.5	58	51.7	67.1	36	No rotation	3
1.2		6.5	58	49.4	67.6	37	12h rotation/12 h stop	3
2.1	20	5.7	57	54.5	66.7	38	24h rotation	10
2.2	20	5.7	57	48.3	62.9	35	24h rotation	10

Sample (b)	Compost	MC (%)	TOC ($g.kg^{-1}$)	TKN ($g.kg^{-1}$)	NH_4 ($g.kg^{-1}$)	pH	WEOC ($g.kg^{-1}$)	WEN ($g.kg^{-1}$)
	raw	81 (0.14)	531 (0.7)	17.6 (1.8)	7.2 (0.30)	8.9	35 (0.7)	3.5 (0.01)
	initial	58 (0.16)	529 (3.3)	13 (0.5)	0.9 (0.04)	9.4	29 (0.7)	1.1 (0.01)
1.1	final-static	52 (1.9)	522 (3.4)	6.3 (1)	0.6 (0.04)	9.08	47 (0.4)	2.6 (0.35)
1.2	final- rotary drum	50 (0.3)	523 (0.4)	6.2 (1.4)	0.36 (0.04)	9.07	45 (1.9)	2.2 (0.07)
	raw	81 (0.2)	525 (0.79)	23 (0.4)	4.3 (0.18)	8.97	28 (3.4)	2 (0.01)
	initial	57 (2.9)	525 (0.7)	21 (2.1)	1.25 (0.01)	9.05	46 (2.5)	1.9 (0.04)
2.1	3th day-water added	54 (0.2)	521 (0.9)	15 (1.7)	0.52 (0.02)	9.01	42 (0.16)	3.3 (0.30)
2.2	3th day -no water add	54 (0.3)	521 (0.7)	21 (5.7)	0.82 (0.42)	9.1	39 (0.32)	3.8 (0.30)
2.1	final-water added	55 (0.9)	518 (2)	7.9 (1.6)	0.16 (0.03)	9.35	29 (0.8)	0.9 (0.08)
2.2	final-no water addition	48 (1.1)	518 (1.3)	12.6 (0.8)	0.17 (0.02)	9.43	23 (0.2)	0.7 (0.10)

Carrying on preliminary studies mentioned before, three more compost samples were studied to understand composting performance including DB, DL, D samples. Fresh animal solid waste (B; L, D) samples as well as their mixtures at a certain ratio were characterized with various physicochemical parameters (Table 4.3). Depending upon the results of preliminary composting studies, the initial moisture content of D was adjusted to 63±1% by mixing it with B and L. While the DB mixture was prepared without any drying operations the others (DL and D) were subjected to a rapid drying prior to composting. With all three manure samples peak composting temperature was attained within a short time period. However, the duration of thermophilic phase (≥55^{o}C) and the variations in the concentration of water extractable nutrients of waste mixtures exhibited considerable differences during the primary composting process (Table 4.3 and Figure 4.3).

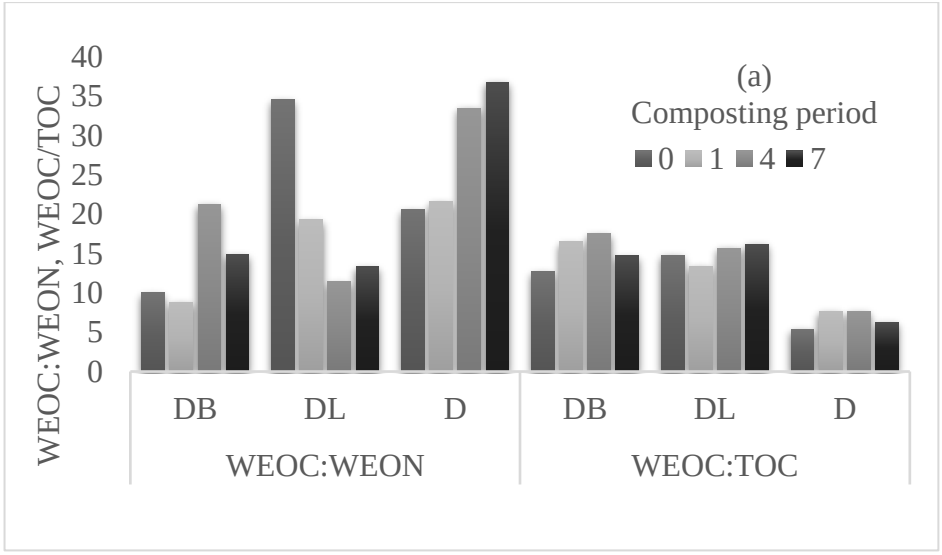
Table 4.3. Characteristics of raw manure samples with their mixtures and composted manure samples in 7 day.

Sample (a)	MC(%)	pH	TOC (g.kg^{-1})	TKN (g.kg^{-1})	NH_4^+-N (g.kg^{-1})
Dairy	79	8.7	527 (0.7)	14.4 (0.9)	1.1 (0.06)
Broiler	31	9.0	480 (2.1)	34.8 (1.3)	2.8 (0.22)
Layer	69	8.4	370 (5.2)	33.0 (2.8)	6.9 (0.24)
DB (3:1, 10 kg)	64	8.9	495 (3.9)	26.6 (3.7)	3.7 (0.11)
DL(3:1, 11.5kg)	63	8.9	415 (13)	27.0 (0.4)	3.6 (0.14)
DD (3:1, 7 kg)	63	9.2	535 (1.7)	14.0 (0.9)	0.7 (0.03)

Sample (b)	Peak T. (^{o}C)	Time to reach peak T. (h)	Duration of ≥55 ^{o}C (h)	Initial OC/ON	Final OC/ON	Final pH	NH_4^+-N (g.kg^{-1})
DB	69.9	22.5	58	22	30	9.1	5.6±0.19
DL	69.5	25	53.5	18	22	9.4	8.4±0.18
DD	68.8	26	40	40	39	9.3	0.2±0.02

*T.; Temperature

The longer duration of thermophilic phase was obviously the result of higher microbial activity due to higher WEOC and WEON contents of DL and DB compare to D. Both thermal and biochemical reactions could be responsible for the increase in water extractable portions of manure nutrients. While the only 3% OC reduction and relatively constant WEOC were observed with DB within 7 d of composting process, 13% TOC reduction and concomitant WEOC/TOC and NH_4^+ increases were the characteristics of DL indicating the higher stability potential of this mixture. The relatively poor efficiency obtained for DB could be attributed to resistance of bedding material (≈40% rice husk) mixed with excreta to biochemical reactions. However, the addition of B to D lead to elimination of drying operation prior to composting.



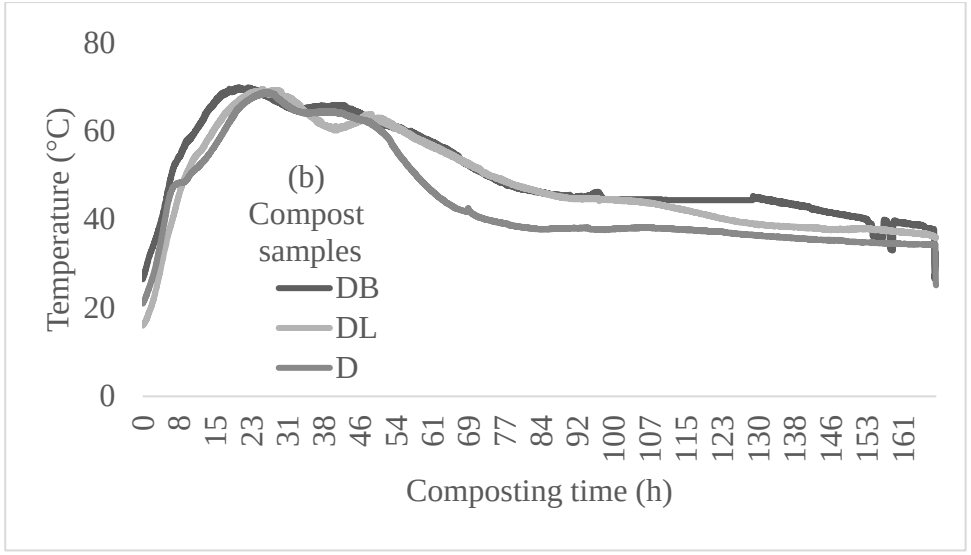


Figure 4.3. Variations of C/N ratios of manure samples during composting (a) and temperature profiles of composting of animal manure mixtures (b).

4.3.2. Characteristics of Manure Samples

The fate of antibiotics in manure was investigated at aerobic composting conditions. The performance of composting process depends upon both the nature of manure (C/N ratio and nutrient content) and the operational parameters (aeration rate, moisture content, temperature) that are interdependent. The characterization studies performed within the scope of this project for various solid samples separated from manure slurry indicated that the moisture (80 ± 1 %) and nutrient contents (C/N >40) of dairy cow manure were not appropriate to achieve efficient composting. Therefore, the moisture content of collected D was adjusted to 60-65% by partial drying or mixing with dried L or B at the ratio of 3:1 (w/w) prior to composting, since the preliminary composting experiments with manure containing 60% moisture exhibited high performance in terms of achieving a temperature above 60 °C. Additionally, to evaluate the contribution of manure blending on composting process B, which has 30% moisture was individually composted after humidification. In table 4.4 the characteristics of manure samples used in different composting experiments are shown.

Table 4.4. Characteristics of raw manure samples.

Manure	OC/ ON	NH_4 ($g.kg^{-1}$)	TOC ($g.kg^{-1}$)	TKN ($g.kg^{-1}$)	pH	EC ($ms.cm^{-1}$)	P ($g.kg^{-1}$)	WEOC ($g.kg^{-1}$)	WEON ($g.kg^{-1}$)
Dairy 1	41	0.7 (0.03)	535 (1.70)	14 (0.90)	9.2		8.4 (0.01)	29 (1.10)	1.2
Dairy 2	79	0.4 (0.02)	537 (1.90)	7 (0.04)	9.3	0.8		18 (0.60)	1.1
Dairy 3	46	1.3 (0.18)	532 (2.80)	13 (2.40)	9.0	1.0 (0.04)	9.9 (0.40)	28 (0.70)	0.7
Broiler 1	15	2.8 (0.20)	478 (2.20)	35 (1.30)	9.0		26 (0.98)	90 (0.40)	7.2
Broiler 2	18	4.8 (0.30)	495 (0.70)	32 (0.90)	8.3	2.7 (0.08)	27 (0.07)		
Layer	14	6.9 (0.20)	370 (5.30)	33 (2.80)	8.4		40 (4.70)	75 (1.50)	13.1

Due to low organic nitrogen concentration of D (13.3 $g.kg^{-1}$), OC/ON ratio was extremely high as shown in table 4. On the other hand, the organic nitrogen concentrations of poultry manure samples were 25-32 $g.kg^{-1}$. Hence, the mixing of dairy and poultry (L or B) manure at 3/1 ratios provided a balanced OC/ON ratio.

4.3.3. Effect of Air Flow Rate on Temperature and Moisture Content

Aeration is an essential requirement to develop the aerobic biological activity. The effect of aeration rate on temperature and MC of manure in the closed rotary drum was investigated in this

study. Both rapid increase of temperature and more than 55 °C temperature was attained in all composting experiments. According to US FDA, (2013), there is a recommendation about maintenance of compost temperature above 55 °C for 3 d for static and 15 d for windrow composting for hygienic safety. The temperature of each manure was reached to peak value of about 70 °C within 1-2 days and subsequently temperature remained above 55 °C for 1.5-4 days (Table 4.5). The shortest time to reach peak composting temperature was achieved with DB-1, DB-2 and DL-1 manure samples that could be attributed to resident microbial abundance and diversity of these manures. Temperature profiles of all compost cases are shown in Appendix H.

Table 4.5. Temperature profiles of composted manure samples.

Aeration rate	Composted manure samples	Peak temperature (°C)	Time to reach peak temperature (h)	Duration of ≥55 °C (h)
Low ($3.6\ m^3.h^{-1}$)	D-1	69	45	42
	DB-1	73	16	58
	DL-1	72	32	84
High ($16.5\ m^3.h^{-1}$)	D-2	60	55	38
	DB-2	68	16	37
	B-2	70	39	45

Aeration can be used as feedback control for the composting temperature in large scale process. D-2, DB-2 and B-2 manures were composted at elevated airflow rate ($16.5\ m^3.h^{-1}$) and at extended composting time (20 d). As expected, high airflow rate through manure composting resulted in heat loss and associated cooling (Table 4.5). In case of dairy manure composting (D-2), increasing airflow rate prolonged the time where the temperature arose to maximum value whereas this effect was not observed for DB-2 manure. However, continuous high airflow rate obviously shortened the time for the temperature remained above 55 ºC that is required to achieve the destruction of pathogenic microorganisms. Therefore, although DB-2 and B-2 composted manure did not contain *E.coli*, D-2 manure still included *E.coli* on 20^{th} day of composted manure because of the lowest attained maximum temperature.

Composting is a highly dynamic process and a decrease in the moisture content of compost is well expected result due to self-heating and continuous aeration especially at high airflow rate. However, during the composting with low airflow rate the decrease of manure moisture content was limited that was not required to the addition of water to the compost. In contrast, water was irregularly added to manures composted with high airflow rate to maintain the moisture content at 40-65%, which can sustain the microbial activity (Herbert et al., 2010). Considering the unfavorable moisture condition (< 30%) for the growth of pathogenic microorganisms (Herbert et al., 2010), humidification

of compost stopped after 12 days of composting process performed with high airflow rate. As can be seen from the Table 4.6 the moisture content of D-2, DL-2 and B-2 composts decreased significantly at extended composting time and hence, it reached to the desired value for their storage.

Table 4.6. Moisture content of composted manure samples.

Aeration rate	Composted manure samples	Initial MC (%)	12 th day MC (%)	20 th day MC (%)
Low ($3.6\ m^3.h^{-1}$)	D-1	61 (0.2)	52 (0.1)	-
	DB-1	64 (0.8)	45 (1.0)	-
	DL-1	60 (0.3)	65 (0.2)	-
High ($16.5\ m^3.h^{-1}$)	D-2	65 (1.0)	42 (0.0)	17 (0.2)
	DB-2	61 (1.9)	52 (0.4)	30 (0.0)
	B-2	57 (0.9)	54 (0.2)	38 (0.5)

4.3.4. Microbial Activity Determination with SOUR

The presence of microbial community, diversity of microbials and biological activity level in blended manure were assessed by SOUR. This measurement was performed in raw and composted DB-2 manure as well as B-2 and D-2 manures (Figure 4.4).

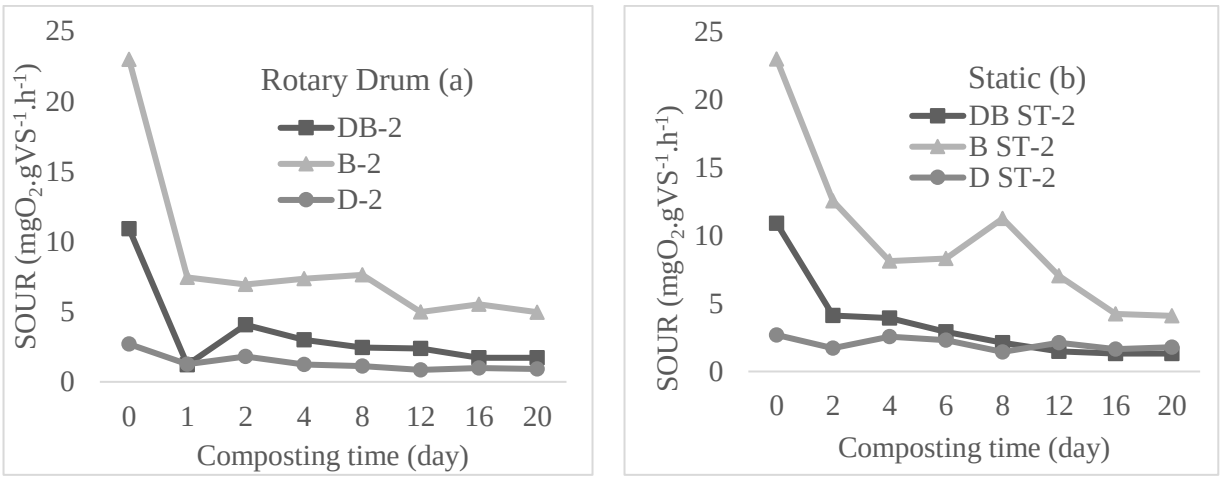


Figure 4.4. SOUR Variation during composting for rotary drum (a) and static (b) compost.

As shown in Figure 4.4, SOUR values of raw D (2.69 $mgO_2.gVS^{-1}.h^{-1}$) and B manure (22.98 $mgO_2.gVS^{-1}.h^{-1}$) were significantly different. Higher SOUR and peak temperature could support the opinion about the higher abundance of microbial community in B compare to D. Furthermore, *E.coli* of B (560,000 cfu) was also approximately two times higher than that of D (320,000 cfu).

During the composting, following the attainment of peak temperature within 24 h of process, a reduction of SOUR was observed in each case although peak composting temperatures of B-2 and D-2 manures were quite different. These results obviously revealed the elimination of biological activity by the reduction of microorganism concentration. However, the blending of D with B provided higher SOUR initially and during the composting. Variation of SOUR for rotary drum and static composts were similar with a slight difference in the first day that was probably resulted from attained peak temperature differences.

4.3.5. Fate of Antibiotics During Composting

In contrast to various studies in the literature (Qui et al., 2012; Mitchell et al., 2015; Dolliver et al., 2008; Amarakoon et al., 2016), antibiotics were not initially spiked to manure samples to investigate their fate in the composting process. SAs, FQs, TCs, ERY, TRI and LIN were predominant in the investigated manures and those of antibiotics found in manure were monitored during composting processes to understand their behavior. Antibiotic reduction was determined by calculating percent reduction (%) and half-life (d) of antibiotics (Table 4.8, Appendix I). In Table 1.3 and 1.4, half-life of analytes and percent reduction given in literature were listed to compare these antibiotic degradation performances between our study results. Fate of antibiotic groups are shown and explained in each following section and comparative discussions with other studies in literature are also written in following sections.

4.3.5.1. Fate of SAs During Composting The analytes of SCP, SFX, STZ and SMH as SAs were detected in manure samples. As shown from Figure 4.5 and Table 4.8 composting could provide the substantial reduction of SAs without depending upon initial concentration, type of manure, turning-static composting or attained maximum temperature of manure samples. Reduction of antibiotics were in the range of 69-100% and half-life of analytes were between 0.8-8.7 days except for SFX-DL-1 rotary drum compost sample, (Half-life, 41.8 days). Although percent reduction of SFX was estimated as 79%, slope of linear regression (k, day^{-1}) of this antibiotic was calculated as 0.0166. It was probably resulted from the incompetency to first order decay of this sample.

In some studies, considerable SAs reduction and elimination were achieved in manure samples during composting (Table 1.3 and 4.7) but in three of them (Dolliver at al., 2008; Zhang et al., 2019., Cessna et al., 2011) SAs could not be degraded due to lack of moisture content. SMZ concentration

Table 4.7. Variation of antibiotic concentration (μg.kg^{-1}) and half-life of analytes in manure and composted samples (n=3).

	Initial Concentration (μg.kg^{-1})	Final Concentration (μg.kg^{-1})		Half-life (d)		Reduction (%)	
		RD	ST	RD	ST	RD	ST
SCP D-1	17 (3)	0	*	0.8	*	100	*
SFX DL-1	22 (3)	4 (0.1)	*	41.8	*	79	*
STZ DL-1	36 (2)	6 (0.2)	*	4.3	*	83	*
STZ DB-2	470 (25)	0	0	1.2	1.2	100	100
SMH DB-1	130 (22)	40 (5)	18 (2)	6.3	4.7	69	86
SMH DB-2	96 (29)	13 (2)	15 (0.1)	7.5	8.7	87	84
SMH B-2	310 (13)	41 (4)	32 (1)	5.4	5.9	87	90
CIP D-1	148 (27)	0	*	0.5	*	100	*
CIP DB-1	6,476 (134)	5,487 (85)	1,681 (100)	36.1	8.9	15	74
CIP D-2	84 (13)	0	0	1.1	1.7	100	100
ENR D-1	277 (41)	271 (32)	*	495.1	*	2	*
ENR DB-1	20,408 (845)	15,007(1,500)	3,926 (240)	26.2	8.1	26	81
ENR DB-2	22,123 (2,400)	4,700 (7)	5,972 (11)	12.2	14.8	79	73
ENR B-2	11,218 (1,000)	5,576 (401)	9,689 (438)	25.6	15.4	50	14
ENR D-2	385 (50)	197 (53)	126 (14)	44.7	40.3	49	67
DAN DB-1	518 (23)	12 (1)	18 (1)	3.0	2.5	98	97
CTC D-1	131 (40)	24 (1)	*	11.4	*	82	*
CTC D-2	1,720 (452)	0	0	1.3	1.2	100	100
OTC D-1	11,575 (880)	8,644 (851)	*	24.2	*	25	*
OTC DL-1	4,260 (45)	1,255 (7)	*	7.3	*	71	*
OTC DB-2	502 (40)	138 (26)	27 (1)	38.5	4.8	67	95
OTC D-2	12,797 (749)	653 (57)	1,113 (72)	17.8	11.7	95	91
LIN D-1	27 (1)	26 (1)	*	216.6	*	3	*
LIN DL-1	28 (3)	22 (1)	*	27.7	*	23	*
LIN DB-1	12,866 (1,500)	5,564 (170)	*	10.5	*	57	*
LIN DB-2	33 (2)	25 (0.2)	25 (0.2)	106.6	84.5	22	23
LIN B-2	518 (50)	0	3.5 (0.1)	1.4	1.5	100	99
LIN D-2	138 (7)	7 (1)	10 (3)	5.7	5.2	95	93
TRI DB-1	116 (6)	77 (20)	88 (8)	28.1	46.8	33	24
TRI DB-2	73 (21)	28 (1)	26 (3)	18.4	13.3	61	64
TRI B-2	1,057 (50)	78 (22)	103 (7)	7.8	7.7	93	90

*ST (Static Compost) was not studied, RD (Rotary Drum).

could not be changed during turkey litter composting by Dolliver et al., (2008) because of low moisture content (37-44%) through composting. In addition, at most 45.1% reduction was barely attained in swine and poultry manure composting by Zhang et al., (2019) which was attributed to the low moisture level (between 30-35%) due to the lack of humidifying through composting after 10 days of compost. In another study, half-life of SMZ was 237 days in beef feedlot manure compost samples and 40% reduction were achieved at the end of composting (126 days) (Cessna et al., 2011).

It was attributed once more to lack of rainfall in the season for windrow composting that could be limiting factor for SMZ reduction. Lack of moisture content leads to oxygen and dissolved nutrient transformation hamper. Insufficient oxygen supply could inhibit microbial activity and biological process.

Although relatively high maximum temperature attained in Zhang et al., (2019) (68 °C), Cessna et al., (2011) (60°C) and Dolliver at al., (2008) (64°C), SAs reduction was not enriched by attaining high temperature. However, it was advocated that thermophilic temperatures are necessary criteria to obtain high antibiotic degradation rates in composting process (Arıkan et al., 2009; Mitchell et al., 2015). On the other hand, elimination of SDZ was attained in broiler manure at 45.7 °C maximum temperature (Ho et al., 2013) which was a clear conflict concerning requirement of thermophilic conditions during SAs reduction through composting.

In preliminary studies of manure compost cases, 55-65 % moisture content was determined as optimum moisture level for composting performance in our study that helped the reduction of SAs. From maximum temperature view, the highest attained temperature case (73°C) was RD-DB-1; however, the lowest degradation rate (69%) was attained in SMH DB-1 in composted manures. It means that our study result complied with the other studies (Table 1.3 and 1.4) in terms of temperature concern. Additionally, 86% reduction was achieved with the same manure sample for SMH ST-DB-1 since static compost maximum attained temperature was at most 60°C (manually measured irregularly, data not shown).

For RD-DB-2 sample, antibiotics were measured for 60^{th} and 90^{th} days during static storage. SMH was reduced at the range of 87% on the 20^{th} day of compost and could not be detected on 60^{th} day whereas it could be found at the range of 37 $\mu g.kg^{-1}$ on the 90^{th} day which was probably resulted from reversible transformation of metabolites to parent compound. It is known that SAs could be transformed to acetyl derivatives with deacetylation and turned back to parent compounds during composting or storage (Lamschóft et al., 2010). Although composting provides substantial benefit for elimination of antibiotic pollution, retransformation to parent compounds poses potential risk for soil that should be considered before fertilization.

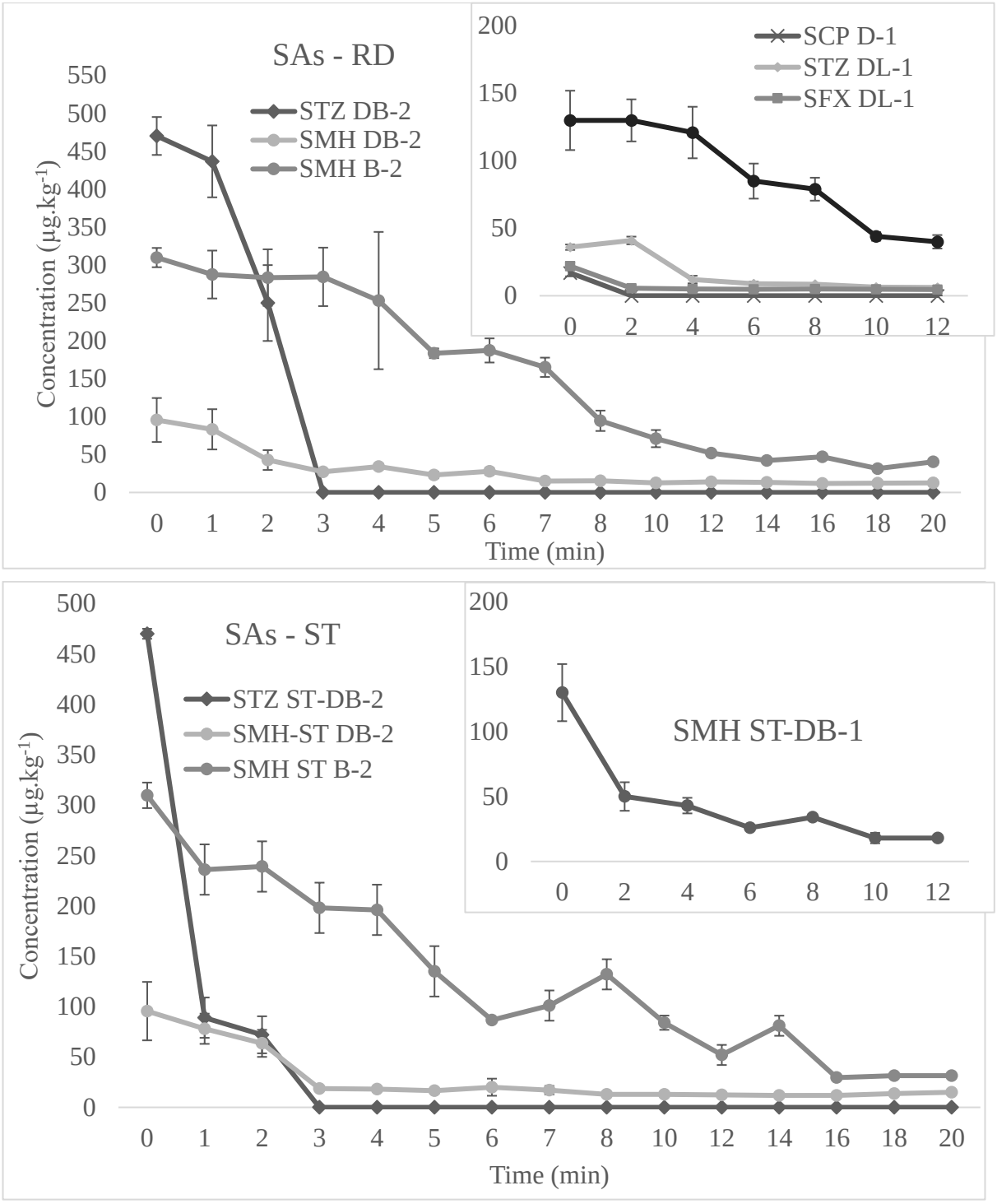


Figure 4.5. Variation of SAs concentration during composting studies (RD: Rotary Drum, ST:Static) (n=3).

4.3.5.2. Fate of FQs During Composting ENR and CIP were detected in all investigated manures whereas DAN was only found in DB-1 manure (Figure 4.6 and 4.7). Contrary to the high reduction result obtained with DAN and ENR, an increase in the concentration of CIP was observed during the composting of DL-1, DB-2, B-2 manure samples. This could be attributed to the transformation of ENR to its active metabolite which is mainly CIP. It is known that CIP is not the only metabolite of

ENR but the others could not pose antibiotic effects (Trouchan and Lefebvre, 2016; Assis et al., 2016). In addition, concentration of ENR increases during DL composting which was clearly known that metabolites of SAs (Sarmah et al., 2006; Lamschóft et al., 2010), MCs (Ray et al., 2017) and LINs (Zhang et al., 2019) antibiotics were able to be bioactive and transformed back to the parent compound during compost; hence, reverse transformation of metabolites of ENR might be possible as occurred for other antibiotic groups. It is also known that ENR was relatively persistent in the manures and could not be transformed (Zhang et al., 2019).

However, half-life of ENR presented a broad range from 8.1 to 495.1 days in our study and for some cases exhibited considerable reduction such as RD-DB-2, which an initial concentration of 22,123 $\mu g.kg^{-1}$ was detected. After 20 days of composting, 79% of ENR was reduced in manure sample and subsequent storage of this compost until 60 days increased the overall degradation rate up to 89%. On the other hand, there was a substantial conflict for ENR and CIP reduction character for DB-1 samples between ST and RD compost. The most successful half-life was attained for ENR (8.1 days) by implementing ST-DB-1 compost; however, half-life of ENR for the same compost sample was taken much more time (26.2 days) by RD compost. What is more, different reduction performance was similar in terms of CIP, which half-lives of DB-1 compost was 36.1 for RD and 8.9 days for ST compost samples, respectively. For another case, ENR reduction efficiency was nearly the same for RD and ST compost during DB-2 compost. To explain this conflict temperature regime was analyzed and attained maximum temperature differences between RD (68, 73°C) and ST (60°C-manually irregularly measured) and RD-DB-1 (73°C) and RD-DB-2 (68°C) compost samples might be effective for antibiotic reduction performances as SAs condition explained in previous section.

Some previous studies in the literature reported almost complete degradation of FQ antibiotics found in manure (Wang et al., 2016; Ho et al., 2013). Ho et al., (2013) achieved ≥99% reduction of 36,770 $\mu g.kg^{-1}$ ENR in 40 days of composting where peak temperature was 45.7 °C. Similarly, in the study of Wang et al., (2016) maximum composting temperature was lower than 60°C and CIP degradation was between 82.7-98.9%. On the contrary, FQs was persistent in manure compost sample and could not be degraded where attained maximum temperature reached to 68°C (Zhang et al., 2019).

In this study, ENR concentrations in D-1, DB-1, DL-1, D-2 and B-2 manures were much lower than that of DB-2 manure but the degradation rates of FQs were poor although the peak temperature of composting was approximately 70 °C indicated enhanced microbial activity. It can be assumed that the peak temperature of composting could cause the destruction of microorganism as shown by

SOUR measurements. However, this assumption cannot explain the high degradation rate of DAN in DB-1 manure. DAN could be nearly eliminated through both ST and RD compost. There is no doubt that reduction performances of antibiotics are influenced from complex biotic and abiotic conditions (Arıkan et al., 2009; Mitchell et al., 2015; Zhang et al., 2019; Ray et al., 2017) that should be criticized upcoming studies with different antibiotics in combination of different compost samples. It should be also noted sufficiently high temperature is a necessary requirement for the compost to achieve hygienic safety. Furthermore, high composting temperature provides beneficial effects to prevent the increase of antibiotic resistance determinants (Lin et al., 2017).

In conclusion, the obtained results clearly reveal the recalcitrance of ENR and CIP in manure with few exceptions. The strong sorption of FQs on the manure can be responsible for their persistence during composting since it is known that they sorbed to manure with various mechanisms including bond bridge with -COOH group and cation bridging with multivalent cations (Ca, Mg, Al, Fe) (Wang et al., 2015; Thiele-Bruhn, 2003; Tolls, 2001).

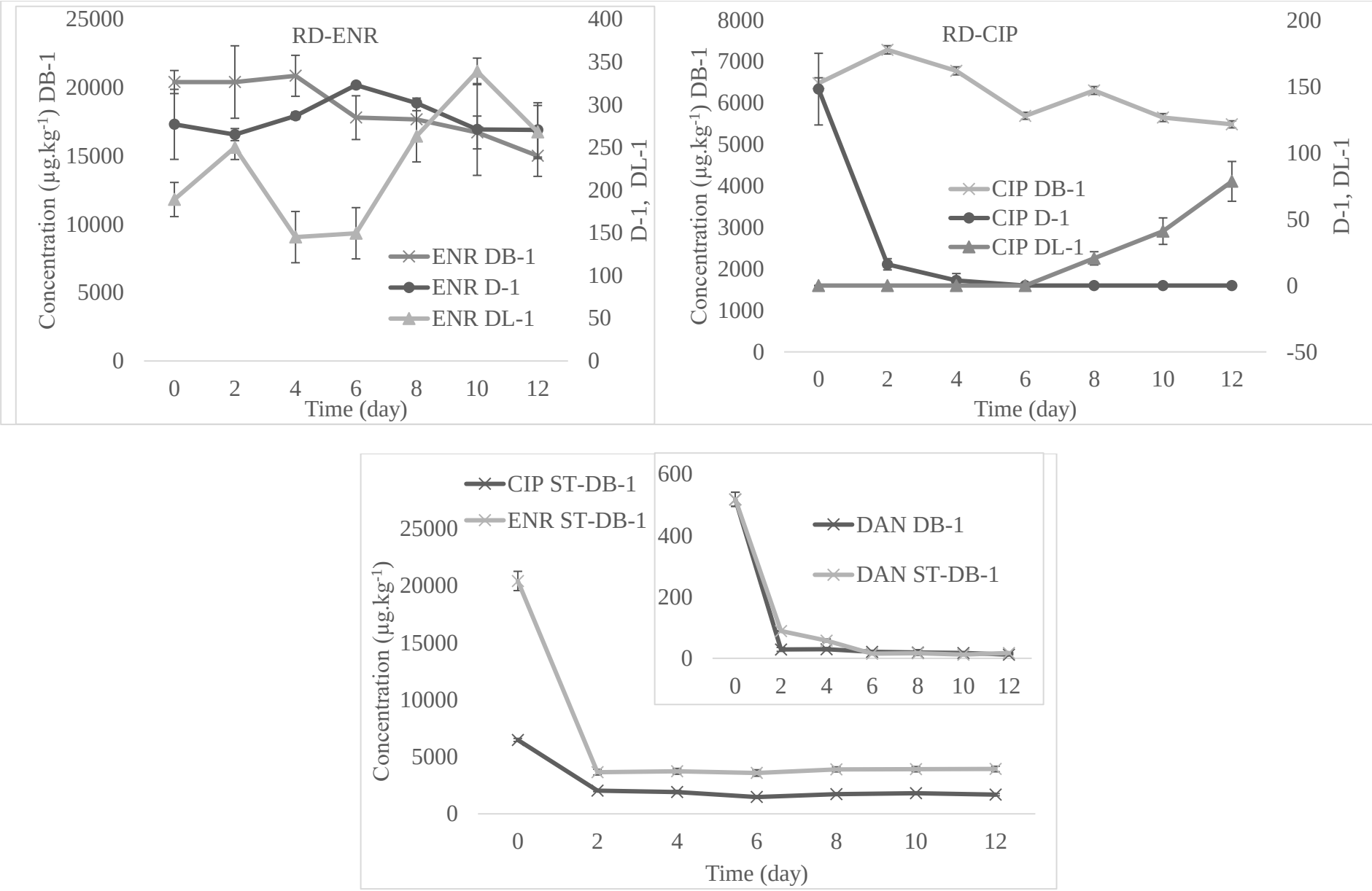


Figure 4.6. Variation of FQs concentration during composting studies for 1st composting cases (RD: Rotary Drum, ST:Static) (n=3).

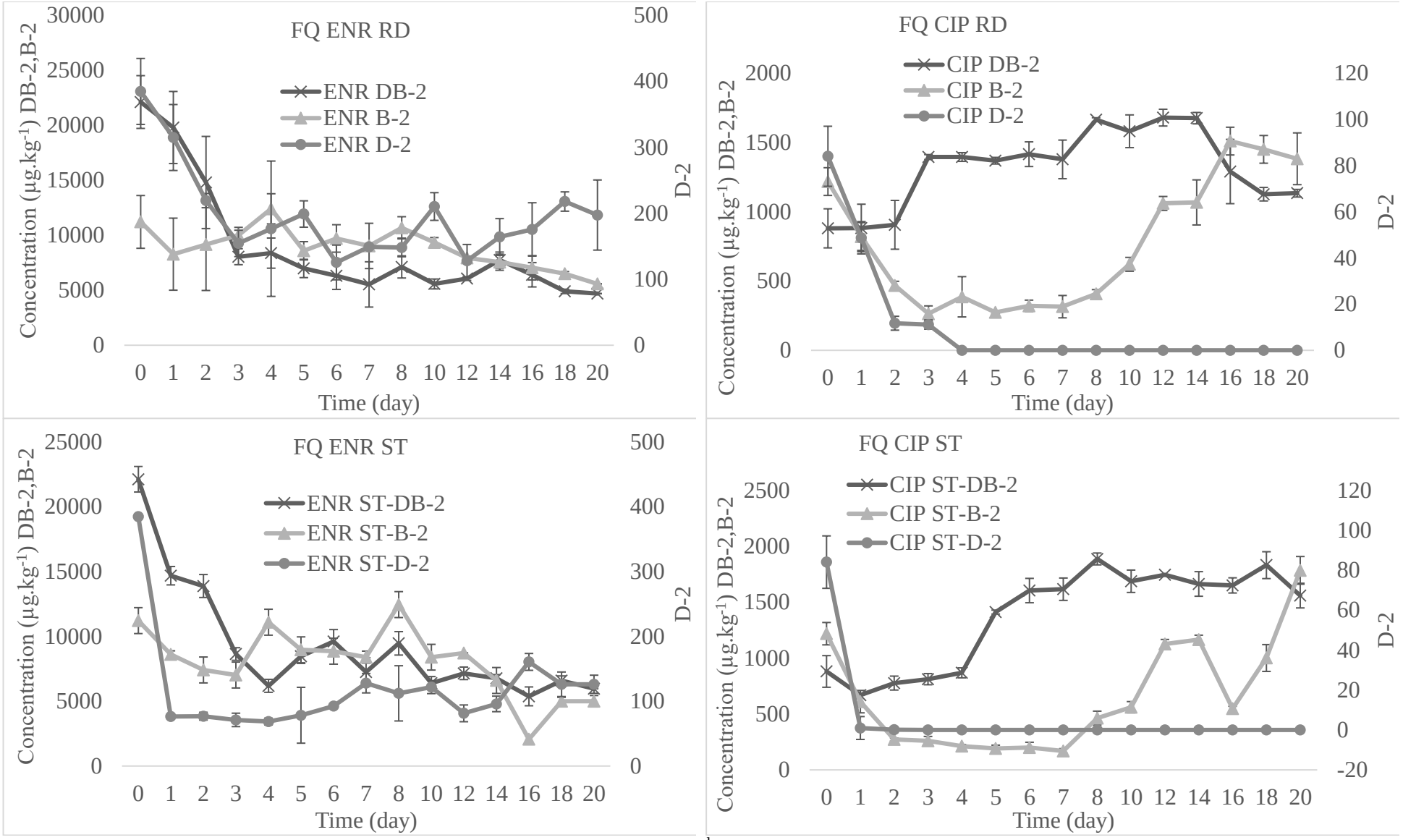


Figure 4.7. Variation of FQs concentration during composting studies for 2nd composting cases (RD: Rotary Drum, ST:Static) (n=3).

4.3.5.3. Fate of TCs During Composting Significantly high concentration of TCs (OTC and CTC) were detected in D-1 and D-2 manure samples (Table 4.8, Figure 4.8). While OTC concentrations of D-1 and D-2 manures were found at mg kg^{-1} level, CTC concentration was comparably low. As in case of TC composting, high airflow rate provided efficient composting of TCs (Table 4.8, Figure 4.8). While the degradation rates of OTC and CTC in D-1 manure were 25% and 82% over the 12 days composting, respectively, 95% and >99.9% degradation rates of these TCs were achieved in D-2 within the 20^{th} day of composting. Half-lives of TCs were between 1.2 to 38.5 days and although initial concentrations of antibiotics and samples were the same, OTC-ST-DB-2 half-life (4.8 days) was lower than RD compost (38.5 days) as some of FQs and SAs compost samples.

TCs are known as instable at high temperature (Pikkemaat et al., 2016). Although high airflow rate caused a decrease in the peak composting temperature of D-2 manure the time to reach this temperature was longer than that obtained by low airflow rate. Therefore, high degradation rates of OTC and CTC in D-2 compare to D-1 manure samples could be attributed to exposure of these antibiotics to lower temperatures for longer time before reaching the peak value. In addition, OTC degradation enhancement during ST-DB compost was only attributed to lower attained peak temperature compare to RD. These results are contrary to what Arıkan et al., (2009); Kim et al., (2012); Chai et al., (2016); Ravindran and Mnkeni, (2016) find. They attributed the removal of TCs to temperature dependent abiotic processes rather than biotic processes. As a support of our study results, TCs showed lower removal rates (74%) in the study performed by Zhang et al., (2019) although the attained maximum temperature reached to 68°C.

Despite high degradation rates of antibiotics, the concentrations of OTC in D-1, D-2, DL-1 manures were high at the end of the composting period. TCs exhibited high sorption tendency like FQs. They can bind to proteins, silanolic groups, humic acids (Thiele-Bruhn, 2003) and/or form complexes with metal cations (Ca, Mg, Al, Fe) found in the composition of manure (Tolls, 2001). High sorption tendency of TCs might prevent their degradation in the compost. There are lots of study about the fate of antibiotics that were fortified to manure before composting and achieved considerably low half-lives. For instance, 1-day half-life was detected for CTC by Dolliver et al., (2008). However, without fortification of antibiotics to manure, Cessna et al., (2011) calculated up to 26.5 days and Ray et al., (2017) found up to 8.7 days half-lives for excreted antibiotics during manure composting. It was probably resulted from the strong sorption of antibiotics to manure during the extended contact time inside the animal body and on the feedlot floor waited for accumulation before treatment (Amarakoon et al., 2016).

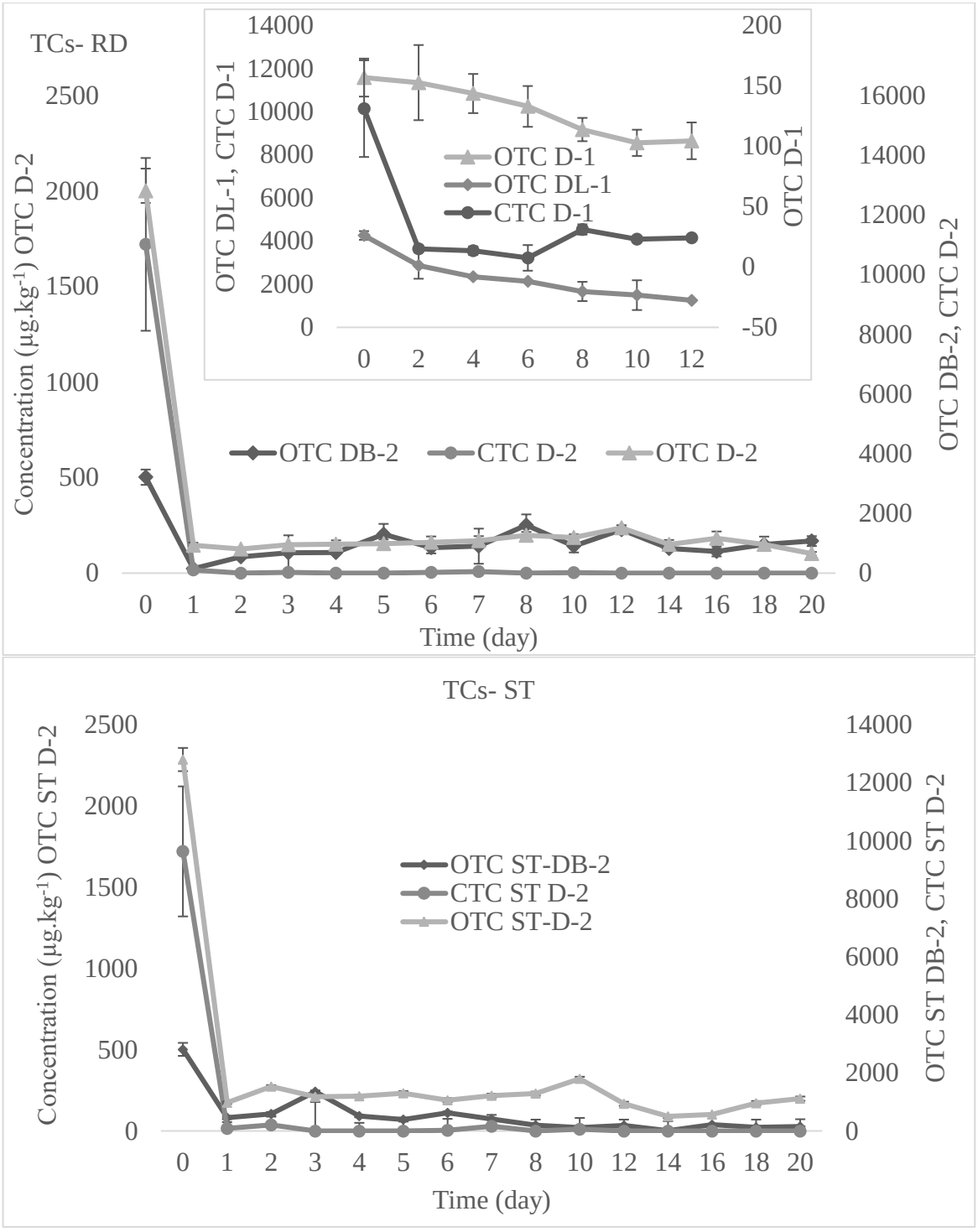


Figure 4.8. Variation of TCs concentration during composting studies (RD: Rotary Drum, ST:Static) (n=3).

4.3.5.4. Fate of MCs During Composting ERY was the only MCs among the investigated antibiotics and was detected in D-1 and DL-1 manures at initial concentrations of 483 and 401 µg.kg^{-1} respectively (Table 4.8 and Figure 4.9). TYL, which is another most commonly administered antibiotic in cattle and poultry production, but it has not been found in any of the manure samples in this study. It should be mentioned that ERY is not used for growth and feed efficiency as opposed to TYL. While the occurrence of ERY in the form of anhydro-erythromycin was reported in wastewater

(Zhou et al.,2013) there is no information for the detection of this transformation product of ERY in manure.

The results of D-1 and DL-1 manures composting revealed that ERY was markedly persistent and the degradation was not achieved in composting period. Aerobic degradation is an important elimination mechanism for ERY while anaerobic conditions lead the persistence of ERY (Schlüsener and Bester, 2006). In the study of Ho et al. (2013), ERY in broiler manure at 15,580 $\mu g.kg^{-1}$ concentrations was degraded through 40 days composting with a half-life of 1.4 days. On the other hand, the persistence of this antibiotic was reported with 41days half-life during storage of pig manure (Massé et al., 2014). Furthermore, the dissipation kinetics of ERY in soil was highly dependent upon the initial concentration of antibiotic (Pan and Chu, 2016) and it is less persistent in soil that has history of antibiotic exposure. Since substantial population of microorganism have resistance the degradation of ERY, it could be degraded even under anaerobic condition (Amin et al., 2006). Therefore, the lack of ERY attenuation in D-1 and DL-1 could be attributed to short composting period. Moreover, attained composting peak temperature might be a second reason of ERY persistence by reduction of microbial community since Motoyama et al., (2011) and Ho et al., (2013) achieved the degradation of ERY with 38 and 45.7 °C maximum composting temperatures, respectively. On the other hand, transformation of metabolites to parent compound might contribute concentration increase of ERY, which was also explained by Ray et al., (2017) for TYL increase during composting.

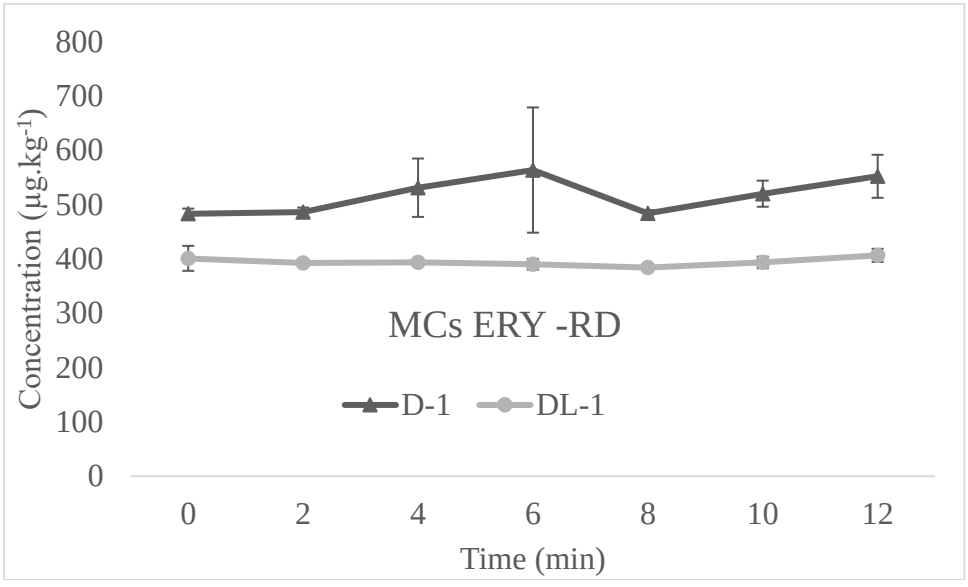


Figure 4.9. Variation of MCs concentration during composting studies (RD: Rotary Drum) (n=3).

4.3.5.5. Fate of LINs During Composting LIN was found in all investigated compost samples in this study and some of the manure samples have relatively high concentration of this antibiotic (Table 4.8

and Figure 4.9). It is clearly known that LIN is one of the most frequently detected antibiotics in the soil and water environments (Barceló and Petrovic, 2008) and a possible source of this pollution is the livestock manure (Kuchta and Cessna, 2009). However, its concentration in the manure was scarcely reported (Berendsen et al., 2015) and less information regarding LIN's fate is known (Zhang et al., 2019).

As shown in Table 4.8, although initial concentration of LIN was in the range of 27-30 $\mu g.kg^{-1}$ in D-1, DL-1 and DB-2 manure samples their degradation efficiency were very poor within the first 12 days of composting period (Figure 4.9). For D-1 manure sample, there was almost no attenuation for LIN. However, the extension of composting period caused an acceleration of LIN degradation. For instance, %71 and 79% degradation rates were achieved for DB-2 manure samples in 60 and 90 days, respectively (data not presented). Contrary to these results obtained with manure samples including low initial LIN concentration, the composting of DB-1 (12,866 $\mu g.kg^{-1}$), B-2 (518 $\mu g.kg^{-1}$) and D-2 (138 $\mu g.kg^{-1}$) manure samples provided drastic decrease of antibiotics (DB-1=57%, B-2= >99%; D-2=95) after first two days when the peak temperature was attained, which showed 1.4-10.5 days half-lives. Although LIN was a recalcitrant antibiotic and persisted more than 4 weeks (Kuchta and Cessna et al., 2009) in dugouts and ephemeral wetlands and 6 months in lagoon storage LIN was successfully reduced through our composting studies.

Besides, ST and RD compost did not demonstrate differences in reduction performance of antibiotics for DB-2, B-2 and D-2 samples. Additionally, the accumulation of LIN in ST-DB-1 manure in thermophilic and mesophilic phase might result from the metabolite transformation to the parent compound that mentioned by Zhang et al., (2019), which was known as a first report that showed the accumulation of LIN during composting in mesophilic phase. Metabolites of LIN were *N*-desmethyl LIN and LIN sulfoxide transformed in metabolism of animal, which were probably found in excreted manure samples.

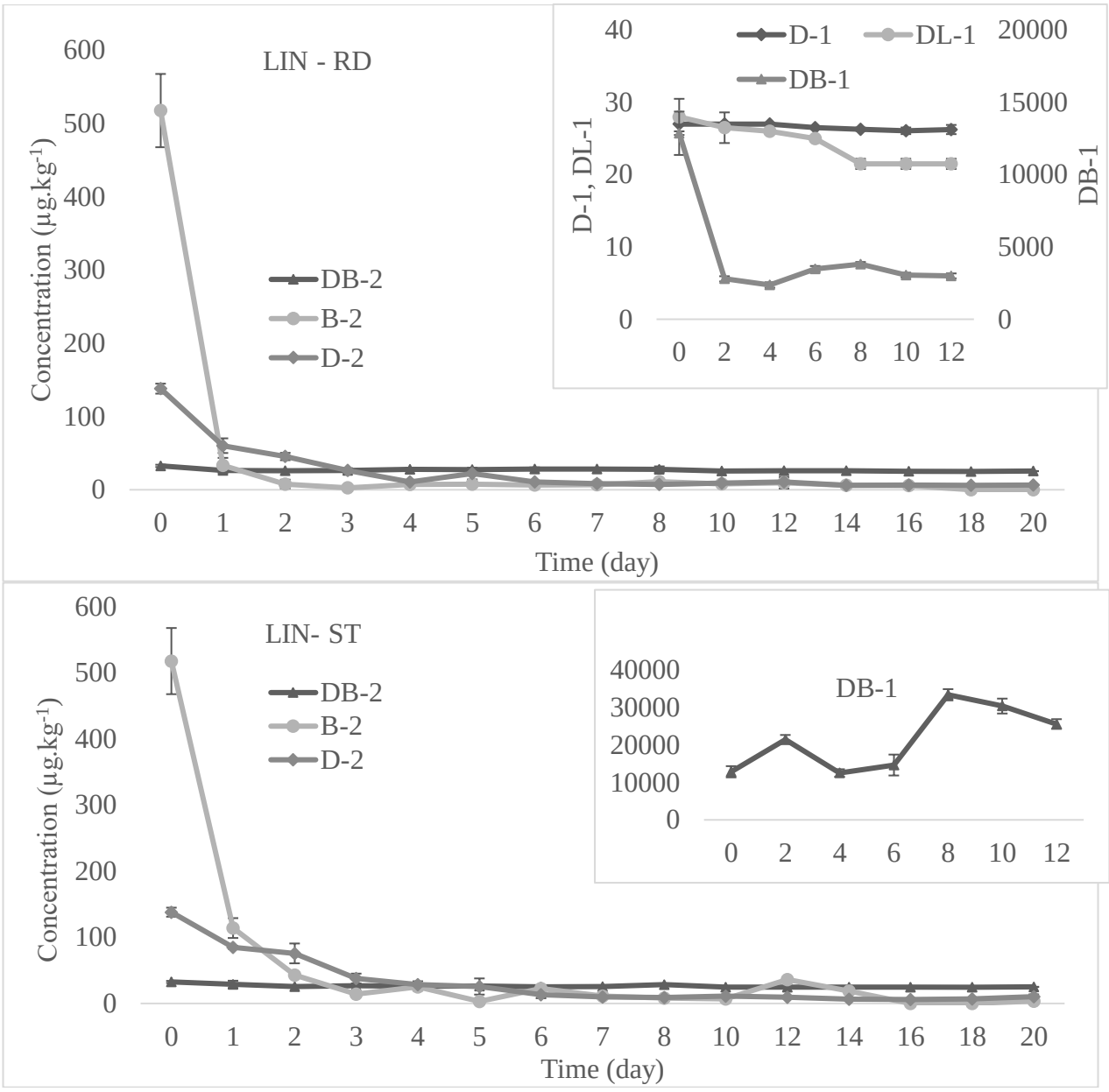


Figure 4.10. Variation of LIN concentration during composting studies (RD: Rotary Drum, ST:Static) (n=3).

4.3.5.6. Fate of DAPs During Composting As administered veterinary medicine to animal, TRI is generally applied together with SMH at a ratio of 1:5 to obtain a synergistic therapeutic effect (Liu et al., 2010). Herein, simultaneous occurrence of SMH and TRI were observed in DB-1, DB-2 and B-2 (Table 4.8 and Figure 4.10). Accordingly, B-2 manure sample has the highest initial TRI concentration (1,057 µg.kg^{-1}), which probably means the source of TRI was B manure in DB manure samples.

Although DB-1 and DB-2 manure samples have similar initial TRI concentrations, the degradation behavior of this antibiotic was different during the composting. A fluctuation in the concentration of TRI in DB-1 was detected and lower degradation rate was observed at the end of the

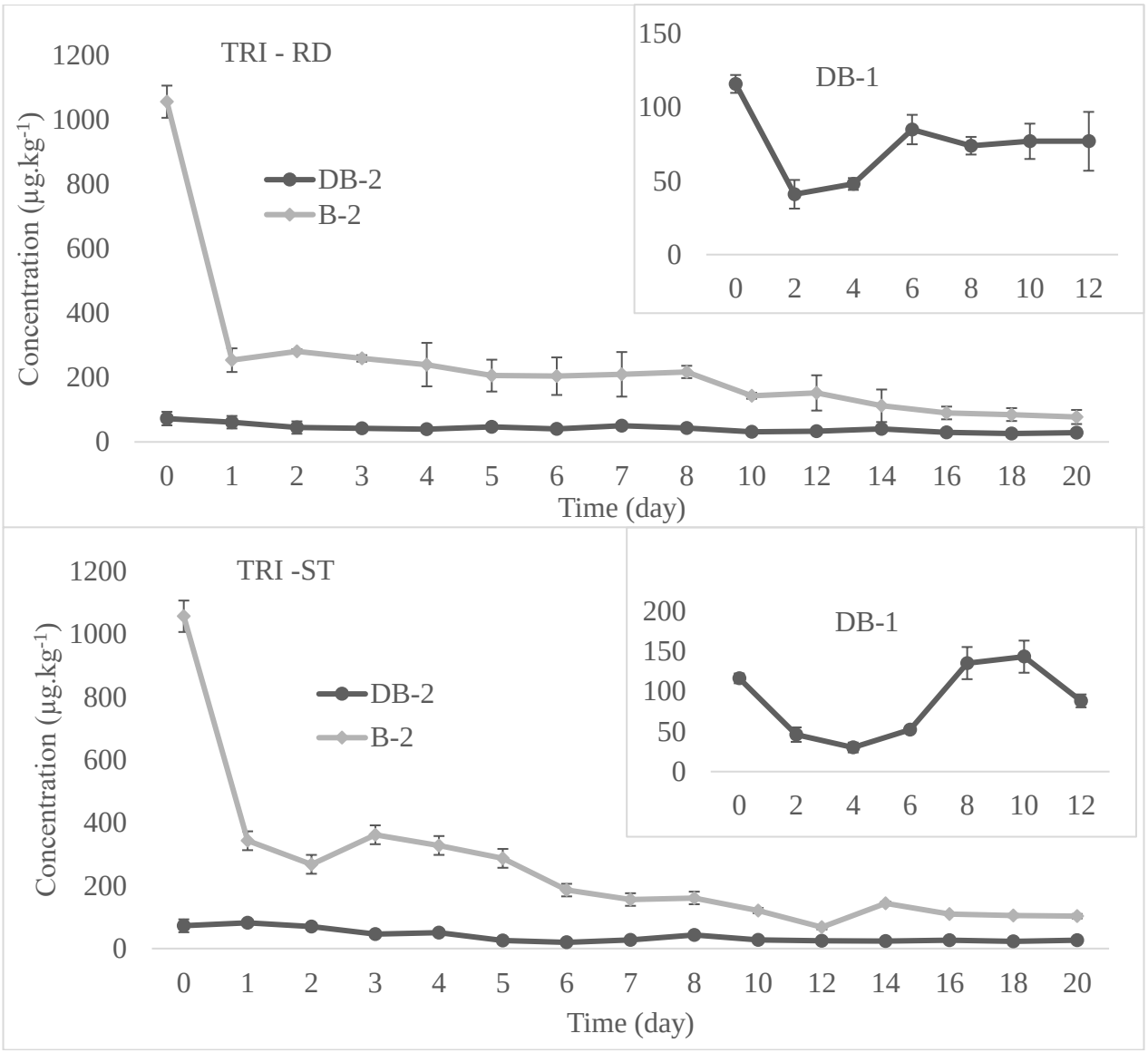


Figure 4.11. Variation of LIN concentration during composting studies (RD: Rotary Drum, ST:Static) (n=3).

12 days composting period for both RD (33%) and ST (24%) compost. In a previous study, even an increase in the concentration of TRI was observed in soil under anaerobic condition (Lin and Gan, 2011). Furthermore, higher peak composting temperature and longer exposure time to more than 55°C of DB-1 did not provide its sufficient degradation (33%) due to high thermal stability of TRI (Svahn and Björklund, 2015). Contrary to DB-1 composting result, relatively efficient degradation of TRI (61%) was achieved in DB-2 manure sample during the first 12 days of compost. This result is consistent with those of other antibiotics investigated in this study and the positive influence of high airflow rate was also observed for the degradation rate of TRI. However, the dissipation rate of TRI was 93% and half-life of this antibiotic decreased to 7.8 days in B-2 manure that has higher SOUR values than the other manure samples. Additionally, reduction performances of TRI were similar between ST and RD compost. As for reduction behavior of almost all antibiotic groups in this study,

biotic degradation was probably occurred for TRI that was also declared by Liu et al., (2010) and Wu et al., (2012).

4.4. Conclusions

Antibiotics are widely used for disease treatment and prevention although its usage as growth promoter was entirely forbidden in the world by legislation. High amount of manure production poses critical risk for agricultural land in developed countries. Additionally, variety of antibiotics have been detected not only in manure but also in agricultural soil in the vicinity of manure laden areas via fertilization effect. 33 antibiotics were monitored in three types of manure samples collected from CAFOs and the fate of detected antibiotics were investigated in this study. Predominant detected antibiotics were SAs, FQs, SAs, ERY, TRI, and LIN in manure samples. Although the blending of D and B or L manures resulted in higher and divergent antibiotic pollution load in the mixture, the time to attain the peak composting temperature (60-73°C) was reduced and higher peak temperatures were achieved in mixed manures compared to the composting of D manure alone because of the enrichment of microbial community.

While the composting with high aeration rate ($17\pm3 m^3.h^{-1}$) caused a decrease in composting temperature, the elevated temperature achieved with low aeration rate (3.6 ± 0.2 $m^3.h^{-1}$) did not improve antibiotic reduction indicating the importance of biotic processes. Practicing high aeration rate during the composting reduced the half-life of antibiotics remarkably regardless of manure blending. For instance, half-life of ENR was reduced from 26.2 to 12.2 days for in DB manure sample and from 11.4 to 1.4 days for CTC in D manure sample. Compare to static composting the rotation of the drum with high aeration rate provided enhanced antibiotic reduction obviously by providing enough oxygen for biotic process. Contrarily, rotary drum harnessed with low aeration showed lower reduction rate for antibiotics concerning both lack of aeration and great composting temperature, which lead to diminish microbial activity.

5. EVALUATION FOR PHYSICOCHEMICAL CHARACTERISTICS OF MANURE SAMPLES THROUGH COMPOSTING

In this section, variation of physicochemical characteristics of composting studies are represented in detail.

5.1. Composting Experiments

Efficient composting of manure depends upon balanced moisture content and C/N ratio. Considering this fact dairy manure was mixed with poultry waste. The moisture content of D was 80% while the B and L were 31% and 69%, respectively. Furthermore, the C/N ratio of dairy manure was too high (>40) to achieve efficient composting. Therefore, blending of layer hen (DL) and broiler manure samples (DB) at a certain ratio (3/1) is known that provides uniform C/N ratio (20-40) and optimum required moisture content (40-65%) (Herbert et al.,, 2010). (Layer hen manure was dried to 30% moisture content under the hood to obtain 60% moisture content for initial day of composting). Individual D and B were also composted to reveal out the differences in manure blending and antibiotic degradation performances. 3/1 ratio of dairy manure was dried under the hood (30%) to attain initial 60% moisture content for individual D compost whereas B was watered to elevate initial moisture content of compost to 60%.

Three groups of composting experiments were investigated which were zero case (D-0, DL-0, DB-0) took for 7 days that is called preliminary studies, 1st case (D-1, DL-1, DB-1) for 12 days and 2nd case (D-2, B-2, DB-2) for 20 days. For zero and first group low aeration rate (3.6±0.2 $m^3.h^{-1}$) was used as an aeration source whereas high aeration rate was performed for second group (17±3 $m^3.h^{-1}$). Static compost was also investigated as control with the second cases of compost manures and DB compost from first case concurrently. Identical composting conditions were carried out for static and rotary drum compost to compare the variations of characterization parameters. Composting efficiency and characterization parameters were monitored for zero case, but the fate of antibiotics was not investigated in this case; hence, its results were only given as preliminary studies (4.3.1).

5.2. Characterization Parameters

5.2.1. Relationship among the Basic Characteristics in Manure Samples

The pH of manure is typically above 8 which promotes the volatilization of ammonia (Bernal et al., 2009; Magrí and Teira-Esmatges, 2015). In our study, initial pH values of composting trials were ≥8 (Table 5.1) that resulted in alkalinity increase and promotes the loss of nitrogen but not resulted in slowing the composting process (Herbert et al., 2010). Due to high manure pH, bulking agent addition was generally preferred to decrease ammonia volatilization (Bernal et al., 2009; Arıkan et al., 2009) however bulking agent addition increases amount of waste that must be treated before land applied as fertilizer. pH value increases are resulted from oxidation of nitrogenous organic compounds to ammonia that means the mineralization of proteins, amino acids and peptides, (Pullicino et al., 2017; Dui-an et al., 2013) and this degradation indicates existence of aerobic process through composting. However, it was not observed for only D-1 and D-2 in our study because of the presence of low nitrogenous compound of dairy manure compare to poultry manure.

When dairy manure was composted alone, ammonia content exhibited a continuous decreasing trend. In contrast to this result, ammonia concentration increased by mixing of dairy manure with broiler or layer-hen manure at low air flow rate. On the other hand, high aeration flow rate could accelerate the stripping of ammonia from manure especially for RD compost cases and it causes the decreases of ammonia content through composting. Although pH and ammonia increases were higher than RD compost on 12^{th} days for ST compost, comparable decrease in ammonia concentration was observed on the 20^{th} days because of its opened air design. Ammonia vaporization during DL-1 manure compost experiment was quite higher than other compost samples because of its high nitrogen content and low aeration rate. On the other hand, although DB-2 and B-2 compost samples compromised the highest nitrogen content, extreme ammonia stripping effect of high aeration rate for second compost lead to decrease ammonia concentration instead of accumulation like DL-1 compost.

ON (Organic Nitrogen) content is expected to decrease through composting concerning ammonia volatilization that resulted from transformation of ON forms to ammonia and nitrate by biological processes (Paradelo et al., 2010). However, there have been numerous studies that found total ON increase through composting (Selvam et al., 2012; Ho et al., 2013; Zhang et al., 2019; Bhatia et al., 2012; Varma and Kalamdhad, 2015; Pullicino et al., 2007; Dui-an et al., 2013). Increasing trend of ON was attributed to concentration effect by the degradation of high OC content that lead to loss of higher carbon dioxide compare to N loss (Pullicino et al., 2007, Zhang et al., 2019) According to

the another opinion, increase of the same characterization parameter was attributed to inhibition of N-mineralization because of antibiotic content of manure (Selvam et al., 2012). In our study, ON increase was substantial in DL-1 compost, moisture content increased steadily in this compost resulted in also anaerobic parts in manure sample; hence, nitrification and denitrification occurred through composting (Torres-Climent et al., 2015) that probably led to ON variation. Moisture content affects composting performances that high moisture content could produce anaerobic conditions and low moisture content could curb the microbial activity; hence, 55-65% of moisture content was provided by adjusting with addition or evaporation of water in initial phase and watered through composting in our study.

Table 5.1. Variations of pH, Moisture Content, ON and Ammonia during composting experiments (n=3).

	RD					ST		
Day	D-1	DL-1	D-2	DB-2	B-2	D-2	DB-2	B-2
				pH				
0	9.25	8.65 (0.01)	9.00 (0.01)	8.67 (0.01)	8.26 (0.04)	9.00 (0.01)	8.67 (0.01)	8.26 (0.04)
12	9.03	9.31 (0.04)	9.14 (0.01)	9.13 (0.01)	9.27 (0.01)	9.17 (0.07)	9.13 (0.01)	9.28 (0.02)
20			8.85 (0.04)	9.23 (0.01)	9.24 (0.04)	8.89 (0.07)	9.17 (0.01)	9.32 (0.01)
				Moisture Content (%)				
0	61 (0.2)	60 (0.3)	65 (1.0)	61 (1.9)	57 (0.9)	65 (1.0)	61 (1.9)	57 (0.9)
12	52 (0.2)	64 (0.2)	41 (0.3)	52 (0.4)	54 (0.7)	49 (1.1)	52 (2.0)	52 (3.8)
20			12 (0.2)	14 (0.7)	38 (1.6)	32 (1.0)	32 (0.8)	41 (3.3)
				Ammonia ($g.kg^{-1}$)				
0	0.42 (0.02)	2.5 (0.2)	1.34 (0.18)	3.70 (0.2)	4.80 (0.3)	1.34 (0.18)	3.70 (0.2)	4.80 (0.3)
12	0.18 (0.01)	10.4 (0.2)	0.12 (0.01)	2.50 (0.04)	4.90 (0.3)	0.18 (0.01)	3.30 (0.3)	5.60 (0.1)
20			0.09 (0.01)	0.90 (0.01)	2.20 (0.02)	0.13 (0.01)	2.30 (0.01)	3.80 (0.07)
				ON ($g.kg^{-1}$)				
0	6.8 (0.02)	13.3 (0.16)	11.5 (2.2)	15.3 (0.2)	27.6 (0.9)	11.5 (2.2)	15.3 (0.05)	27.6 (0.9)
12	11.9 (2.1)	21.0 (0.2)	5.5 (1.0)	24.2 (1.4)	22.4 (1.0)	5.8 (0.26)	19.7 (3.7)	26.3 (2.5)
20			3.6 (0.5)	16.3 (1.8)	20.7 (1.5)	5.6 (0.8)	17.7 (2.5)	22.5 (4.5)

5.2.2. Variation of Carbon to Nitrogen (C/N) Ratio

Nutritional balance is necessary for microbial activity during composting and defined as C/N ratio (Bernal et al., 2009). It is well known that nutrients must be available in adequate amounts to achieve the proper function of a biological system. Decomposition of manure as a waste is a microbial process and C, N, P is required to synthesis of microbial cells. For microbial growth, available nutrient is widely measured by the carbon to nitrogen and carbon to phosphorus ratio (C/N, C/P ratio), which are major macro nutrients. C/N ratio for living organisms is accepted at about 30/1 and C/P ratio is about 100/1 (Satyanarayana, et al., 2012). In our study, OC/ON ratio in D compost had higher than 40 which means excess of degradable substrate for microbial process whereas OC/ON ratio was lower than 20 for B compost means there was an excess of inorganic N; hence, led to increase of ammonia volatilization (Table 5.2) (Bernal et al., 2009; Herbert et al., 2010). However, blending of dairy and poultry manure provided uniform OC/ON ratio which promoted microbial activity therefore this ratio showed a decreasing trend during composting processes especially for DB and DL compost samples.

Table 5.2. Variation of OC/ON ratio during composting experiments.

	OC/ON Ratio							
Day	DL-1	D-1	D-2	DB-2	B-2	ST-D-2	ST-DB-2	ST-B-2
0	40.4	79.2	46.4	32.7	17.9	46.4	32.7	17.9
12	25.3	45.8	61.8	19.4	17.7	90.7	24.0	22.0
20			85.9	25.9	17.4	93.7	26.4	19.5

To understand the process involved through composting water extractable portions of OC and ON were proposed for some studies (Hue and Liu, 1995, Bernal et al., 1997; Zmora-Nahum et al., 2005; Pullicino et al., 2007). Since biochemical reactions of organic matter and nutrients were carried out by microorganisms in water soluble phase WEOC and WEON were measured during composting besides OC and ON (Figure 5.1).

More labile compounds were solubilized in the first two days of D-1, D-2 and 12 days of DL-1 compost that led to increase of the concentrations of WEOC. This initial increase of WEOC agrees with Zmora-Nahum et al., (2005) that WEOC increased initial phase of separated cow manure composting then it showed decline. In our study, WEOC was generally reduced during composting except DL-1 that might indicate existence of active composting on 12 days of compost. On the contrary, OC content of DL-1 was quite stable on that day. Problem could be related to low WEOC, high nitrogen content and the volatilization of high amount of ammonia.

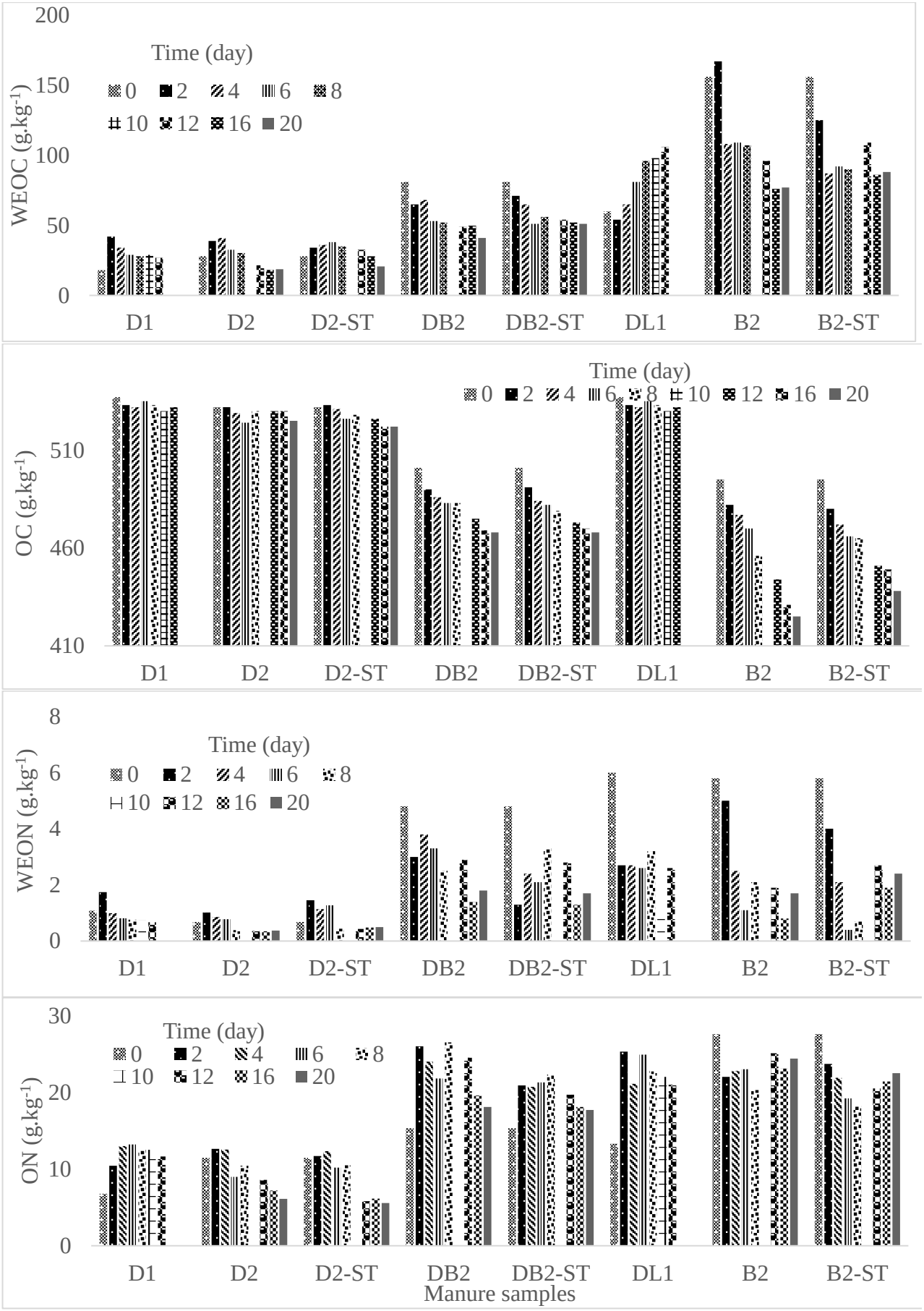


Figure 5.1. Variations of C and N during composting experiments.

Higher degradation percentage of OC was observed for DB and B compost (8-15%) trials compare to D compost (1-2%). Not only available carbon but also ON concentrations were high for broiler manure that enhanced OC degradation depended upon accelerated microbial activity with uniform C and N ratio (Bernal et al., 2009). ON increased on the second day of D-1, D-2, DB-2 and DL-1 compost and 8th day of B compost that might result from the inhibition of aerobic microbiota somehow anaerobic conditions occurred because of excess moisture content through composting. However, it is known that high nitrogen loss compare to OC leads to ON increase in literature (Fang et al., 1999; Bernal et al., 2009; Pullicino et al., 2007; Dui-an et al., 2013), which was not a clear explanation that microbial degradation of organic matter resulted in a decrease both of WEOC and WEON in our study. Although WEON demonstrated increase and then decrease pattern similar to WEOC for D compost trials, it decreased gradually during DB, DL and B compost trials (Figure 5.1). WEOC and WEON increase in the first phase of composting could be explained as solid liquid transfer of nutrients through consumption of amino acids and proteins by microorganisms (Pullicino et al., 2007).

5.2.3. Electrical Conductivity (EC)

The EC was the measure of dissolved minerals in the compost. Solubilization of manure components by microbial and thermal reactions caused an increase for the electrical conductivity of manure slurry. As well as dissolved organic minerals can enhance the microbial activity in manure. However, it indicates the salinity level of final compost might pose a risk in the environment. Hence, maximum limit value of EC (10 ds.cm^{-1}) was determined by compost notification (2015). Although EC values increased during composting in our study for all trials (Table 5.3) final EC values was lower than the determined maximum value. In addition, EC increase was also measured by Irshad et al., (2013) and by Lyimo et al., (2012) and attributed to mineralization through composting.

Table 5.3. Variation of EC during composting.

	EC(ms.cm^{-1})							
Day	DL-1	D-1	D-2	DB-2	B-2	ST-D-2	ST-DB-2	ST-B-2
0	2.4 (0.01)	0.8	1.0 (0.01)	2.2 (0.06)	2.7 (0.08)	1.0(0.01)	2.2 (0.06)	2.7 (0.08)
12	3.1 (0.05)	0.7	1.0 (0.43)	3.3 (0.08)	3.2 (0.01)	1.0 (0.05)	3.7 (0.26)	3.4 (0.21)
20			1.7 (0.50)	5.7 (0.21)	3.3 (0.01)	1.6 (0.01)	5.3 (0.01)	3.9 (0.18)

5.2.4. Total and Soluble Metals

It is known that soluble metal concentrations increased during composting process, (Hsu and Lo., 2001; Amir et al., 2005; Larney et al., 2014; Wu et al., 2017). Because of microbial degradation of organic matter; low molecular weight organics, minerals, metals, volatile organics and inorganics releases. Adverse environmental impact of manure about metal content is not related with the solid phase of metals but their soluble concentrations in terms of leachability to ground water, inhibitory effect on microbial activity in soil and bioavailability for plants, (Hsu and Lo, 2001; Bolan et al., 2003; Hazarika et al., 2017). In addition, bioavailability and mobility of metals are correlated with soluble organic matters and humification in manure and manure amended soil (Jackson et al., 2003; Larney et al., 2014; Hazarika et al., 2017). Hence, soluble metal concentrations are correlated with WEOC concentrations during composting to evaluate their behavior (Figure 5.2).

Solubility of metals generally increased with decreasing WEOC concentration through composting except for D manure, which changes in solubility of metal were mild compare to DB and B samples. It supports the SOUR results that DB and B compost was probably had higher microbial activity compare to D manure. Especially, soluble Fe concentrations arose while organic decomposition was increased by empowered microbial activity. Total metal and nutrient concentrations for raw manure and compost samples and soluble portions (%) of metals to total amounts were listed in Table 5.4. Poultry manure contained higher concentration of Cu, Zn and Mn metals compare to dairy manure that poses pollution risk for environment by leaching of these metals due to their solubilization during compost. Initially in the compost samples, Ca, Mg, and Zn were relatively insoluble for dairy manure whereas they were soluble for B and DB manures. Besides, Cu, Na and K exhibited greater solubility in the initial day of three compost samples, which are DB-2, B-2, D-2 (Table 5.4). Jackson et al., (2003) attributed higher Cu solubility with the affinity of dissolved organic carbon that could be responsible for increase of solubility in DB compost samples for our study. Our results were consistent by Hsu and Lo, (2001) that found Mn and Zn solubility (<2%) were lower than Cu (3-16%) in swine manure and Irshad and Sabir, (2012) exhibited solubility of trace metals in the order of Cu>Zn for poultry litter and cow manure.

Solubility of Ca and Mg decreased during both rotary drum and static compost except for Ca during B-2 compost. On the other hand, solubility of Na and K were comparatively stable. Antibiotics can chelate with divalent cations such as Ca and Mg (Park et al., 2000; Karcı and Balcıoğlu, 2009) and solubility decrease of those metals might be attributed to their complexation with antibiotics. For instance, TCs-metal complexes are known as a new class of emerging contaminants and have a scarce information about their adsorption, desorption and bioavailability (Pulicharla et al., 2017) that might cause to concentration change of metals through composting.

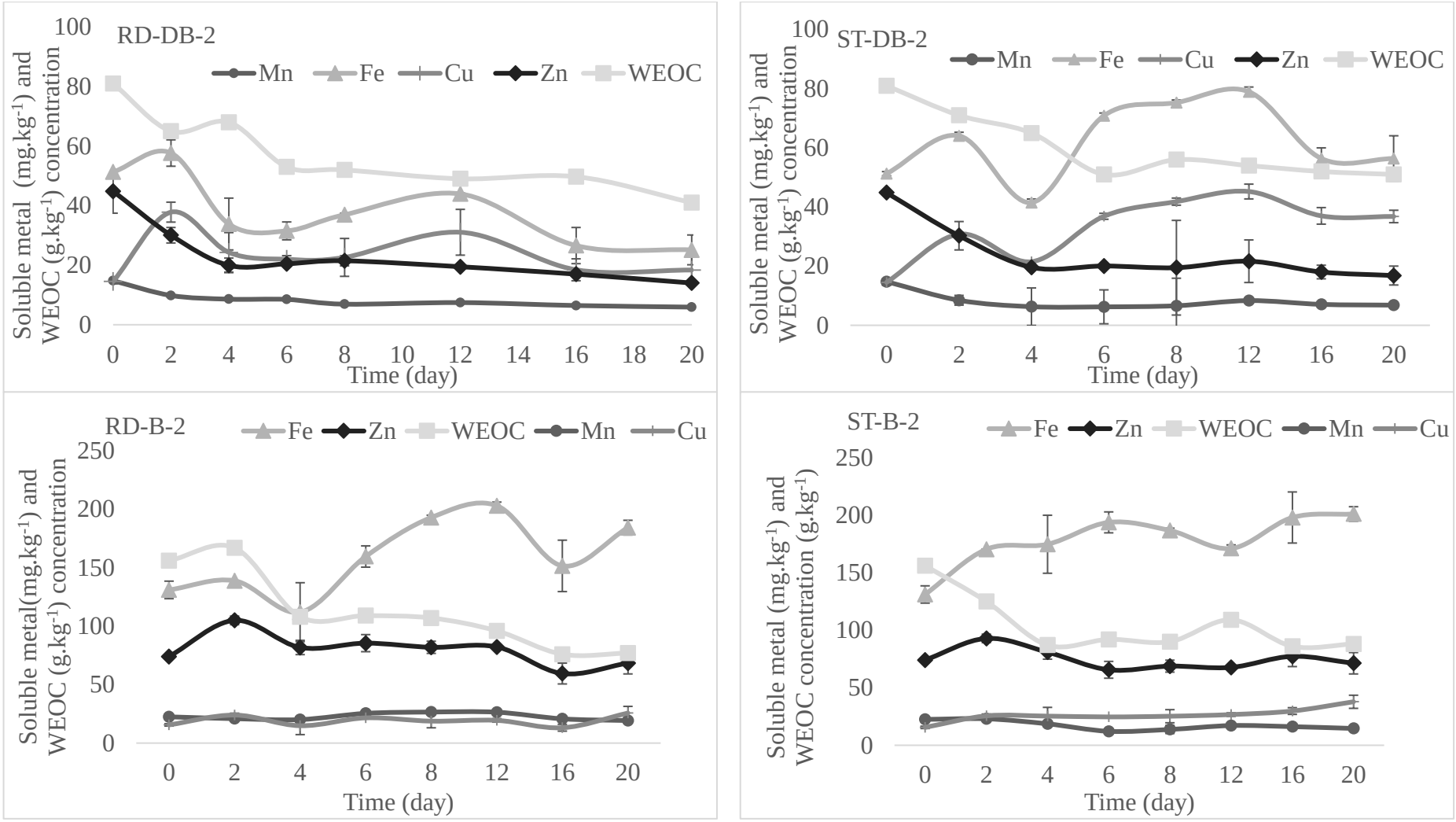


Figure 5.2. Variation of soluble metal concentrations during composting.

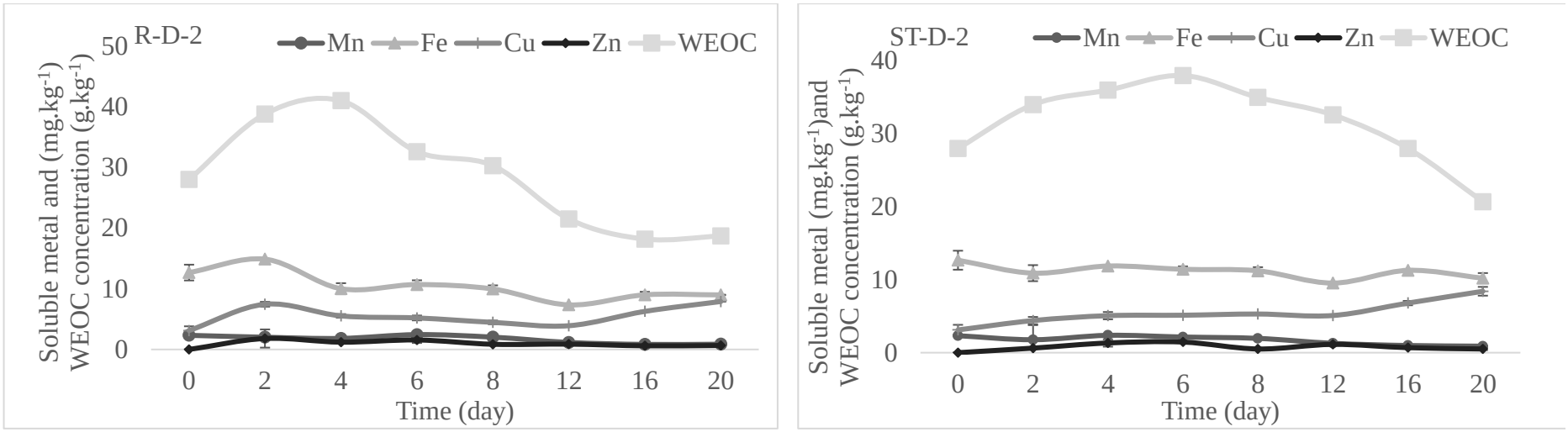


Figure 5.2. Continued. Variation of soluble metal concentrations during composting

Table 5.4. Total metal and nutrient concentrations ($mg.kg^{-1}$) with percent amount of soluble metal concentrations.

		Mn	Fe	Cu	Zn	Al	Ca	Mg	Na	K
Manure samples	D	73 (5)	586 (20)	34 (0.1)	96 (14)	398 (14)	162,857 (15,282)	117,403 (1,172)	31,711 (3,874)	11,334 (1,827)
	B	1,065 (48)	2,657 (28)	172 (12)	1,140 (26)	721 (242)	324,083 (12,630)	20,513 (2,961)	24,163 (944)	17,187 (2,352)
	L	1,451(0.1)	5,448(512)	117 (7)	790 (60)	14,145(532)	148,750 (4,214)	7,982 (602)	25,015 (1,364)	50,860 (1,164)
	DB-2	681 (81)	2,627(1,381)	312 (22)	1215(267)	402 (117)	54,318 (5,343)	10,017 (69)	19,688 (4,425)	52,097 (299)
	DL-1	936 (14)	3,011 (239)	106 (16)	644 (30)	504 (61)	129,214 (100)	2,012 (100)	28,221 (762)	38,886 (761)
(%) amount soluble metal	D-2	3.2	2.2	9.1	0.1	0.2	1.2	0.5	39.1	41.5
	DB-2	2.2	1.9	4.7	3.6	0.2	5.8	7.0	18.2	24.0
	B-2	2.1	4.9	9.0	6.5	0.2	3.6	8.5	39.2	71.2

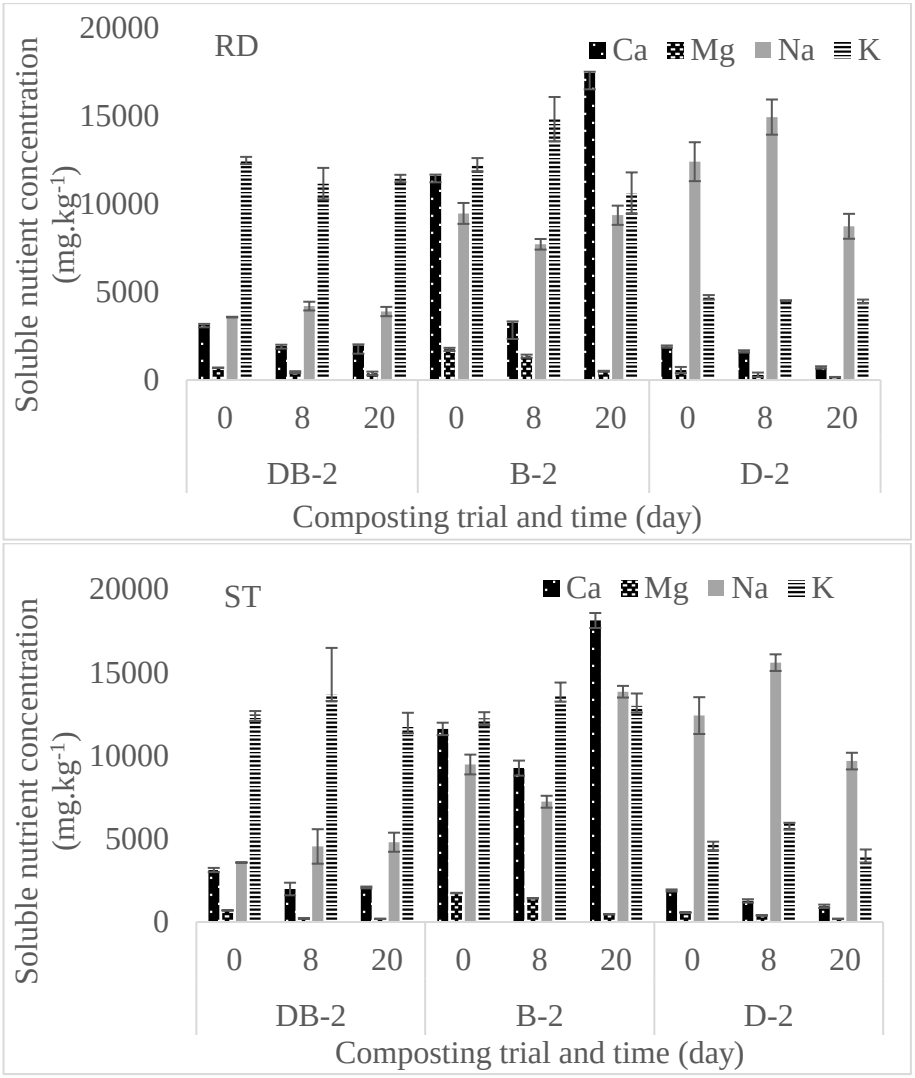


Figure 5.3. Variation of soluble nutrient concentration during composting experiments.

5.2.5. Phosphorus Sequential Fractionation

Besides nitrogen, phosphorus (P) is the second major element for plant growth. P are given feedstock by dietary applications and excess amount of P leads to redundant amount for agricultural implications by manure fertilization, cause to eutrophication and potential risk for water quality (Li et al., 2014; Wei et al., 2015). However, amount of total P data gives limited knowledge about the bioavailability and solubility of P to evaluate the fate of this compound in receiving environment. Hence, fractionation of manure provided labile (H_2O, $NaHCO_3$) (available form), organically bound (NaOH) and inorganically bound (recalcitrant form) (HCl) portions of P (Dui-an et al., 2013; Li et al., 2014; Wei et al., 2015).

Poultry manure contained high total P compare to dairy manure (Table 5.5). Hence, total P was uniformed by blending D with L and B manure. Sequential fractionation of P was performed for D-1 and DL-1 compost (Figure 5.4 and 5.5). Total, organic and inorganic distribution of P from several

manure studies was listed by He at al., (2006). Total P was detected between the range of 2.8-18.3 and 8.6-30.4 g.kg^{-1} for dairy and poultry manure respectively. Complying with these results, total P values were detected in the range of 8.4-10 and 27-40 g.kg^{-1} (Table 5.5) for dairy and poultry manure in this study, respectively. In addition, inorganic and organic portions of P for dairy manure were listed between the range of 28-96% and 8-61%, 32-84% and 14-68% for poultry manure, respectively (He et al., 2006). Also, inorganic and organic portions agree with He et al., (2006) in this study, organic portion of dairy manure was of 89% and 79% for poultry manure.

Table 5.5. Total phosphorus content for manure samples.

Manure samples	L-1	D-1	D-2	B-2	DL-1	DB-2
Concentration (g.kg^{-1})	40 (4.7)	8.4 (0.1)	10 (0.4)	27 (0.1)	34 (2.3)	27 (0.1)

Sequential fractionation was carried out through D-1 and DL-1 compost. Fractionation did not much variate through composting for dairy manure (only 3% inorganic portion increase) whereas inorganic portion of P increased up to 26% for DL-1 compost (Figure 5.4 and 5.5). Additionally, 13% of inorganic P increase of DL-1 manure sample was resulted from HCl-extractable portion. These results were consistent with Ngo et al., (2013) that composting of buffalo manure led to increase HCl-extractable inorganic portion (20%) of P. Increase of non-labile recalcitrant form (HCl) provided immobilization of P that is required to prevent the leaching of P; hence, it reserves P for plant nutrition (Dui-an et al., 2013; Irshad et al., 2013; Wei et al., 2015).

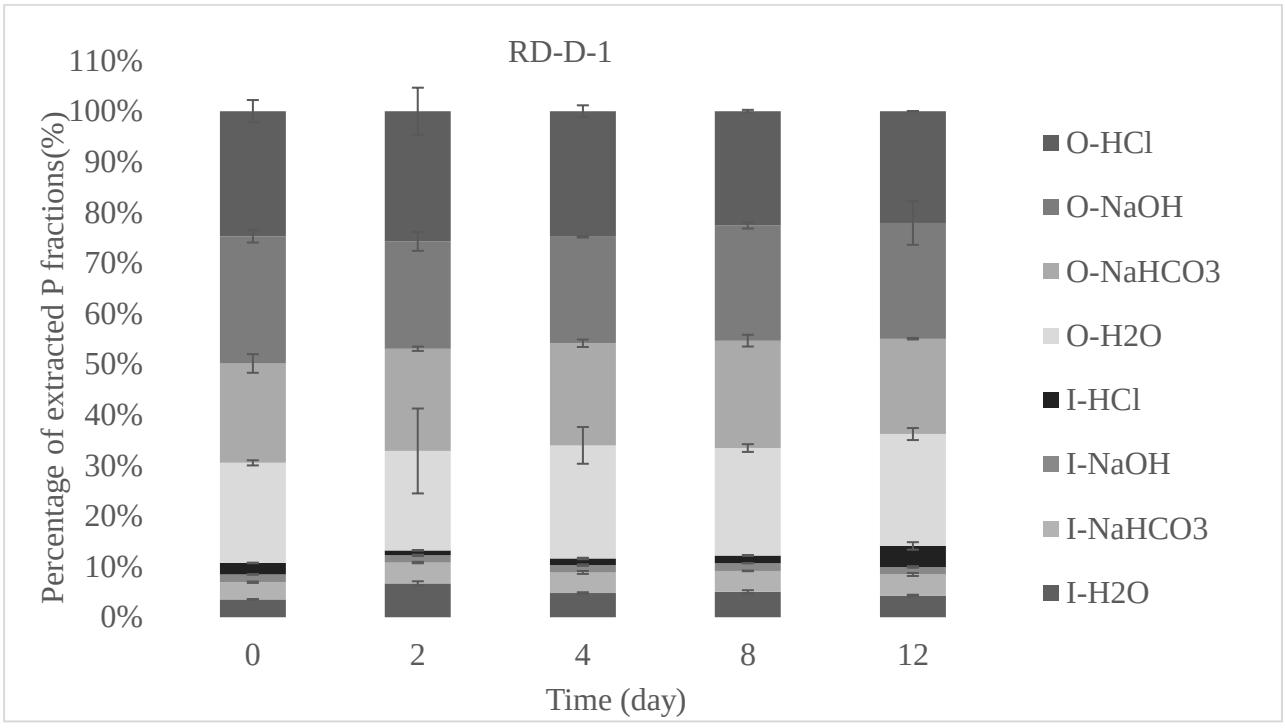


Figure 5.4. Sequential phosphorus fractionation results of D-1 compost.

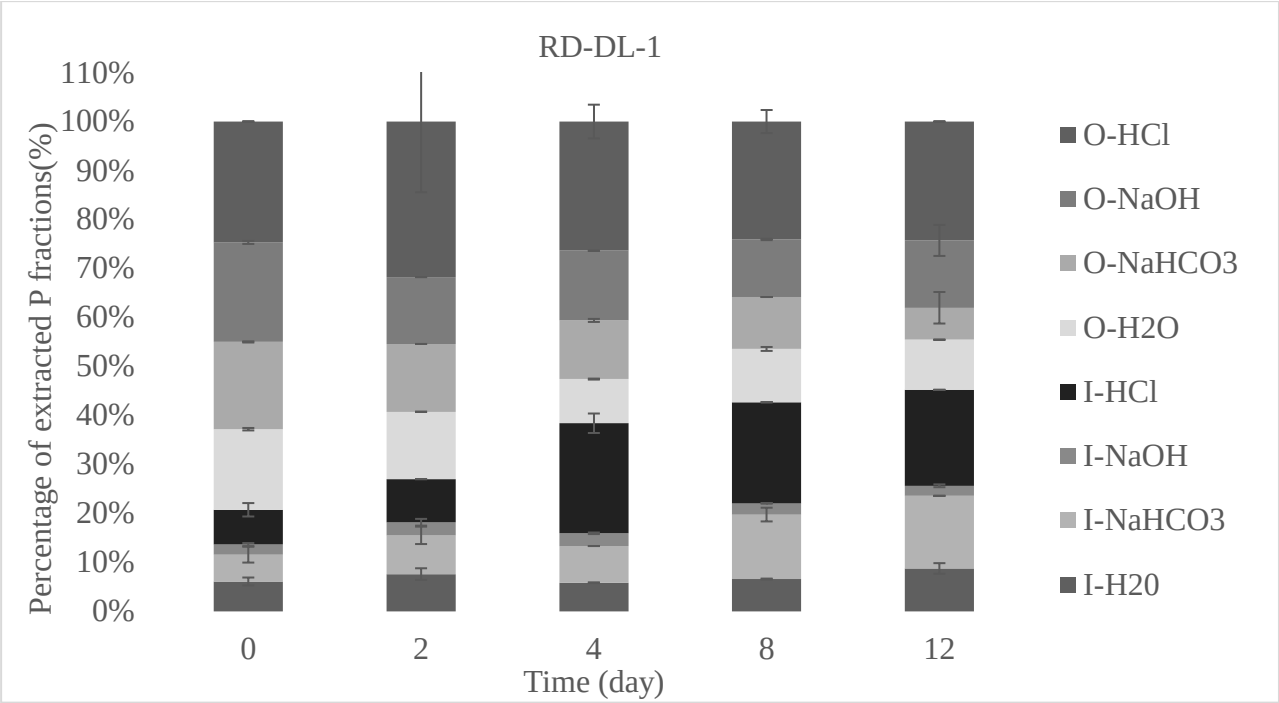


Figure 5.5. Sequential phosphorus fractionation results of DL-1 compost.

5.3. Conclusions

Blending different type of manure samples prevent the usages of bulking agent that causes the increase of manure waste. Without using bulking agent optimum moisture and nutrient content in the initial phase of composting were provided by taking the advantage of extreme moisture content of dairy manure and nutrient content of poultry manure, which led the average level parameters by blending them with each other. Although excessive amount of ammonia generation was a common results of manure composting (Herbert et al., 2010), extreme ammonia vaporization in poultry manure was reduced by blending dairy manure samples. Additionally, the problems of limited nutrient content and high moisture content of dairy manure could be eliminated by blending of these two different manure samples. Microbial activity decreased considerably when conditions were at steady state in terms of low temperature in our study. Therefore, nutrient loses were not substantial amount and compost samples probably contained necessary nutrient for plant growth. Changes of metals and phosphorus content was similar with the literature. High divalent cation solubilization through compost could be attributed to their complexation with antibiotics. On the other hand, increases of recalcitrant phosphorus helped the immobilization of phosphorus and provided safe manure for fertilization.

6. CONCLUSIONS AND RECOMMENDATIONS

The uncontrolled release of antibiotics to the environment by manure because of the inevitable fertilization for plant growth was a critical issue that have been tried to solve by different authorities in all over the world. At first, its extensive consumption would like to be cut down to prevent commonly known problem which is antibiotic resistance that blocked the effectiveness of antibiotic even for surgery. In this regard, effective and reliable analysis of these antibiotics in manure samples is the first step to cope with this crucial problem. In other words, continuous measuring of antibiotics in source and receiving environment is necessary to control antibiotic pollution. Hence, a reliable and easily performed method was developed for a simultaneous analysis of 33 antibiotics belonging to seven different classes (SAs, TCs, MCs, FQs, DAPs, APs, and LINs) for both poultry and dairy manure samples by UHPLC-ESI-MS/MS.

Existed methods were modified at first regarding the composition of mobile phase, and then extraction conditions were selected by applying different extraction solvents and agitation methods and decided the most successful one to increase the method performances for seven groups of antibiotics. Salting-out-induced extraction of antibiotics from manure samples with a MeCN/EDTA/McIlvaine buffer solvent mixture (50:25:25; v:v:v) enabled successful application of the method without the use of SPE. To maximize the signal intensities of antibiotics, priority those of TCs, Oxalic Acid addition was performed in mobile phase and sensitivity of quantification was enhanced for almost all antibiotic classes. Sample preparation time was substantially reduced, and preparation step was simplified by subtracting the SPE step. Additionally, produced method performance was considerably successful compare to studies that used SPE through antibiotic analysis. The influences on the pH of the McIlvaine buffer and ultrasound on the extraction performances were investigated. In the end, method performances were developed by the addition of acidic buffer and using of mechanical agitation during extraction.

As a second part of the solution of antibiotic pollution, antibiotics were monitored during composting before its application to the soil as a fertilizer. There have been numerous studies about the manure and manure laden soil pollution in literature (Ho et al., 2012; Zhao et al., 2010; Karcı and Balcıoğlu, 2009). Compost has been a proposed treating method to eliminate the antibiotic pollution by realizing both biotic and abiotic processes in its nature. 33 antibiotics were scanned in poultry and dairy manure samples collected from different CAFOs. Fate of detected antibiotics during the treatment with composting were determined and results were given for turning and static compost.

Predominant detected antibiotic classes were SAs, FQs, TCs, LINs and DAPs for manure samples. The actual purpose for blending of D, B and L manure was to uniform the moisture and nutrient content. However, time to reach peak temperature was reduced and the attained peak temperature was arisen by blending the manure samples that resulted from the increased microbial performance. Although the high aeration rate decreased the attained peak temperature and reduced the duration of high temperature through composting, it most probably led to the enrichment of the microbial activity since it reduced the half-life of antibiotics remarkably. For instance, half-life of ENR was reduced from 26.2 to 12.2 days in DB manure and from 24.2 to 17.8 days for OTC in D manure samples. It has been known that antibiotic degradation was attributed to temperature dependent abiotic processes in composting; however, our study proves that the higher temperature is not the unique necessary requirement for antibiotic degradation without considering the facts of biotic processes. Amount of aeration, turning frequency and attained temperature regime are required to control to increase the removal rate of antibiotics.

Rotation effect of antibiotics were analyzed in our study with rotary drum reactors and static compost, which was performed as a control. However, manure samples were turned frequently for sampling (1-2 days) of static compost that resulted in nearly identical antibiotic degradation performance with rotary drum reactors. Further studies can be performed for static compost by applying sampling as a less frequent way to represent actual static compost performance to compare turning effect regarding antibiotic degradation. In addition, static compost performances can be measured by continuous recording temperature data logger in following studies. Although the static versus turning compost effect on degradation of antibiotics were not clear, aeration effect on antibiotic reduction was elaborately determined by performing all compost samples and it became evident that passive aeration must be performed at least by opening a cap on rotary drums to provide necessary aeration.

Excessive amount of ammonia generation is the common issue for manure samples during composting since it is generally solved by bulking agent addition to manure samples. An optimum solution was suggested in our study to eradicate disadvantages of different manure samples and used their differences as a benefit for composting process. Available nitrogen of poultry manure samples is incredibly higher than dairy manure and it was decreased to a certain amount by blending with dairy manure, which led to diminish ammonia volatilization and loss of nitrogen content before applying to the soil. Besides, high moisture content of dairy manure was uniformed with this blending performance to initiate the composting process. As a result, compost samples reached to steady state

conditions regarding temperature regime without losing much nutrient contents, which is necessary for nutrition of plant and biota in receiving environments.

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APPENDIX A: PHYSICOCHEMICAL PROPERTIES, PRODUCERS AND DISSOLUTION SOLVENT OF TARGET ANALYTES

Compounds	Exact mass ($g.mol^{-1}$)*	log p*	log kow*	Solubility ($mg.L^{-1}$)*	Pka*
SCP, (99%)	284.013	1.00	0.31	35	2.0, 6.5
SDZ, (99%)	250.052	-0.09	-0.09	77	2.1, 6.4
SDX, (99.9%)	310.074	0.70	0.70	296	2.3, 6.1
SMZ, (99.7%)	278.080	0.89	0.89	1,500	2.1, 7.7
SMT, (99%)	270.025	0.54, 0.90	0.54	1,050	2.1, 5.3
SMH, (99.8%)	253.052	0.89	0.89	610	1.6, 5.7
SMX, (95.6%)	280.063	0.30	0.32		2.0, 6.8
STZ, (98%)	255.010	0.05	0.05	373	2.1, 7.2
SQX, (99%)	300.068	1.68	1.68	8	5.1
SFX, (99.9%)	267.068	1.01	1.01	300	1.5, 5.0
CIP, (≥98%)	331.133	0.3, 2.3	0.28	30,000	6.1, 8.7
DAN, (98%)	357.149	-0.3	0.44		5.7, 6.7
DIF, (98.3%)	399.139	1.6	1.28		5.6, 6.5
ENR, (≥98%)	359.165	-0.2	0.7	>54	5.7, 6.7
FLU, (98%)	261.080	1.6	2.6	Insoluble	6.0
MAR, (99%)	362.139	-0.5	-2.92		5.4, 6.2
NOR, (≥98%)	319.133	1.0	-1.03	178,000	6.3, 8.8
OXO, (98%)	261.064	-0.2	0.94		5.6
SAR, (99%)	385.124	0.3	1.07		5.8, 8.6
ERY, (96%)	733.461	3.1	3.06	2,000	8.9
JOS, (≥98%)	827.467	2.9	3.16	54	7.9, 12.7
SPI, (99%)	842.514	2.1		Slightly soluble	7.9
TIL, (98%)	868.566	3.8	3.80	0.015	8.2
TYL, (98%)	915.519	1.6	1.63	5	7.7
CTC, (99%)	478.114	-1.3	-0.62		3.3, 7.4, 9.3
DXC, (≥98%)	444.153	-0.0	-0.02	630	3.5, 7.7, 9.5
OTC, (97%)	460.148	-0.9	-0.90	313	3.3, 7.3, 9.1
TC, (95%)	444.153	-1.3	-1.37	231	3.3, 7.7, 9.7
CHL, (≥98%)	322.012	0.7, 1.1	1.14	2,500	7.5
FLF, (99%)	357.000	0.8	-0.04		6.8
THI, (98%9	355.005	-0.3	-0.27	>53.4	7.7
LIN, (≥90%)	406.214	0.6	0.56	927	7.6
TRI, (≥98%)	290.138	0.6, 0.9	0.73	400	7.12
SMH D4, (99%9	257.077	0.9			
NOR D5,(98.5%)	324.165	-1.0			
ROX,(≥90%)	836.525	1.7		0	9.1, 12.5
DEM,(≥92.3%)	464.099	0.2		1,520	3.3, 7.2, 9.2

(*https://pubchem.ncbi.nlm.nih.gov, qiang et al., 2004)

APPENDIX B: METHOD PERFORMANCE PARAMETERS FOR DAIRY MANURE IN ANTIBIOTIC CONCENTRATION RANGE OF 25-200 µG.KG^{-1} (MeCN/EDTA/pH 8 MCllVAINE BUFFER, MECHANICAL AGITATION, AQUA II MOBILE PHASE A)

	Mean recovery (%) (intra-day RSD%) n=3				RT (min)	Linearity (R^2)	ME (%)
	25 (µg.kg^{-1})	50 (µg.kg^{-1})	100 (µg.kg^{-1})	200 (µg.kg^{-1})			
SDZ	119 (39.3)	149 (42.7)	110 (10.6)	102 (2.7)	4.2	0.998	98.2
STZ	128 (26.0)	134 (31.8)	107 (12.6)	102 (2.3)	5.3	0.999	97.9
SMZ	117 (6.3)	125 (35.1)	120 (23.6)	98 (18)	11.7	0.998	93.8
SMT	109 (30.2)	136 (30.8)	118 (11.8)	98 (1.4)	8.8	0.998	96.2
SMX	109 (2.9)	121 (33.6)	128 (17.4)	107 (10.1)	8.8	0.998	96.3
SCP	130 (15.2)	118 (31.6)	122 (26.1)	104 (2.9)	11.0	0.998	96.4
SDX	117 (11.1)	123 (31.6)	114 (17.2)	99 (1.9)	11.7	0.999	92.8
SMH	146 (36.8)	120 (28.9)	114 (17.8)	103 (2.7)	12.1	0.999	100.0
SQX	106 (5.5)	104 (4.1)	104 (4.8)	100 (0.6)	14.0	0.999	99.8
SFX	102 (5.9)	62 (86.9)	102 (3.5)	102 (2.2)	12.8	0.999	84.3
DIF	104 (7.1)	105 (9.7)	111 (7.0)	100 (3.5)	11.8	0.999	93.7
MAR	98 (1.7)	108 (4.0)	103 (5.8)	100 (4.6)	7.9	0.999	75.5
OXO	103 (4.1)	109 (12.9)	101 (1.4)	98 (7.4)	13.2	0.999	89.8
FLU	108 (9.0)	107 (7.5)	105 (2.9)	101 (2.2)	15.5	0.999	90.2
SAR	119 (5.2)	100 (6.9)	100 (2.9)	106 (8.3)	11.6	0.998	97.3
NOR	91 (11.3)	99 (4.3)	97 (7.3)	101 (6.2)	8.9	0.998	39.8
ENR	104 (5.4)	100 (1.6)	106 (7.0)	100 (4.1)	10.6	0.999	61.3
DAN	96 (70.2)	102 (11.4)	109 (3.0)	97 (0.7)	10.2	0.981	75.6
CIP	86 (10.3)	106 (6.3)	96 (8.9)	104 (3.8)	9.4	0.998	82.1
SPI	128 (4.4)	97 (2.7)	92 (0.4)	102 (0.4)	12.5	0.992	>150
ERY	150 (9.4)	105 (4.8)	79 (6.4)	98 (10.8)	14.6	0.958	100.0
TYL	170 (24.7)	120 (14.1)	104 (8.9)	99 (8.2)	14.9	0.961	14.2
JOS	104 (9.4)	97 (1.6)	103 (3.3)	108 (6.7)	16.8	0.999	93.8
TIL	155 (24.5)	94 (1.5)	104 (2.7)	95 (10.4)	13.7	0.992	45.5
OTC	325 (69.3)	64 (5.1)	105 (6.7)	98 (4.3)	8.5	0.648	32.9
TC	120 (37.7)	73 (17.4)	96 (9.9)	108 (4.2)	9.9	0.979	>150
DXC	78 (0.4)	106 (16.0)	90 (7.2)	104 (3.3)	9.9	0.993	30.9
CTC	106 (10.4)	97 (8.1)	105 (5.7)	103 (6.9)	12.6	0.999	27.9
THI	100 (10.1)	59 (16.8)	64 (42.0)	150 (40.7)	8.0	0.952	80.0
FLF	40 (28.3)	56 (49.2)	62 (47.9)	99 (1.8)	12.1	0.964	>150
CHL	123 (0.2)	91 (8.6)	83 (12.9)	102 (0.7)	13.2	0.991	>150
TRI	126 (3.6)	87 (9.7)	84 (16.1)	103 (1.2)	7.7	0.994	22.2
LIN	37 (95.6)	57 (57.3)	28 (77.7)	103 (0.1)	5.7	0.985	57.1

APPENDIX C: METHOD PERFORMANCE PARAMETERS FOR POULTRY MANURE IN ANTIBIOTIC CONCENTRATION RANGE OF 25-200 µG.KG^{-1} (MeCN/EDTA/pH 3 MCllVAINE BUFFER, MECHANICAL AGITATION, AQUA I MOBILE PHASE A)

	Mean recovery (%) (intra-day RSD%) n=3				RT (min)	Linearity (R^2)	ME (%)
	25 (µg.kg^{-1})	50 (µg.kg^{-1})	100 (µg.kg^{-1})	200 (µg.kg^{-1})			
SDZ	82 (1.5)	112 (13.5)	106 (1.6)	110 (15.0)	3.6	0.995	91.4
STZ	98 (20.2)	117 (1.2)	107 (12.6)	114 (17.6)	4.5	0.993	92.5
SMZ	95 (25.7)	107 (7.0)	99 (2.5)	98 (2.5)	10.7	0.993	96.5
SMT	105 (12.1)	158 (12.5)	105 (5.2)	105 (7.0)	7.5	0.999	95.2
SMX	84 (10.5)	110 (4.6)	104 (9.2)	97 (1.7)	9.6	0.995	97.2
SCP	82 (2.1)	103 (21.0)	104 (17.1)	104 (3.4)	9.9	0.981	96.1
SDX	85 (23.6)	103 (10.8)	97 (1.5)	96 (5.3)	10.8	0.991	95.4
SMH	88 (35.4)	102 (4.3)	111 (3.6)	103 (8.0)	11.0	0.985	94.1
SQX	145 (31.5)	83 (18.8)	89 (41.4)	89 (14.5)	13.1	0.974	97.7
SFX	98 (34.3)	102 (13.4)	113 (4.3)	105 (10.7)	11.8	0.976	138.5
DIF	112 (19.2)	96 (16.0)	96 (5.1)	97 (4.7)	10.8	0.994	98.2
MAR	104 (15.4)	82 (16.3)	80 (36.0)	78 (40.9)	6.6	0.998	87.2
OXO	106 (32.6)	68 (42.5)	74 (41.2)	68 (68.8)	14.6	0.993	90.8
FLU	99 (36.3)	64 (46.7)	77 (42.5)	81 (79.2)	14.6	0.994	79.0
SAR	111 (9.7)	80 (32.2)	76 (32.8)	72 (57.9)	10.7	0.995	96.5
NOR	157 (7.4)	162 (33.2)	131 (30.2)	113 (18.3)	7.5	0.982	84.7
ENR	138 (6.2)	73 (25.2)	84 (21.2)	71 (59.8)	9.4	0.992	91.3
DAN	60 (13.9)	39 (14.8)	83 (23.1)	82 (34.7)	9.0	0.992	92.3
CIP	76 (67.0)	79 (19.7)	91 (18.7)	87 (21.5)	8.0	0.997	89.9
ERY	144 (0.1)	78 (14.5)	83 (34.0)	124 (27.1)	17.4	0.981	100.0
JOS	93 (141.0)	143 (38.6)	100 (9.1)	74 (31.2)	15.8	0.747	99.3
TIL	98 (10.8)	129 (7.4)	80 (14.0)	92 (14.4)	13.1	0.984	99.7
OTC	86 (3.3)	103 (1.4)	117 (16.4)	119 (23.5)	7.2	0.998	>150
TC	126 (29.2)	80 (27.9)	92 (20.0)	92 (19.7)	6.8	0.962	>150
DXC	82 (1.4)	119 (9.0)	110 (13.1)	89 (16.8)	6.8	0.996	>150
CTC	129 (43.0)	110 (3.0)	125 (30.2)	83 (28.2)	11.8	0.998	>150
THI	93 (53.6)	72 (8.2)	108 (5.1)	270 (90.0)	6.7	0.975	>150
FLF	110 (13.3)	78 (9.1)	100 (16.8)	213 (76)	11.1	0.981	52.3
CHL	114 (7.2)	83 (1.0)	99 (7.9)	216 (76.3)	12.4	0.993	>150
TRI	114 (6.4)	89 (28.0)	100 (12.7)	190 (68.3)	6.4	0.977	56.1
LIN	118 (5.3)	103 (48.1)	111 (10.9)	189 (68.8)	4.8	0.964	>150

APPENDIX D: METHOD PERFORMANCE PARAMETERS FOR POULTRY MANURE IN ANTIBIOTIC CONCENTRATION RANGE OF 25-200 µG.KG^{-1} (MeCN/EDTA/pH 3 MCllVAINE BUFFER, ULTRASONIC AGITATION AQUA II MOBILE PHASE A)

	Mean recovery (%) (intra-day RSD%) n=3				RT (min)	Linearity (R^2)	ME (%)
	25 (µg.kg^{-1})	50 (µg.kg^{-1})	100 (µg.kg^{-1})	200 (µg.kg^{-1})			
SDZ	94 (11.2)	111 (14.5)	109 (12.1)	103 (3.5)	3.4	0.999	97.5
STZ	122 (16.9)	95 (2.7)	95 (10.3)	97 (4.4)	4.3	0.998	97.6
SMZ	93 (4.6)	99 (12.7)	96 (5.6)	99 (3.2)	10.7	0.997	96.2
SMT	80 (1.0)	108 (2.6)	105 (4.4)	97 (2.3)	7.3	0.996	95.8
SMX	108 (6.6)	100 (7.9)	107 (4.8)	97 (3.6)	7.4	0.998	97.1
SCP	58 (34.5)	110 (10.0)	100 (2.3)	101 (0.9)	9.7	0.993	97.7
SDX	88 (8.0)	101 (12.6)	98 (3.6)	99 (3.1)	10.7	0.998	96.0
SMH	90 (9.1)	90 (13.1)	102 (2.6)	102 (4.0)	11.0	0.999	87.2
SFX	157 (4.1)	105 (44.2)	98 (6.1)	100 (6.5)	11.8	0.973	85.5
DIF	80 (66.8)	50 (121.4)	90 (13.7)	91 (15.6)	10.7	0.997	95.7
MAR	116 (5.6)	84 (8.8)	103 (5.2)	94 (9.4)	6.4	0.997	79.2
OXO	84 (21.9)	88 (16.5)	96 (9.9)	99 (1.2)	14.6	0.999	88.6
FLU	102 (10.9)	104 (11.3)	102 (6.9)	101 (0.9)	14.6	0.998	75.4
SAR	64 (141.4)	123 (45.0)	94 (9.5)	98 (4.4)	10.6	0.993	95.2
NOR	114 (5.2)	50 (141.4)	92 (0.8)	101 (0.9)	7.3	0.994	46.4
ENR	65 (132.8)	45 (109.0)	99 (7.1)	92 (12.0)	6.0	0.991	92.5
CIP	17 (141.4)	76 (62.2)	112 (13.7)	95 (2.4)	7.9	0.952	87.0
SPI	134 (15.7)	67 (24.5)	80 (49.7)	112 (15.8)	11.8	0.989	8.4
ERY	0	52.3 (141)	118 (37.9)	49 (115.0)	13.7	0.821	97.9
TYL	125 (8.4)	101 (16.9)	116 (17.8)	102 (3.0)	14.0	0.997	64.3
JOS	118 (16.8)	75 (24.3)	99 (5.4)	100 (2.8)	15.7	0.993	91.5
TIL	116 (10.0)	70 (40.4)	96 (1.5)	99 (3.6)	13.0	0.995	64.3
OTC	99 (16.2)	95 (3.9)	115 (5.7)	102 (5.6)	7.0	0.991	64.0
TC	107 (26.7)	78 (24.6)	98 (4.9)	100 (2.0)	6.6	0.994	85.3
DXC	97 (20.1)	80 (16.9)	99 (4.7)	102 (3.9)	10.7	0.997	89.6
CTC	82 (52.7)	81 (29.7)	95 (17.5)	93 (5.6)	6.7	0.996	65.1
FLF	67 (125.3)	70 (141.4)	143 (4.2)	128 (43.2)	11.1	0.779	>150
CHL	81 (50.6)	174 (55.3)	105 (14.9)	81 (26.5)	12.4	0.974	>150
TRI	93 (20.5)	107 (4.0)	95 (11.9)	128 (32.0)	6.3	0.996	>150
LIN	105.1 (29)	135 (46.8)	99 (0.4)	116 (18.4)	4.8	0.997	>150

APPENDIX E: METHOD PERFORMANCE PARAMETERS FOR DAIRY MANURE IN ANTIBIOTIC CONCENTRATION OF 100 µG.KG^{-1} (MeCN/EDTA/pH 3 MCllVAINE BUFFER, ULTRASONIC AGITATION AQUA II MOBILE PHASE A)

	Mean recovery (%) (intra-day RSD%) n=3 100 (µg.kg^{-1})	RT (min)	Linearity (R^2)	ME (%)
SDZ	92 (1.5)	3.5	0.996	99.2
STZ	98 (9.8)	4.3	0.991	99.6
SMZ	95 (1.0)	10.9	0.997	98.2
SMT	100 (7.4)	7.4	0.996	100.5
SMX	91 (3.9)	7.6	0.996	99.4
SDX	94 (3.7)	10.9	0.998	98.3
SMH	97 (0.1)	11.0	0.999	94.5
SQX	89 (35.0)	13.3	0.986	99.6
SFX	88 (30.5)	11.9	0.997	94.2
DIF	101 (14.8)	10.7	0.991	91.5
MAR	88 (2.4)	6.3	0.988	76.2
OXO	109 (33.2)	14.8	0.978	89.2
FLU	110 (36.8)	14.8	0.972	78.7
SAR	105 (8.1)	10.6	0.991	95.7
NOR	102 (8.8)	7.2	0.994	36.8
ENR	106 (4.3)	9.3	0.996	75.4
DAN	100 (6.7)	8.8	0.994	66.5
CIP	92 (3.1)	7.8	0.996	75.0
SPI	95 (3.7)	12.0	0.991	110.4
ERY	99 (12.9)	14.0	0.991	99.6
TYL	95 (19.4)	14.4	0.989	99.4
JOS	94 (23.4)	16.0	0.984	98.7
TIL	99 (24.3)	13.1	0.974	102.5
OTC	105 (8.8)	7.1	0.998	3.7
TC	99 (5.3)	8.3	0.997	39.9
DXC	98 (2.0)	8.3	0.999	60.0
THI	55 (9.1)	8.3	0.831	>150
TRI	99.5 (51.9)	6.2	0.844	>150
LIN	79 (30.0)	4.8	0.894	>150

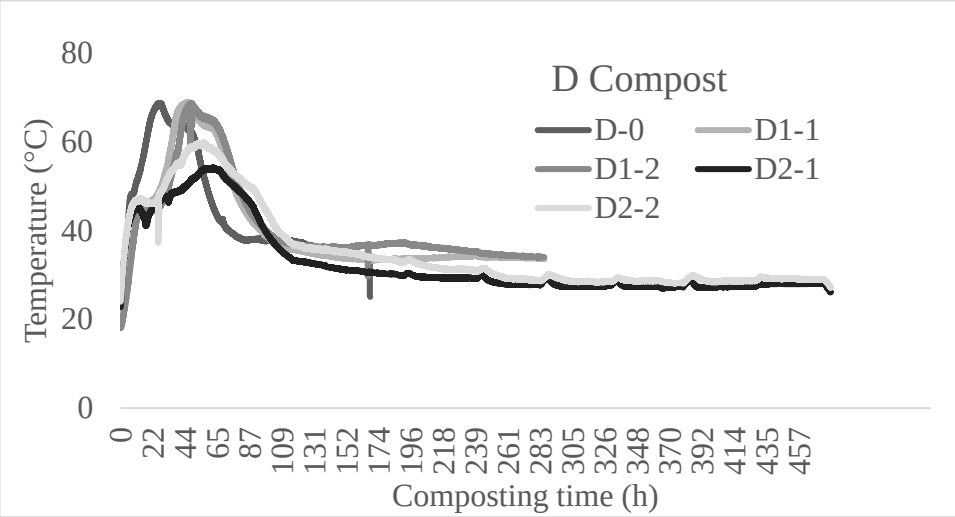
APPENDIX F: METHOD PERFORMANCE PARAMETERS FOR DAIRY MANURE IN ANTIBIOTIC CONCENTRATION OF 100 µG.KG^{-1} (MeCN/EDTA/pH 3 MCllVAINE BUFFER, MECHANICAL AGITATION, AQUA I MOBILE PHASE A)

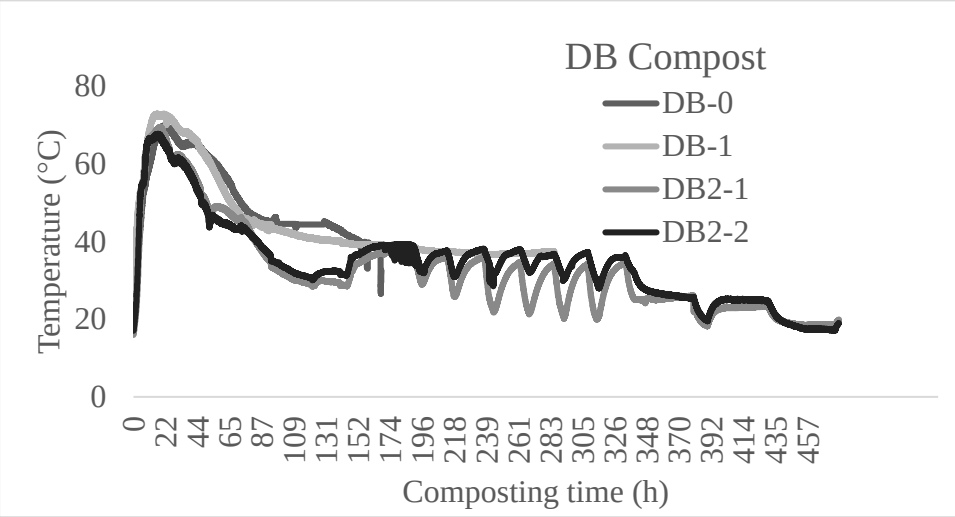
	Mean recovery (%) (intra-day RSD%) n=3 100 (µg.kg^{-1})	RT (min)	Linearity (R^2)	ME (%)
SDZ	104 (2.8)	3.5	0.997	97.4
STZ	112 (6.7)	4.3	0.998	98.7
SMZ	105 (3.4)	10.8	0.993	97.4
SMT	108 (9.9)	7.3	0.999	98.3
SMX	112 (10.8)	7.5	0.997	99.6
SCP	104 (6.8)	9.7	0.991	99.1
SDX	105 (1.1)	10.8	0.998	99.0
SMH	98 (1.4)	11.0	0.999	98.1
SFX	110 (5.1)	11.8	0.998	106.4
DIF	109 (10.4)	10.7	0.998	98.5
MAR	103 (6.4)	6.2	0.993	89.8
OXO	96 (0.7)	14.8	0.997	90.1
FLU	96 (3.7)	14.8	0.997	75.7
SAR	108 (9.2)	10.5	0.999	97.2
NOR	106 (3.3)	7.1	0.994	75.7
ENR	102 (12.1)	9.2	0.985	79.0
DAN	99 (17.1)	8.7	0.979	89.0
CIP	101 (3.0)	7.6	0.999	93.4
SPI	74 (37.5)	11.9	0.996	98.2
ERY	83 (21.4)	13.9	0.997	99.8
TYL	95 (8.9)	14.3	0.998	99.0
JOS	171 (59.0)	15.9	0.998	99.7
TIL	98 (5.1)	13.1	0.995	89.5
DXC	134 (10.1)	8.2	0.861	>150
THI	86 (3.3)	6.7	0.969	>150
TRI	88 (20.9)	6.1	0.999	12.3
LIN	101 (8.4)	4.6	0.996	>150

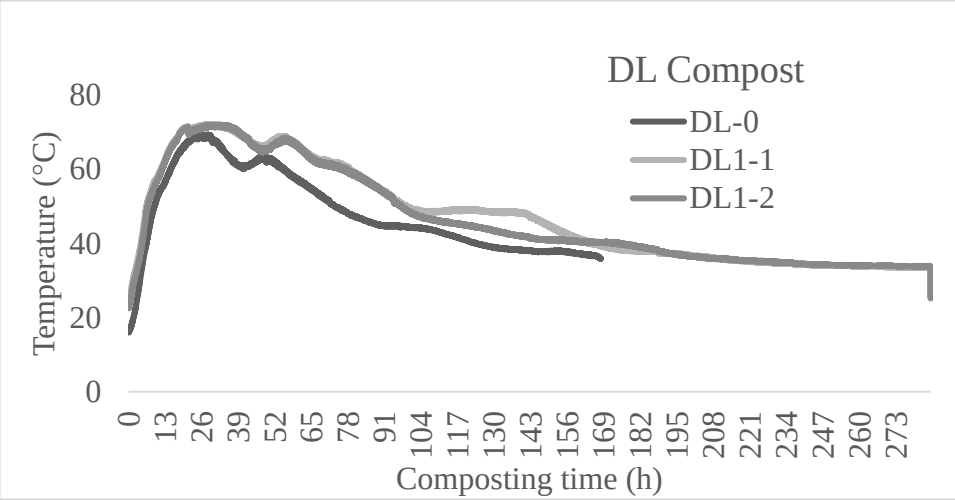
APPENDIX G: METHOD PERFORMANCE PARAMETERS FOR POULTRY MANURE IN ANTIBIOTIC CONCENTRATION OF 100 µG.KG^{-1} (MeCN/EDTA/pH 8 MCllVAINE BUFFER, MECHANICAL AGITATION, AQUA II MOBILE PHASE A)

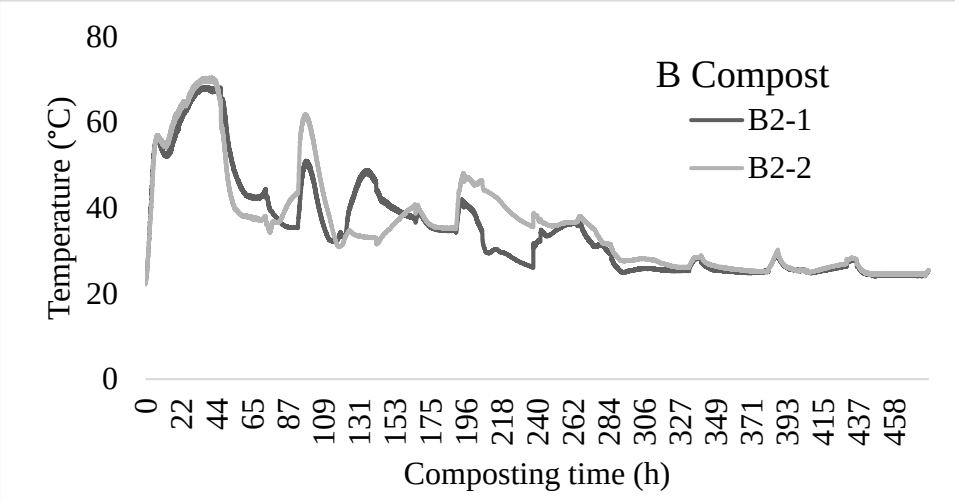
	Mean recovery (%) (intra-day RSD%) n=3 / 100 (µg.kg^{-1})	RT (min)	Linearity (R^2)	ME (%)
SDZ	95 (2.9)	3.5	0.998	91.7
STZ	98 (1.2)	4.3	0.998	92.1
SMZ	98 (2.5)	10.9	0.997	84.4
SMT	102 (3.0)	7.4	0.999	72.6
SMX	100 (7.8)	7.6	0.997	85.7
SCP	99 (1.5)	9.8	0.999	86.8
SDX	97 (1.3)	10.9	0.998	83.6
SMH	95 (3.4)	11.0	0.999	65.3
SQX	98 (11.5)	13.3	0.987	94.9
SFX	100 (4.5)	11.9	0.997	59.2
DIF	90 (7.1)	10.8	0.976	91.1
MAR	100 (4.5)	6.4	0.999	61.1
OXO	85 (2.5)	14.8	0.986	91.2
FLU	100 (19.2)	14.8	0.948	85.9
SAR	90 (17.3)	10.6	0.999	93.7
NOR	106 (12.7)	7.3	0.998	10.6
ENR	97 (4.2)	9.3	0.994	81.7
DAN	92 (10.1)	8.9	0.981	78.3
SPI	89 (2.0)	12.0	0.993	>150
ERY	107 (2.0)	14.0	0.996	97.2
TYL	95 (4.5)	14.3	0.999	>150
JOS	100 (2.8)	15.9	0.999	75.7
TIL	103 (0.7)	13.2	0.999	>150
OTC	53 (4.0)	7.1	0.846	62.5
TC	55 (3.9)	8.4	0.859	57.0
DXC	55 (3.9)	8.4	0.861	72.1
CTC	55 (3.2)	11.8	0.864	97.6
FLF	56 (3.8)	11.4	0.841	>150
CHL	142 (31.5)	15.8	0.975	>150
TRI	105 (2.5)	6.3	0.995	>150
LIN	100 (8.0)	4.8	0.992	43.9

APPENDIX H: TEMPERATURE PROFILES OF COMPOST SAMPLES: ROTARY DRUM WITH TWO REACTOR TEMPERATURE VARIATION RESULTS









APPENDIX I. EQUATION AND LINEAR REGRESSION VALUES OF ANTIBIOTICS IN COMPOST SAMPLES

	Equation	R^2	Slope (k)
SCP D-1	y=-0.8508x-5.8763	0.549	-0.8508
STZ DL-1	y=-0.1628x-0.3989	0.754	-0.1628
SFX DL-1	y=-0.0166x-1.4027	0.791	-0.0166
SMH DB-1	y=-0.1094x+0.1914	0.906	-0.1094
STZ DB-2	y=-0.5949x-7.3405	0.364	-0.5949
SMH DB-2	y=-0.092x-0.6727	0.706	-0.092
SMH B-2	y=-0.129x+1.2771	0.93	-0.129
CIP D-1	y=-1.3722x-0.6985	0.806	-1.3722
CIP DB-1	y=-0.0192x+0.0733	0.62	-0.0192
ENR D-1	y=-0.0014x-0.0137	0.375	-0.0014
ENR DB-1	y=-0.0265x+0.05	0.873	-0.0265
DAN DB-1	y=-0.2279x-1.4034	0.61	-0.2279
CIP D-2	y=-0.617x-5.1231	0.466	-0.617
ENR DB-2	y=-0.0569x-0.5515	0.565	-0.0569
ENR B-2	y=-0.0271x-0.0275	0.659	-0.0271
ENR D-2	y=-0.0155x-0.5843	0.103	-0.0155
CTC D-1	y=-0.0608x-1.3749	0.0856	-0.0608
OTC D-1	y=-0.0287x+0.0241	0.947	-0.0287
OTC DL-1	y=-0.095x-0.1292	0.966	-0.095
OTC DB-2	y=-0.018x-0.9749	0.134	-0.018
OTC D-2	y=-0.0389x-2.0375	0.132	-0.0389
CTC D-2	y=-0.5477x-7.3266	0.318	-0.5477
LIN D-1	y=-0.0032x+0.0039	0.842	-0.0032
LIN DL-1	y=-0.025x+0.0022	0.902	-0.025
LIN DB-1	y=-0.0663x-0.8359	0.257	-0.0663
LIN DB-2	y=-0.0065x-0.1383	0.371	-0.0065
LIN B-2	y=-0.4965x-1.2508	0.552	-0.4965
LIN D-2	y=-0.1213x-1.2464	0.636	-0.1213
TRI DB-1	y=-0.0247x-0.1923	0.487	-0.0247
TRI DB-2	y=-0.0376x-0.2679	0.721	-0.0376
TRI B-2	y=-0.0892x-0.9766	0.777	-0.0892
STZ ST- DB-2	y=-0.56x-7.8224	0.366	-0.56
SMH ST- DB-2	y=-0.0795x-0.8634	0.505	-0.0795
SMH ST B-2	y=-0.1175x-0.1386	0.914	-0.1175
SMH ST DB-1	y=-0.1466x-0.4014	0.842	-0.1466
CIP ST-DB-1	y=-0.0783x-0.6451	0.454	-0.0783
DAN ST-DB-1	y=-0.275x-0.9259	0.763	-0.275
ENR ST-DB-1	y=-0.0851x-0.935	0.331	-0.0851
ENR ST-DB-2	y=-0.0467x-0.5639	0.568	-0.0467

APPENDIX I. (CONTINUED) EQUATION AND LINEAR REGRESSION VALUES OF ANTIBIOTICS IN COMPOST SAMPLES

	Equation	R^2	Slope (k)
ENR ST-B-2	y=-0.045x-0.0342	0.423	-0.045
ENR ST-D-2	y=-0.0172x-0.8739	0.151	-0.0172
CIP ST-D-2	y=-0.4013x-8.2853	0.351	-0.4013
OTC ST-DB-2	y=-0.1445x-1.0629	0.486	-0.1445
OTC ST-D-2	y=-0.0593x-1.824	0.273	-0.0593
CTC ST-D-2	y=-0.594x-6.822	0.33	-0.594
LIN ST-DB-2	y=-0.0082x-0.1495	0.433	-0.0082
LIN ST-B-2	y=-0.4717x-0.9132	0.446	-0.4717
LIN ST-D-2	y=-0.1345x-0.8934	0.701	-0.1345
TRI ST-DB-1	y=-0.0148x-0.1319	0.428	-0.0148
TRI ST-DB-2	y=-0.0523x-0.2937	0.529	-0.0523
TRI ST-B-2	y=-0.0898x-0.9375	0.68	-0.0898