Effect of Ceftiofur on Mesophilic Anaerobic Digestion of Dairy Manure and the Removal of the Cephalosporin-Resistance Gene *CMY-2*

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ABSTRACT

This research study evaluated the effect of ceftiofur (CEF), one of the most commonly used antibiotics on dairy farms, on the performance and stability of mesophilic anaerobic digestion of dairy manure, in terms of methane production, organic matter removal (COD, dCOD, TS, and VS) and intermediates (VFAs) profile. Additionally, this study also determined the fate of the antibiotic-resistance gene (ARG) *cmy-2*, a cephalosporin-resistance marker, in a mesophilic fed-batch anaerobic digester over a 600-day period and assessed how the presence of CEF alters the *cmy-2* removal rate in 15-day batch anaerobic digestions. A semi-continuous lab-scale anaerobic digester was set up and operated under mesophilic conditions, with a hydraulic retention time of 30 days and fed with dairy manure from a commercial farm. The sludge of the digester was analyzed for *cmy-2* levels and used as inoculum for batch experiments where different CEF concentrations (0.2-250 mg/L) were tested. The results indicated that low CEF concentrations (0.2 – 2 mg/L) do not have any significant effect on either methane production or the stability of the process. CEF concentrations of 10 mg/L can reduce methane productivity by more than 10% in anaerobic digesters operated at HRTs below 20 days. The presence of high CEF levels (50-250 mg/L) can cause a decrease in methane production of up to 60%, although the overall stability of the process is not compromised. The biochemical analyses suggested that hydrolytic microorganisms were the most affected by the presence of the antibiotic, while acetogens seemed to have temporarily slowed down, which was reflected in lower organic matter removal and slightly greater levels of butyrate and valerate accumulation during the anaerobic digestion process, respectively. Methanogens, on the other hand, were not affected, since no acetate or hydrogen accumulation was detected. Regarding the levels of the antibiotic resistance gene *cmy-2*, this study demonstrated that anaerobic digestions is a good alternative for the removal (> 90%)

of the high *cmy-2* levels found in dairy manures (>100 copies/ng DNA), and that the presence of ceftiofur (50-250 mg/L) does not substantially increase levels of the gene during 15-day batch anaerobic digestions.

Keyword: Anaerobic Digestion; Dairy Manure; Ceftiofur; Methanogenesis; Antibiotic Resistance Genes;

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CHAPTER 1 - INTRODUCTION

1.1 Need for renewable energy sources

With the increase of societal needs such as food, housing, transport, and energy, the use of sustainable sources of material and energy has become one of the most important challenges of our century. Until now, the societal demands have been satisfied by using fossil sources, which are limited and eventually will be depleted. Besides, the extensive use of fossil fuels has provoked an alarming rise in the atmospheric carbon dioxide (CO_2) leading to fast global warming. Therefore, the development of new technologies capable of harvesting energy from natural processes is urgently required (Panwar et al., 2011). The launch of the Dow Jones Sustainability World Index (DJSI) in 1999, the first global sustainability benchmark, motivated the creation of different platforms to support companies and investors interested in adopting sustainable practices (De Meester et al., 2012). Nonetheless, the transition from fossil sources towards renewable sources can be only achieved by the development of profitable technologies aligned to bio-economy strategies.

1.2 Biogas as a renewable source of energy

Biogas is an energy-rich end-product of the natural degradation of organic matter under anaerobic conditions. It is mainly composed of methane (CH_4) and carbon dioxide (CO_2) (Awe et al., 2017). Methane is a secondary energy carrier with high heating values (> 34,000 kJ/m^3) that can be combusted to generate heat and electricity (Cheng, 2010; Hosseini and Wahid, 2014). Methane is the principal component of natural gas, which is widely utilized worldwide. The estimated natural gas consumption in 2017 was around 4,000 billion m^3, which represented 22% of the total energy consumed and the demand is expected to grow in the future (Enerdata Yearbook, 2018). Although

natural gas is significantly cleaner than other fossil fuels, it is not exempt of environmental drawbacks. The drilling, extraction, and transportation often result in methane leakage, contributing to the emission of greenhouse gases (GHG). Hence, biogas stands as a good natural gas substitute because it is produced in a cleaner process, recycling biological wastes and mitigating natural GHG emissions.

The use of biogas as an alternative source of energy is promising. Although biogas produces slightly less energy (13,000-27,000 kJ/m^3) than natural gas, its usage has increased considerably in the last few decades. In Europe alone, more than 14.5 gigawatts are generated with biogas and this is expected to double in the near future (Pike Research, 2012). Globally, much effort is dedicated to increasing biogas production capacity with the ultimate aim to respond to foreseeable growth in the renewable energy market over the next decade (Messenger, 2017).

1.3 Anaerobic digestions for biogas production

Biogas is naturally produced in environments where oxygen is not available (such as bogs, lakes, oceans, and some digestive tracts) throughout a series of biochemical reactions collectively known as anaerobic digestion. Anaerobic digestion (AD) has been applied to treat different high-strength biowastes, such as municipal sewage solids, animal manures, food, agricultural wastes and petrochemical wastes (Amani et al., 2010; Hamza et al., 2016; Verstraete and Vandevivere, 1999). Anaerobic digestion offers more benefits than other traditional treatments. It is an efficient process capable of reducing organic pollution with relatively low residual biomass (also referred as sludges), while generating biogas, which, under controlled conditions, can be collected, enriched, and then used to produce energy (Cheng, 2010).

The implementation of AD not only as a treatment of biowastes, but for biogas production has given rise to significant interest globally. During the last decade, thousands of new anaerobic

digestion plants have been commissioned worldwide. Indeed, more than 17,000 biogas plants are operating in Europe and the number is expected to grow further (European Biogas Association, 2018).

In the United States, this technology has not been as popular as in Europe, but there are increasing numbers of biogas plants being developed. In the US, about 1,100 anaerobic digesters were operating in 2018 (U.S. EPA, 2018), whereas in Canada approximately 120 anaerobic digesters were reported to operate where the biogas is mostly used to produce heat and steam for internal processes (Kelleher Environmental, 2013). The low temperatures in winter and the relatively low price paid for energy in Canada are the principal reasons that have limited the expansion of this technology. Nonetheless, stakeholders are committed to significantly increase the production and usage of biogas by 2030 (Kelleher Environmental, 2013).

1.3.1 Anaerobic digestion of livestock manures

The treatment of livestock manures is one of the most common applications of AD due to the different benefits it offers compared to other treatments. It can reduce odors up to 80%, remove most of the pathogens (up to 99%), and concentrate nutrients such as phosphorus and nitrogen (Parkin and Owen, 1986). The sludges (digestate) generated during AD of manures can be used as crop fertilizer or recycled animal bedding (Kelleher Environmental, 2013). The application of digestate on crop fields has been shown as a good alternative to synthetic fertilizer to improve nutrient availability (Möller and Müller, 2012).

Regarding the environmental impact, AD helps to alleviate GHG emissions related to manure management (Kelleher Environmental, 2013; Labatut et al., 2011). The natural microbiome found in manure can degrade the organic matter and convert it into methane, which is a powerful GHG, considered 28-36 times stronger than CO_2. The United States Environmental Protection Agency

(U.S. EPA) reported that agricultural activities such as livestock production and manure management contributed to up to 9% of the global GHG emissions in 2016 (USEPA, 2018). Hence, AD is a feasible alternative to reduce GHG emissions through the collection and utilization of methane. The possibility of recovering the residual energy not used by livestock through AD of animal manures can contribute to sustainable economic development. It is estimated that up to 2% of the annual energy demand in Canada could be fulfilled with biogas obtained from anaerobic digestion of half of the manure generated (Kelleher Environmental, 2013).

There are a few economic and technical challenges while implementing anaerobic digestions of animal manures for biogas production. On the one hand, the initial capital investment required for setting up the bioreactors, biogas containers, biogas filters, and the operational costs, are considerably greater compared to traditional management systems such as lagoons, tanks, or aerobic digesters (Awe et al., 2017; Surendra et al., 2013). Hence, subsidy programs are often required to incentive the implementation of anaerobic digesters. On the other hand, the stability of the process can be affected by small changes on operational parameters, such as temperature, organic loading rates (OLR), and hydraulic retention times (HRT) (Amani et al., 2010), or by the presence of toxic compounds that alter the sensitive syntrophic interactions between the microorganisms involved in AD (Chen et al., 2008). Besides, the biogas that can be obtained from manure digesters is not always sufficient to render it a profitable technology.

The biogas yield depends greatly on the type and source of manure. While cattle manures generally produce between 180-260 L/kg of volatile solids (VS, a measure of organic strength), hog manures yields are in the range of 400-600 L/kg VS (Nasir et al., 2012). However, an easy and suitable alternative to improve biogas yields is the use of energy-rich crop residues and food processing waste as co-substrate. Different studies have shown that corn, alfalfa, switchgrass, and other crop

residues can significantly boost biogas production and improve the stability of the process (Demirel, 2014; Jordaan and Çiçek, 2014; Moody et al., 2011).

1.4 Anaerobic digestion fundamentals

The understanding of the biochemical reactions, the microbial interactions, and how operational parameters affect them is essential to successfully implement AD for biogas production. A brief description of the fundamentals of AD and some of the most important operational parameters are described in the following sections.

1.4.1 Microbiology of anaerobic digestion

Anaerobic digestion is a complex biological process where several interacting microorganisms transform biodegradable organic matter into biogas in an oxygen-free environment. The principal product of AD is energy-rich biogas, mainly composed by methane (CH_4), carbon dioxide (CO_2), and trace amounts of hydrogen (H_2) and hydrogen sulfide (H_2S). In AD, the electron transfer is regulated by thermodynamically suitable secondary metabolites produced in the different metabolic pathways (Barua and Dhar, 2017). Cell-to-cell electron transfer between some particular bacteria and methanogens, known as direct interspecies electron transfer (DIET), has been recently discovered (Morita et al., 2011; Rotaru et al., 2014). Therefore, the balance of the sensitive relationships of the different microorganisms is critical for the stability of the process.

The anaerobic digestion process is generally divided into four fundamental steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Figure 1.1). Although defined as a step-wise process, it is well known that all these reactions occur in parallel because the different groups of microorganisms depend on each other (Parkin and Owen, 1986).

1.4.1.1 Hydrolysis:

The first step of AD is hydrolysis, where complex molecules such as lignin, cellulose, hemicellulose, proteins, and fats are broken down by extracellular enzymes into simpler compounds, such as mono- and disaccharides, amino acids, and long-chain fatty acids, which are utilized in the next steps (Amani et al., 2010; Parkin and Owen, 1986). The initial substrate composition is a key factor that determines not only the hydrolysis rate but also the total biogas yield. High levels of recalcitrant materials such lignocellulosic materials, are associated with a slow hydrolytic activity, less biodegradability and thus, less biogas production (Demirel, 2014). Because of that, hydrolysis is usually considered the rate-limiting step of the AD process. Some of the typical microorganisms responsible for the hydrolytic activity in AD include species belonging to the genera *Bacillus*, *Bacteroides*, *Clostridium, Desulfovibrio, Geobacter, Mycobacterium, Peptococcus, Proteiniphilum, Staphylococcus,* and *Vibrio* (Amani et al., 2010).

1.4.1.2 Acidogenesis:

During acidogenesis (also known as fermentation) the solubilized organic molecules are consumed by a group of microorganisms collectively known as acidogens. Under stable conditions, the principal products of acidogenesis are H_2, CO_2 and reduced metabolites, such as lactate, volatile fatty acids (VFAs) and some alcohols (Cheng, 2010). These molecules play an essential role in the electron transfer to methanogens in the absence of an external inorganic electron acceptor (Barua and Dhar, 2017). The bacterial consortia responsible for acidogenesis are mainly composed of species of the genera *Acetobacterium, Bacillus, Clostridium, Lactobacillus*, *Pseudomonas, Syntrophomonas*, *Micrococcus, Pseudomonas, and Zymomonas*. These microorganisms have considerably high growth rates, up to 40 times greater than methanogens, and tolerate extreme

conditions such as low pH, high temperature and high organic loading rates (OLRs)(Amani et al., 2010).

1.4.1.3 Acetogenesis:

The acetate production from the reduced metabolites of the previous stage is known as acetogenesis. During this step, CO_2, H_2, and formate are also produced. The conversion of VFAs and alcohols in acetogenesis, which involves interspecies hydrogen transfer, is not thermodynamically favorable under standard conditions, thus, syntrophic interactions are required to remove the acetogenic products and allow the reactions to proceed. Usually, acetogens are coupled in syntrophic association with hydrogenotrophic methanogens that can consume H_2 and CO_2, keeping the H_2 pressure below 10^{-4} atm and making the reactions thermodynamically feasible (Cirne, 2006; Leng et al., 2018). Hence, minor disturbances to the syntrophic interaction between acetogens and methanogens can severely affect the AD process. Some of the typical syntrophic acetogens found in AD include microorganisms of the genera *Clostridium, Pelotomaculum, Smithella, Syntrophobacter, Syntrophus, Syntrophomonas, Syntrophothermus* (Amani et al., 2010; Leng et al., 2018). They grow slowly, with a maximum specific growth rate (μmax) of $1h^{-1}$ and are sensitive to OLR's fluctuations and environmental disturbances (Amani et al., 2010; Cirne, 2006).

1.4.1.4 Methanogenesis:

The final stage of AD is methanogenesis, where acetate, CO_2, and H_2 are consumed and CH_4 is produced. There are two main metabolic pathways for methane production in AD: hydrogenotrophic (hydrogen-consuming) and acetotrophic (acetate-consuming) methanogenesis. In hydrogenotrophic methanogenesis, H_2 is utilized to reduce CO_2 to CH_4 in an energetically favorable reaction:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (\Delta G^{\circ\prime} = -135 \text{ kJ}) \tag{1.1}$$

On the other hand, the acetotrophic methanogenesis utilizes acetate to produce CH_4 and CO_2:

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (\Delta G^{\circ\prime} = -31 \text{ kJ}) \tag{1.2}$$

Although hydrogen-consuming methanogenesis (-135 kJ at 25°C) is thermodynamically more favorable than acetate-consuming methanogenesis (-31 kJ at 25°C), it contributes only up to 30% of the total CH_4 production in anaerobic digesters because only around a third of the fermented glucose goes to H_2 and CO_2,whereas the rest goes to acetate (Amani et al., 2010; Cirne, 2006). The microorganisms responsible for methanogenesis belong to the domain Archaea, mainly from the genera *Methanosaeta* and *Methanosarcina*. The former are obligate acetotrophic methanogens while the latter are facultative acetotrophic methanogens that can produce CH_4 through both pathways (Leng et al., 2018). Methanogens require very low redox potential since they are strict anaerobes and even low oxygen concentrations can severely affect their growth (Sirohi et al., 2010). Typical grow rates of mesophilic methanogens are in the range of 0.05 to 4.07 d^{-1} (Leng et al., 2018).

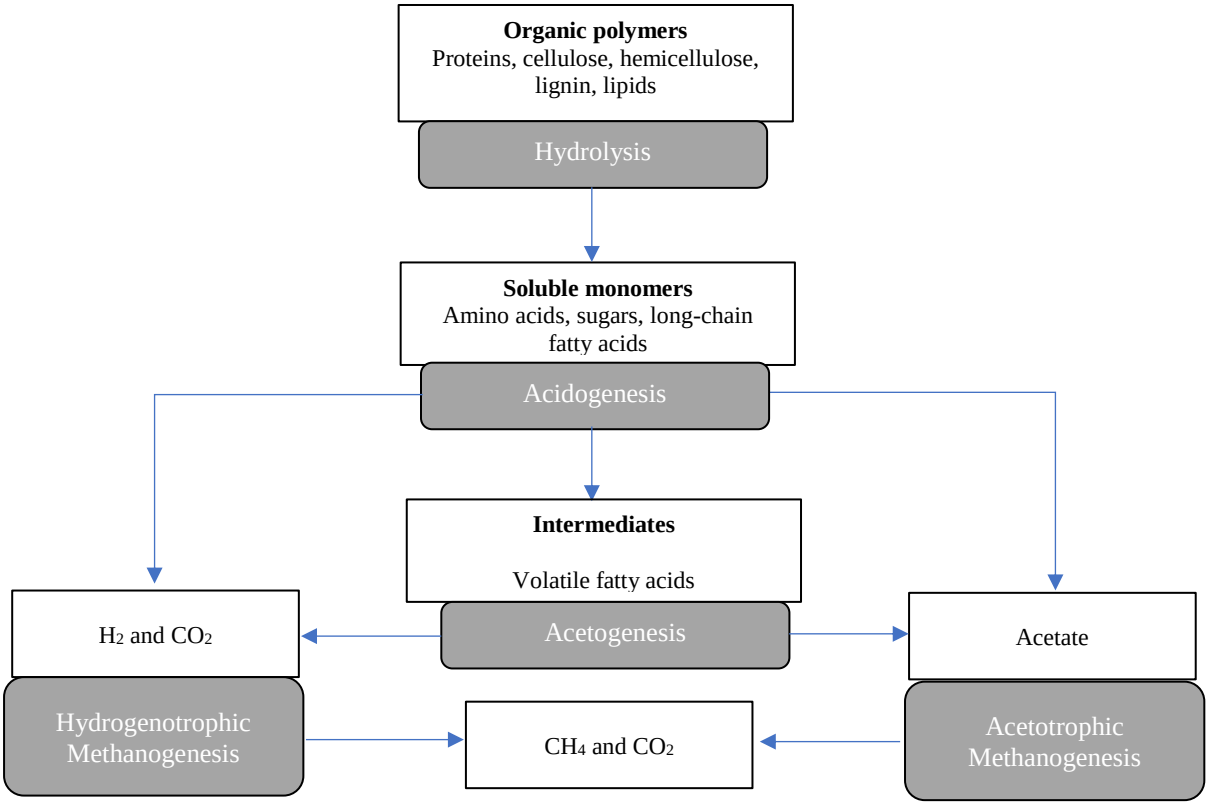


Figure 1.1 Simplified anaerobic digestion process scheme.

(Based on Amani et al., 2010)

1.4.2 Operational parameters

1.4.2.1 Hydraulic retention time

The hydraulic retention time (HRT) is the time that a molecule of substrate spends inside the digester and is approximately the time during which the substrate is consumed in the AD process. Conceptually, HRT is different from the solid retention time (SRT), which is the time that the microbial biomass spends in the digester. However, they are used as synonyms when good mixing and homogeneous digestates are assumed, and solids are not separately removed from the digester (Lindmark et al., 2014). Both must be long enough to avoid washing microbial populations out, thus, they should be 2-fold greater than the lowest microbial growth rate, usually methanogens

whose growth rates range from 0.05 to 0.7 d^{-1} (Leng et al., 2018). The most commonly used HRT in anaerobic digesters are in the range of 10-30 days, but longer HRTs (up to 175 days) have been used (Komilis et al., 2017). No clear correlation between HRT and biogas yield has been found.

1.4.2.2 Organic Loading Rate

The organic loading rate (OLR) can be defined as the amount of organic matter fed to the digester per volume per unit of time. The OLR is used for digesters design and depends on the biowaste characteristics, microbial activity, temperature, pH, toxicity, type of digester, and mass transference between the biowaste and the biomass (Amani et al., 2010; Khanal et al., 2016; Komilis et al., 2017; Speece, 1983). Acetogens and methanogens are especially sensitive to rapid changes in OLR, therefore, major failures can occur after a rapid OLR change (Amani et al., 2010). The OLR can be calculated as follow:

$$OLR = \frac{(VS)\,x\,Q}{V} \tag{1.3}$$

Where: VS is the volatile solids concentration (kg/L); Q is the flow of the substrate (kg/day); and V is the volume of digester (L). Typical OLRs are in the range between 1-5 kg VS/m^3-day (Komilis et al., 2017).

1.4.2.3 pH

The optimal pH of most of the microbial populations involved in AD for biogas production is reported to be between 6.5-8.0 (Amani et al., 2010). Drastic changes in pH may drive methane inhibition or a major failure in the digester. pH lower than 6 leads to hydrogen production because methane production is inhibited (Cheng, 2010). Biowastes like livestock manures can provide natural buffering capacity that can help to avoid extreme pH changes (Amani et al., 2010).

1.4.2.4 Temperature

Temperature plays an important role in the performance of anaerobic digesters because some biochemical reactions can occur only under specific temperature ranges. Also, the operating temperature can influence the microbial community composition and thus, affect the productivity of the process (Tian et al., 2018; Zinder et al., 1984). Temperature fluctuations greater than 3 °C are associated with process instability, so they should be avoided (Amani et al., 2010). Based on the temperature at which the digestion is performed, AD is classified as psychrophilic (10-25°C), mesophilic (30-40°C), and thermophilic (45-60°C).

1.4.2.4.1 Psychrophilic anaerobic digestion

Naturally, methanogenesis occurs in cold environments, even in arctic and sub-arctic peatlands. Under low temperatures, the thermodynamics of AD reactions is less favorable, thus, the syntrophic interactions become even more important (McKeown et al., 2011). Psychrophilic anaerobic digesters are usually operated at the ambient temperature in zones with tropical or subtropical climates with average temperatures around 10-25°C. Generally, the systems are simple, and the operation costs are relatively low because no heat exchangers are required. Although psychrophilic anaerobic digesters have been successfully used to treat carbohydrate-rich bio-wastes, industrial applications have been limited (Cheng, 2010). The efficiency of psychrophilic AD is low because the biochemical reaction rates are low, and thus, large digester volume and long HRT (>50 days) are required (McKeown et al., 2011). The start-up of this type of digestions is more complicated because the inoculum must acclimate to sub-optimal conditions. Furthermore, since methane is more soluble in the liquid phase at low temperatures, its recovery is more complex (McKeown et al., 2011).

1.4.2.4.2 Mesophilic Anaerobic digestion

Mesophilic anaerobic digestion is the most commonly used system for the treatment of several biowastes such as food-processing wastes, winery wastewater, municipal sewage, and manures (Cheng, 2010). Usually, they operate at temperatures between 30-40 °C where microbial activity is close to the optimal (Amani et al., 2010). Mesophilic anaerobic digesters are more stable and easier to start up than psychrophilic and thermophilic digesters (Cheng, 2010). They have shown to be more robust and resilient due to their high microbial diversity which is a key factor to preserve stability (Wang et al., 2018). The HRT of this type of digestion varies from a few days up to 40 days, with most systems operating at around 25-30 days (Amani et al., 2010). Additionally, higher OLR (4,000-12,800 mg/L) can be supported in mesophilic anaerobic digesters (Bayr et al., 2012).

1.4.2.4.3 Thermophilic anaerobic digestion

Thermophilic anaerobic digestion is performed at temperatures between 50-65°C where the maximum metabolic rates are often found, therefore, the HRT (10-15 days) and the volume of the digesters are lower than mesophilic systems (Cheng, 2010). Also, higher biogas yields and higher organic matter reduction have been reported (De la Rubia et al., 2012; Wang et al., 2018). One of the greatest advantages of thermophilic anaerobic digesters is the ability to reduce most of the pathogens present in the sludges, which allows the safe use of the digestate (residual biomass) on croplands as a fertilizer (Cheng, 2010).

Thermophilic AD faces some challenges. The start-up phase of an anaerobic digester is long and complicated because a mature and healthy inoculum is required. The inoculum must contain the key microbial populations which are not always present in mesophilic anaerobic digesters (De la Rubia et al., 2012). Also, higher VFA concentrations are found in thermophilic anaerobic digesters because, at high temperature, acetogenesis is thermodynamically more favorable, while

hydrogenotrophic methanogenesis is less favorable. This results in the accumulation of some VFAs such as propionate and butyrate, which can be toxic for some anaerobes and cause instability at high concentrations (Amani et al., 2010).

1.5 Anaerobic digestion inhibitors

Toxic compounds, such as metals, organic and inorganic molecules, and pharmaceuticals, are often found in considerable concentrations in wastewaters and livestock manures (Cheng, 2010; Halling-Sorensen et al., 1998). These compounds can adversely affect the different microbial communities involved in AD and lead to instability and sometimes even complete digester failure (Chen et al., 2008). They represent a serious threat to biogas producing plants since the CH_4 yield, and thus, the energy yield can be compromised, affecting not only the profitability but the sustainability of the process. This section is devoted to present some of the AD inhibitors most commonly found in biowastes, especially in livestock manures.

1.5.1 Organic compounds

Several organic compounds have toxic effects on AD, especially some insoluble molecules and substances that can be adsorbed to the surface of sludge solids, or those whose hydrophobicity can disrupt the cell membrane. These compounds can accumulate to toxic levels or cause microbial biomass flotation (Cheng, 2010). Among the organic inhibitors, are long-chain fatty acids (LCFA), phenols, halogenated aliphatics, alcohols, and nitro-aromatics (Chen et al., 2008; Cheng, 2010).

1.5.1.1 Long-chain fatty acids

Long-chain fatty acids (LCFA) are intermediates of the lipid degradation process and their accumulation has been associated not only with microbial toxicity but with sludge flotation and biomass washout (Chen et al., 2008; Cirne, 2006; Zonta et al., 2013). LCFAs have shown adverse effects in all the stages of the AD process (Cirne, 2006). Adsorption of LCFAs onto substrates

reduces their surface available for hydrolytic activity (Cirne, 2006), while the adsorption onto microbial biomass interferes with transport and protective functions (Chen et al., 2008). Some of the LCFAs that affect AD are oleic acid, lauric acid, cyprylic acid, capric acid and myristic acid (Chen et al., 2008). Their inhibitory concentrations vary depending on the temperature, feeding pattern, acclimation and pH (Cheng, 2010). Common half maximum inhibitory concentration (IC_{50}), a measure of the toxicant concentration that causes 50% reduction of the cumulative methane production, are found in the range of 50 – 200 mg/L (Cirne, 2006).

1.5.1.2 Lignin and derivates

As mentioned in previous sections, the use of crop residues as co-substrates in AD is a growing practice. Some of these crops are rich in lignin, a recalcitrant compound that has a complex structure hard to decompose in AD. Moreover, phenolic aromatics are structural units of lignin and they have shown to be inhibitory to some anaerobic bacteria (Chen et al., 2008). Lignin derivates with aldehyde groups have been reported as highly toxic to some anaerobes due to their high hydrophobicity (Koyama et al., 2017).

1.5.2 Inorganic compounds

1.5.2.1 Ammonia

Ammonia (NH_3) is an essential nutrient for microbial growth, and it is generated through the biodegradation of nitrogenous material such as proteins and urea. In anaerobic digesters, the ion ammonium (NH_4^+) and free NH_3 are the principal forms of inorganic nitrogen (Cheng, 2010; Yenigün and Demirel, 2013). At high concentrations, free NH_3 can cause intracellular proton imbalance by passively diffusing into microbial cells (Chen et al., 2014). In contrast, NH_4^+ inhibits the enzymes responsible for methane synthesis (Chen et al., 2014). Among the microorganism involved in mesophilic AD, methanogens have shown the highest sensitivity to NH_3, whereas

acidogens are the most tolerant (Chen et al., 2008; Yenigün and Demirel, 2013). Concentrations above 1.7 g NH_3/L have been associated with inhibition of AD microorganisms (Chen et al., 2014).

1.5.2.2 Sulfate and sulfide

Some wastewaters and manures can contain significant levels of sulfates (SO_4^{-2}); thus, they are often found in anaerobic digesters. Sulfide (S^{-2}) is produced throughout the anaerobic degradation of material containing sulfate (amino acids such as cysteine and methionine) by a group of microorganism known as sulfate-reducing bacteria (SRB) (Cheng, 2010). Sulfates can affect methanogenesis in two different ways. On the one hand, the SRB compete directly with acetogens and methanogens for inorganic and organic substrates such as hydrogen, lactate, VFAs and acetate (Chen et al., 2014; Cheng, 2010). The reduction of sulfate to sulfite is thermodynamically more favorable than methanogenesis (Eq 1.1 and Eq.1.2); thus, methanogenesis is diminished (Chen et al., 2014):

$$4H_2 + SO_4^{2-} \rightarrow H_2S + 4H_2O + 2OH^- \quad (\Delta G = -154 \text{ kJ}) \tag{1.4}$$

$$CH_3COOH + SO_4^{2-} \rightarrow H_2S + 2HCO_3 \quad (\Delta G = -154 \text{ kJ}) \tag{1.5}$$

On the other hand, the reduced sulfide is associated with the inhibition of cellular functions of the microbial population involved in AD. Since sulfide is highly reactive, it can affect cellular components, affect protein structures and interfere with electron transfer (Chen et al., 2014). Sulfate concentrations higher than 2,000 mg/L have been shown as toxic for thermophilic anaerobic digestion of pig and cattle manure (Moset et al., 2013), whereas the IC_{50} of hydrogen sulfide (H_2S) for methanogens is usually found between 160-220 mg H_2S/L (Yamaguchi et al., 1999).

1.5.3 Inorganic salts

Inorganic salts are commonly found in agricultural, municipal and food-processing waste streams since they are used as food additives. Although some of these salts are required for the optimal microbial growth in AD, high concentrations cause an increase of the osmotic pressure leading to dehydration of the cell wall (Cheng, 2010).

1.5.3.1 Calcium

Calcium is an essential nutrient for anaerobic bacteria and it is also responsible for the formation of microbial aggregates. Concentrations in the range between 1-3 g/L have been shown as the optimum for biogas production, whereas higher than 5 g/L are considered toxic (Ahn et al., 2006; Chen et al., 2008). High calcium concentration causes carbonate and phosphate precipitation which leads to a loss of buffering capacity (Chen et al., 2008). Also, a reduction of microbial activity as a product of biomass scaling is also related to high Ca^{2+} levels (Chen et al., 2008).

1.5.3.2 Sodium

Sodium is another essential nutrient for anaerobic microorganism because it is related to the generation of adenine triphosphate (ATP) and the oxidation of nicotinamide adenine dinucleotide (NADH) (Cheng, 2010). The osmotic pressure caused by high Na^{+} levels is usually responsible for its inhibitory effect. Sodium concentrations of 100-350 mg/L are reported as the optimal for the microbiome of anaerobic digesters (Chen et al., 2008). The different microorganism involved in AD have different tolerance but concentrations greater than 3.5 g/L are usually associated with moderate inhibition (Chen et al., 2008). Feijoo et al. (1995) found microbial populations can significantly increase their sodium tolerance after an adaptation period.

1.5.4 Heavy metals

Heavy metals have been found in different waste streams including municipal sewage and animal manures. Since these compounds are not degraded by any biological process, they tend to accumulate in anaerobic digesters, where they can cause severe harm to the microbial populations (Cheng, 2010). Heavy metals can potentially disrupt the enzymatic activity by binding the active site of the enzymes causing problems in the main metabolic pathways (Chen et al., 2008). Some of the heavy metals that have been associated with AD inhibitions are iron, cobalt, copper, and zinc (Cheng, 2010).

1.5.4.1 Copper

Although copper (Cu) is an essential element for enzymatic activity in AD, it is also considered one of the most toxic heavy metals to methanogens and might lead to a complete digester failure (Paulo et al., 2015). A common practice in cattle and hog farms is the use of Cu, in the form of copper sulfate ($CuSO_4$), as an antimicrobial agent in foot-bath solutions to prevent infections. Generally, it ends up being flushed to the manure pit, thus, high Cu concentrations are found in manures during application periods (Guo et al., 2012). The Cu concentrations reported in cattle manure ranges between 5 to 352 mg/kg (Xiong et al., 2010), while in pig manure it can range between 50 to 3,300 mg/kg (Hölzel et al., 2012; Xiong et al., 2010). The toxicity of Cu in AD has been widely studied and several inhibitory concentrations have been reported. However, it varies depending on the Cu form used in each study. Ke et al. (2014) reported that 200 mg Cu/L in the form of $CuSO_4$ caused 15% reduction of the cumulative methane production in AD of cattle manure, although 120 mg Cu/L stimulated methane production up to 20%. Luna-delRisco et al. (2011) reported 103 mg Cu/L, in the form of cupric oxide (CuO), as the half inhibitory concentration (IC_{50}) in AD of cattle manure. In contrast, improvement in the stability of AD and

methane production yields at similar Cu concentrations but in the form of cupric chloride ($CuCl_2$) were reported by Hao et al. (2017). Methanogenesis inhibition of $CuCl_2$ has been observed at 300 mg Cu/L or higher (Hao et al., 2017; Wong and Cheung, 1995).

1.5.5 Pharmaceuticals

Pharmaceuticals are chemical compounds intended to perform biological effects either as growth promoters to increase feed efficiency or as treatment of several diseases in both, humans and livestock animals (Kumar et al., 2012). Recently, some studies have shown that anaerobic digesters can be affected by residual antibiotics in manures and municipal sewage (Arikan et al., 2018; Beneragama et al., 2013; Mai et al., 2018; Mitchell et al., 2013). The severity of these effects depends on the type of antibiotic, the concentration, substrate, AD conditions, and the resilience of the microbial population in each digester. A general overview of the problems related to the use of antibiotics is discussed in the following sections.

1.6 Antibiotics and anaerobic digestion

The use of antibiotics for human and veterinary purposes has dramatically increased in the last few years. In Canada alone, more than 1 million kg of veterinary antibiotics is distributed annually (Public Health Agency Canada, 2017). Their extensive use has caused the release of considerable amounts of these compounds to the environment. It is estimated that only approximately 30% of the antibiotic dose is metabolized in the body and the rest is secreted in urine and feces in their original form or in secondary forms that remain active (Jjemba, 2002; Wohde et al., 2016). Therefore, residual antibiotics are commonly found in manures and municipal sewage (Wohde et al., 2016). This represents a serious problem because the sludges obtained after the treatment of these streams are regularly used as field fertilizers, potentially spreading the antibiotics into the

environment, affecting natural processes, and driving the generation of antibiotic-resistant microorganisms (Kumar et al., 2012).

Recent studies have suggested AD as a feasible alternative to degrading some of the residual antibiotics in biowastes such as manures and municipal sewage (Mitchell et al., 2013; Panseri et al., 2013; Wallace et al., 2018). However, many of these antibiotics can disturb the homeostasis in anaerobic digesters and lead to a decrease in biogas yields or even to complete process failure. This might become a problem to small AD plants producing biogas for energy purposes since CH_4 yields can be compromised. The severity of the effect of antibiotics in AD depends on the type of antibiotic, chemical structure, mode of action, AD conditions (Temperature, HRT, OLR, etc.), and antibiotic concentration, although the dose-response relationship is not clear. Some of the most frequent class of antibiotics used in veterinary medicine, their mode of action (Table 1.1), and their effect on AD (Table 1.2) are discussed below.

1.6.1 Tetracyclines

Tetracyclines are a class of broad-spectrum antibiotic against different gram-positive and gram-negative microorganisms. They inhibit protein synthesis by binding reversibly to the bacterial 30S ribosomal subunit and preventing the association of the incoming aminoacyl tRNA (Chopra and Roberts, 2003). Tetracyclines are one of the most commonly used antibiotics in the veterinary industry given their properties, low cost, and the lack of major side effects (Chopra and Roberts, 2003; Public Health Agency Canada, 2017). They are used as a treatment of several infectious diseases and as growth promoters (Chopra and Roberts, 2003). Tetracyclines have been found in hog, cattle, and poultry manures in concentrations as low as 0.05 mg/L and as high as 330 mg/L (Wohde et al., 2016).

The effect of tetracyclines on anaerobic digestion has been widely studied. However, the results are very varied (Table 1.2). While Álvarez et al. (2010) reported 50% methane reduction (IC_{50}) under 9 mg/L of both chlortetracycline (CTC) and oxytetracycline (OTC) in mesophilic batch anaerobic digesters fed with hog manure, Kitazono et al. (2015) found no significant effect on the cumulative methane production after 20 days of mesophilic AD of dairy manure containing 50 mg CTC/L. Nevertheless, in both studies, more than 85% of the antibiotics were degraded after the first 20 days of digestion. In a more recent study, CTC improved methane yield up to 20% in mesophilic AD of swine manure (Wang et al., 2018).

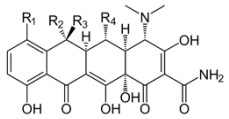
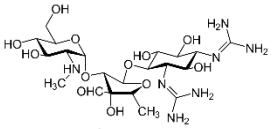
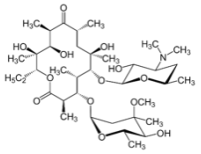
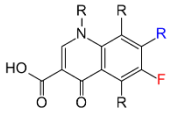
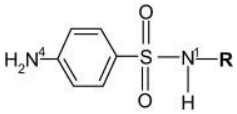
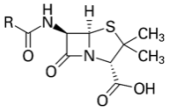
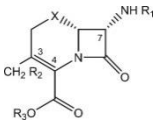
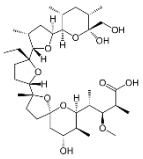
1.6.2 Quinolones/Fluoroquinolones

Quinolones are a group of broad-spectrum antibiotics with a bi-cyclic core structure derived from the compound quinine (Andersson, 2003). Quinolones were improved shortly after their discovery by adding a fluorine atom to the structure and since then they are known as fluoroquinolones. These molecules interrupt DNA synthesis by inhibiting the activity of the topoisomerases II and IV, thus blocking microbial cell division (Alexandrino et al., 2017). They are used to treat several infections of gram-positive and gram-negative bacteria and can act even against intracellular pathogens (Boothe, 2019). Despite the FDA issuing a warning of the risk they represent to human health, fluoroquinolones are still used in human and veterinary medicine (Public Health Agency Canada, 2017). Fluoroquinolones are chemically stable and resistant to natural hydrolysis but are susceptible to photodegradation (Zhou et al., 2012). They have been found in concentrations as high as 70 mg/L in pig, cattle, and poultry manures (Wohde et al., 2016).

Given the mode of action, fluoroquinolones are toxic for most of the microorganism in AD (including methanogens), thus, they represent a risk to anaerobic digesters. Some of the studies that have evaluated the effect of fluoroquinolones in AD are presented in Table 1.2. Most of the

results agree that concentration as low as 0.5 mg/L can potentially affect the process stability and lead to lower methane yields in AD of livestock manures (Bauer et al., 2014; Guo et al., 2012; Mai et al., 2018; Panseri et al., 2013). A more recent study reported a increase of methane production in high-solids AD of dewatered municipal solids under the presence of > 100 mg/L of different fluoroquinolones (Zhi and Zhang, 2019).

Table 1.1. Antibiotic class, mode of action, and chemical structure

Antibiotic	Mode of action	Structure
Tetracyclines	Protein synthesis binding to ribosomal 30S subunit	
Aminoglycosides	Protein synthesis binding to ribosomal 30S subunit	
Macrolides	Protein synthesis binding ribosomal 50S subunit	
Quinolones	DNA synthesis inhibiting topoisomerases II and IV *	
Sulfonamides	DNA synthesis blocking folic acid production *	
Penicillins	Cell-wall synthesis by binding to the penicillin-binding proteins	
Cephalosporins	Cell-wall synthesis by binding to the penicillin-binding proteins	
Ionophores (Monensin)	Ion gradient disruption *	

* Directly affect methanogens

1.6.3 Sulfonamides

Sulfonamides are a group of broad-spectrum antibiotics that interrupt the DNA replication by inhibiting the dihydropteroate synthase involved in the production of folic acid, which is essential for the synthesis of the nucleotide thymine (Bhattacharjee and Bhattacharjee, 2016). They have been used for more than 50 years in livestock farms to treat different diseases caused mainly by gram-positive bacteria, although some gram-negatives are also sensitive (Baran et al., 2011). Sulfonamides are chemically stable under environmental conditions with half-life values higher than 30 days (Białk-Bielińska et al., 2012). Not surprisingly, they have been found in cattle, swine and poultry manures in concentration in the order of µg/L to mg/L (Spielmeyer, 2018).

Sulfonamides have been shown to be toxic to some archaea, including methanogens, at concentrations as low as 5 mg/L (Khelaifia and Drancourt, 2012). Several studies have measured the effect of sulfonamides in AD (Table 1.2). In general, concentrations in the range of 0.1 – 280 mg/L have been shown as safe for thermophilic and mesophilic anaerobic digesters (Spielmeyer, 2018). Sulfonamides can be partially degraded in AD, although the residual antibiotic keeps its antimicrobial effect (Mohring et al., 2009; Spielmeyer et al., 2014).

1.6.4 Macrolides

Macrolides are secondary metabolites naturally produced in some microorganisms from the actinomycete family and are considered broad-spectrum antibiotics. They consist of a large macrocyclic lactone ring substituted with one or more unusual deoxysugar residues and can be classified as 14 or 16-membered macrolides (Katz and Ashley, 2005). Macrolides disrupt the protein synthesis by binding to the ribosome 50S, thereby preventing cell growth (Hansen et al., 2005; Katz and Ashley, 2005). They are mainly used to treat infections caused by gram-positive pathogens of the genera *Staphylococcus*, *Streptococcus*, *Chlamydia*, *Chlamydia*, and *Mycoplasma*

(Arsic et al., 2018; Katz and Ashley, 2005). Gram-negative bacteria are less sensitive to these compounds because they have some protective mechanisms (Hansen et al., 2005; Katz and Ashley, 2005). Macrolides have half-life values in the range of few days (Zhu et al., 2014) and have been found in manures and municipal sewage in concentrations between 0.01 to 12 mg/L (Wohde et al., 2016). Although methanogens are known to have good tolerance to macrolides (Khelaifia and Drancourt, 2012), some studies have shown both adverse and positive effects on AD (Table 1.2). Similar to sulfonamides, macrolides are partially degraded during AD and the residues might keep their biological effect (Zhu et al., 2014).

1.6.5 Ionophores

Ionophores are biological molecules toxic not only to microorganisms but to higher organisms (Kart and Bilgili, 2008). They have been used as broad-spectrum antibiotics, especially to prevent and treat coccidiosis (Azzaz et al., 2015; Kart and Bilgili, 2008). However, they are mainly used as a feed additive in ruminant feeds. Ionophores alter the ruminal anaerobic fermentation by affecting directly methanogens and that way reducing hydrogen and formate levels, inducing more propionate and less acetate production which leads to a reduction in methanogenesis (Azzaz et al., 2015). Besides, ionophores can improve nitrogen utilization and mitigate morbidity and mortality by reducing the acute and subacute ruminal acidosis (Ranga Niroshan Appuhamy et al., 2013). Ionophores act by disrupting the ion gradient and altering the cation transference across the cell membrane (Azzaz et al., 2015).

Based on the mode of ion transfer, they are classified into two groups: channel formers and ion carriers. Channel formers create a hydrophilic channel structure in the membrane that allows the pass of ions from the outside into the cell, whereas ion carriers bind and cover ions, move them across the lipid bilayer and release them inside the cell (Kart and Bilgili, 2008). Ionophores doses

are in the range of 10-40 mg/kg of dry matter fed (Ranga Niroshan Appuhamy et al., 2013) and it is estimated that about 50% is excreted in active forms, therefore, they can be found in manures at concentrations up to 3 mg/L (Wildenauer et al., 1984).

The effect of ionophores on AD has been poorly studied and only a few articles are available (Table 1.2). Wildenauer et al. (1984) found that 2-5 mg/L monensin, a common ionophore, can inhibit up to 45% methanogenesis. In a more recent study, Arikan et al. (2018) reported that 10 mg/L monensin caused 75% reduction of the cumulative CH_4 production and accumulation of acetate, propionate, butyrate, and valerate during AD of dairy manure. On the other hand, ionophores were partially degraded under anaerobic conditions, with elimination rates between 25% and 78% in the studies carried out by Arikan et al. (2018) and Varel et al. (2012). Nevertheless, more studies are needed to have a better understanding of the fate and impact of ionophores in anaerobic digesters.

1.6.6 Beta-lactams

Beta-lactams are a class of antibiotics with the distinctive β-lactam ring. They interrupt the bacterial cell-wall synthesis by binding to the penicillin-binding proteins (PBP), which are responsible for the peptidoglycan cross-linking (Bush and Bradford, 2016; Ghooi and Thatte, 1995), thus, rapidly multiplying microorganism are more sensitive. Beta-lactams were the first agents used to treat infectious diseases (Ghooi and Thatte, 1995). Currently, they are the most used antibiotic in both humans and animals (Bush and Bradford, 2016; Public Health Agency Canada, 2017) and they have been found in different concentrations in municipal wastewaters and manures (Wohde et al., 2016). Their excessive use has driven the development of more beta-lactam resistant microorganism that are capable of hydrolyzing the distinctive β-lactam ring (Kong et al., 2010).

Depending on the structure and the target, beta-lactams can be classified as penicillins, cephalosporins, carbapenems, and monocyclic beta-lactams.

1.6.6.1 Penicillins

Penicillins have a characteristic 6-aminopenicillanic acid with a particular side chain that determines the pharmacological properties (DrugBank, 2019). They are naturally produced by different microorganisms and were the first antibiotics utilized for clinical application. Nowadays, semisynthetic derivates with wider spectrum are in use. Residual penicillins have been found in manures in lower concentrations (<1 mg/L) than other types of antibiotics since the β-lactam ring is chemically unstable and it is easily hydrolyzed once it is exposed to the environment (Cha and Carlson, 2018).

Since penicillins act by inhibiting the cell wall synthesis, they do not affect Archaea like methanogens, therefore, only a slow down in the AD process should be expected with minimum effect on biogas production. However, the results are ambiguous. Some studies have demonstrated that even very high penicillins concentrations (> 300 mg/L) can be eliminated in anaerobic digestion processes with minimum impact in the biogas production (Table 1.2). Sanz et al. (1996) and Mitchell et al. (2013) reported < 20% cumulative CH_4 reduction in digester treated with 10 and 350 mg/L ampicillin respectively. On the other hand, 60 mg/L ampicillin reduced 25% of the total CH_4 production in a study conducted by Lallai et al. (2002).

1.6.6.2 Cephalosporins

Cephalosporins are broad-spectrum antibiotics belonging to the semi-synthetic beta-lactam group against gram-positive and gram-negative pathogens. They are more stable and resistant to beta-lactamases than other beta-lactams and less allergic reaction have been associated with their use (Ribeiro et al., 2018). Cephalosporins are classified by generation, depending on the spectrum they

cover. The first and second generations have a narrow spectrum of activity, mainly focused on gram-positive bacteria. Third generation cephalosporins have a higher spectrum and are more effective against gram-negative bacteria. They are less sensitive to beta-lactamase and can be used to treat penicillin-resistant bacteria. Ceftiofur, the antibiotic used in this study, belongs to this category. More details about ceftiofur is provided in sections 1.8. Fourth and fifth generations have the broadest applications against gram-positive and gram-negative bacteria, and they have greater resistance to beta-lactamases (Devansh and Kumar, 2015). The first 2 generations have been used in human and veterinary medicine. However, the last generations have been exclusively utilized for animal diseases. They are the preferred treatment for respiratory and intra-mammary infections in cattle and hog farms (Ribeiro et al., 2018). Similarly to penicillins, cephalosporins are easily degraded under environmental conditions, with an average half-life of less than a week (Kitazono et al., 2015; Ribeiro et al., 2018). Nevertheless, they have been found in municipal wastewater and manures in concentrations as high as 30 mg/L (Ribeiro et al., 2018) and 10 mg/L (Sim et al., 2011; Zhou et al., 2012), respectively.

The effect of cephalosporins on anaerobic digestion has been poorly evaluated compared to the rest of antibiotics. Only a few studies are available, and the results are quite inconsistent (Table 1.2). While some studies reported negligible impact at concentrations up to 90 mg/L in mesophilic and thermophilic AD of dairy manure (Beneragama et al., 2016, 2013; Kitazono et al., 2015), a separate study found more than 40% inhibition with 50 mg/L but an increase in methane production at concentrations as high as 1,000 mg/L in AD of waste activated sludges (Lu et al., 2014). Despite the variation of the results, cephalosporins degradation have been greater than 62% in all these studies.

Table 1.2 Summary of the effect of antibiotics on anaerobic digestion.

Antibiotic	Concentration	Feed	AD conditions	Cumulative CH_4 reduction	Antibiotic removal	VFAs accumulation	Source
Tetracyclines							
Chlortetracycline	500 mg/kg (dw)	Swine manure	Batch; 37°C; 57 days	+ 21.5%	NR	No accumulation	Wang et al. (2018)
	10, 50 mg/L	Dairy manure	Batch; 37°C; 20 days	< 10%	> 85%	NR	Kitazono et al. (2015)
	10, 50, 100 mg/L	Swine manure	Batch; 35°C; 21 days	45%, 57%, 64%	95%	NR	Álvarez et al. (2010)
Oxytetracycline	10, 100, 500 mg/L	Dewatered municipal sludge	Batch; 37°C; 25 days	7%, +33%, +18%	NR	Accumulation	Zhi and Zhang (2019)
	20, 50, 80 mg/L	Cattle manure	Batch; 37°C; 50 days	44%, 65%, 78%	NR	NR	Ke et al. (2014b)
	30, 60, 90 mg/L	Dairy manure	Batch; 55°C; 16 days	21%, 30%, 31%	NR	No accumulation	Beneragama et al. (2013)
	10, 50, 100 mg/L	Swine manure	Batch; 35°C; 21 days	45%, 57%, 64%	95%	NR	Álvarez et al. (2010)
	125, 250 mg/L	Swine manure	Batch; 37°C; 11 days	< 5%	NR	NR	Lallai et al. (2002)
Penicillins							
Ampicillin	350 mg/L	Cattle manure	Batch; 37°C; 40 days	< 10%	100%	NR	Mitchell et al. (2013)
	10 mg/L	Synthetic feed	Batch; 30°C; 15 days	20%	100%	NR	Sanz et al. (1996)
Penicillin	10 mg/L	Synthetic feed	Batch; 30°C; 15 days	20%	100%	NR	Sanz et al. (1996)
Amoxicillin	60, 120 mg/L	Swine manure	Batch; 37°C; 11 days	25%, 32%	NR	NR	Lallai et al. (2002)
Cephalosporins							
Cefazolin	10 mg/L	Dairy manure	Batch; 37°C; 22 days	<5%	NR	No accumulation	Beneragama et al. (2016)
	10, 50 mg/L	Dairy manure	Batch; 37°C; 20 days	< 10%	> 99%	NR	Kitazono et al. (2015)
	30, 60, 90 mg/L	Dairy manure	Batch; 55°C; 16 days	+ 2%, + 3%, + 9%	NR	No accumulation	Beneragama et al. (2013)
Cefalexin	50, 200, 400 mg/L	Waste activated sludge	Batch; 35°C; 157 days	43%, 0%, 70%	> 62%	Accumulation	Lu et al. (2014)
	600, 1000, 2000 mg/L	Waste activated sludge	Batch; 35 °C; 157 days	+30%, +63.8%, 10%	> 84%	No Accumulation	Lu et al. (2014)
Ceftiofur	10 mg/L	Cattle manure	Semi-continuous; 37°C; 50 days	15%	77%	NR	Howes (2017)
	0.2, 0.5, 1, 5, 10 mg/L	Synthetic feed	Batch; 35 °C; 21 days	< 10%	> 90%	NR	Rodríguez et al. (2017)
	1.7, 6.9, 13.8 mg/L	Swine manure	Batch; 37 °C; 60 days	< 5%	74%	NR	Panseri et al. (2013)
Aminoglycosides							
kanamycin	15 mg/L	Waste activate sludge	Batch; 37 °C; 10 days	< 5%	NR	No Accumulation	Mustapha et al. (2016)
	100 mg/L	Synthetic feed	Batch; 30 °C; 15 days	20%	NR	No Accumulation	Sanz et al. (1996)
Neomycin	20 mg/L	Synthetic feed	Batch; 30 °C; 15 days	20%	NR	Accumulation	Sanz et al. (1996)
Streptomycin	18 mg/L	Synthetic feed	Batch; 30 °C; 15 days	20%	NR	Accumulation	Sanz et al. (1996)

NR: not reported; "+" indicates increased in CH_4 production; When more than one value is presented in the *CH_4 reduction* column, it corresponds to the antibiotic *concentration* in the same position.

Antibiotic	Concentration	Feed	AD conditions	CH4 reduction	Antibiotic removal	VFA's accumulation	Source
Fluoroquinolones							
Ciprofloxacin	10, 100, 500 mg/L	Dewatered municipal sludge	Batch; 37 °C; 25 days	4%, +27%, +5%	NR	Accumulation	Zhi and Zhang (2019)
	0.5, 2.5, 5, 50 mg/L	Synthetic feed	Batch; 35 °C; 45 days	8%, 13%, 15%, 33%	> 86%	Accumulation	Mai et al. (2018)
Danofloxacin	1.1, 4.3, 8.5 mg/L	Swine manure	Batch; 37 °C; 60 days	10%, 16%, 17%	< 34%	NR	Panseri et al. (2013)
Difloxacin	0.39 mg/L	Swine manure	Semi-continuous; 38 °C; 67 days	9%	NR	NR	Guo et al. (2012)
Enrofloxacin	10, 100, 500 mg/L	Dewatered municipal sludge	Batch; 37 °C; 25 days	< 5%, +39%, 59%	NR	Accumulation	Zhi and Zhang (2019)
	1.3, 13, 130, 260 mg/L	Swine manure	Semi-continuous; 37.5 °C; 99 days	25%, 25%, 50%, 50%	NR	NR	Bauer et al. (2014)
	2.5, 3.75, 5 mg/L	Swine manure	Semi-continuous; 37.5 °C; 25 days	38%, 46%, 46%	NR	NR	Bauer et al. (2014)
Ofloxacin	10, 100, 500 mg/L	Dewatered municipal sludge	Batch; 37 °C; 25 days	+12%, +60%, 89%	NR	Accumulation	Zhi and Zhang (2019)
Norfloxacin	10, 100, 500 mg/L	Dewatered municipal sludge	Batch; 37 °C; 25 days	9%, +15%, +50%	NR	Accumulation	Zhi and Zhang (2019)
Sulfonamides							
Sulfadimethoxine	10, 100, 500 mg/L	Dewatered municipal sludge	Batch; 37 °C; 25 days	15%, +37%, 85%	NR	Accumulation	Zhi and Zhang (2019)
	25, 50 mg/L	Swine manure	Batch; 25 °C; 20 days	< 35%	100%	NR	Shi et al. (2011)
Sulfamethoxazole	10, 100, 500 mg/L	Dewatered municipal sludge	Batch; 37 °C; 25 days	13%, +18%, +136%	NR	No Accumulation	Zhi and Zhang (2019)
Sulfamethazine	38 mg/L	Cattle manure	Semi-continuous; 41 °C; 63 days	< 5%	48%	NR	Spielmeyer et al. (2014)
	0.28-280 mg/L	Dairy manure	Batch; 37 °C; 40 days	< 5%	0%	NR	Mitchell et al. (2013)
Sulfadiazine	1 mg/L	Swine manure	Batch; 52 °C; 90 days	+ 2%	0%	NR	Feng et al. (2017)
	1,940 mg/L	Swine manure	Semi-continuous; 38 °C; 67 days	10%	NR	NR	Guo et al. (2012)
	38 mg/L	Cattle manure	Semi-continuous; 41 °C; 63 days	< 5%	< 34%	NR	Spielmeyer et al. (2014)
Sulfamethizole	0.5 mg/L	Swine manure	Batch; 52 °C; 40 days	+ 2%	0%	NR	Feng et al. (2017)
Macrolides							
Lincomycin + Spectinomycin	14, 56, 112 mg/L	Swine manure	Batch; 37 °C; 60 days	18%, 22%, 23%	< 34%	NR	Panseri et al. (2013)
Spectinomycin	20 mg/L	Synthetic feed	Batch; 30°C; 15 days	20%	NR	No Accumulation	Sanz et al. (1996)
Azithromycin	15 mg/L	Waste activated sludge	Batch; 37 °C; 10 days	+ 50%	NR	Accumulation	Mustapha et al. (2016)
Erythromycin	1 mg/L	Swine manure	Batch; 52 °C; 90 days	+ 2%	> 99%	NR	Feng et al. (2017)
	1mg/L	Swine manure	Batch; 15 °C; 40 days	NR	20%	NR	Feng et al. (2017)
	250 mg/L	Synthetic feed	Batch; 30°C; 15 days	0%	NR	No Accumulation	Sanz et al. (1996)
Clarithromycin	1 mg/L	Swine manure	Batch; 52 °C; 90 days	+ 2%	< 36%	NR	Feng et al. (2017)
	1 mg/L	Swine manure	Batch; 15 °C; 40 days	NR	33%	NR	Feng et al. (2017)
Tylosin	130 and 913 mg/L	Cattle manure	Batch; 37 °C; 40 days	10, 38%	100%	NR	Mitchell et al. (2013)
	25 and 250 mg/L	Synthetic feed	Batch; 30°C; 15 days	35%, 45%	NR	Accumulation	Sanz et al. (1996)
Ionophores							
Monensin	1 and 10 mg/L	Dairy manure	Semi-continuous; 30 °C; 50 days	12.5%, 75%	70%	Accumulation	Arikan et al. (2018)
	1 mg/L	Dairy manure	Batch; 35 °C; 28 days	50%	NR	NR	Zitomer et al. (2013)
	2 and 5 mg/L	Cattle manure	Semi-continuous; 35 °C; 10 days	45%	NR	Accumulation	Wildenauer et al. (1984)

1.7 Anaerobic digestion and antibiotic resistance

1.7.1 Antibiotic resistance

Although the first antibiotic resistance mechanism was observed more than 70 years ago (Franco et al., 2009), it was not until recently when antibiotic resistance has emerged as a global threat to human health (WHO, 2018). The development of antibiotic resistant bacteria (ARB) is a consequence of the evolutionary response to the selective pressure of great amounts of antibiotics in the environment (Franco et al., 2009). Bacteria have the remarkable ability to adapt to the presence of toxic compounds (Kaufman, 2013). When first exposed to a new antibiotic, only very few bacteria will survive thanks to random mutations that provide them with the required mechanisms to mitigate the antibiotic effect. When some generations are exposed to the antibiotic, these resistance mechanisms will spread into the offspring generating a population resistant to that particular antibiotic (Franco et al., 2009; Kaufman, 2013). The antibiotic-resistance mechanisms are encoded in the so-called antibiotic resistance genes (ARG) usually contained in mobile genetic elements (MGE), which can be interchanged between bacterial species. Hence, bacteria that have not been exposed to the antibiotic can potentially develop resistance (Kaufman, 2013). The transference of ARG to the next generation is known as vertical transference while the transference within species is known as horizontal transference (Kaufman, 2013).

Antibiotic resistance can be developed by 4 different mechanisms:

- *Degradation or inactivation of the antibiotic*. A new enzyme capable of hydrolyzing or inactivating the antibiotic is produced. This mechanism is commonly found in beta-lactams resistance by the productions of beta-lactamase enzymes such as penicillinase and cephalosporinases (Franco et al., 2009; Howes, 2017; Kaufman, 2013);

- *Antibiotic's target modification*. Changes in the target molecule can prevent the recognition of the antibiotic. For example, alterations to penicillin-binding protein (PBPs) avoid the binding of beta-lactams antibiotics (Kaufman, 2013);
- *Efflux*. This mechanism involves protein structures that pump antimicrobial agents out of the bacterial cells, preventing the antibiotic from reaching the minimum intracellular concentration to exert its effect (Kaufman, 2013). This is a common mechanism that bacteria use to resist different toxic compounds such as antibiotics, heavy metals, and toxins and play an important role in producing multidrug resistance (Howes, 2017; Nikaido, 2009). The ARG *mefA* encodes this mechanism and is often found in macrolide-resistant bacteria (Hansen et al., 2005).

1.7.2 Multidrug resistance

Antibiotic resistance genes (ARG) tend to group and accumulate in MGEs that can be transferred vertically or horizontally. This has led to the generation of multidrug-resistant bacteria capable of surviving under the presence of antibiotics that they have never been exposed to. Besides, the selective pressure exerted by a specific antibiotic can lead to the preservation and dissemination of very different ARGs or the overexpression of efflux pump proteins (Cantón and Ruiz-Garbajosa, 2011).

1.7.3 Fate of ARG in anaerobic digestion

The transmission of antibiotic resistance is most likely to happen in environments highly populated by different bacteria under stress caused by biochemical compounds (Karkman et al., 2018). Wastewater treatment plants have been suggested as the perfect hotspot for horizontal gene transfer since different bacterial populations from diverse backgrounds meet and interact under the influence of residual antibiotics (Karkman et al., 2018). Very low antibiotic concentrations exert

selective pressure for ARB and drive the dissemination of ARGs in these type of environments (Gullberg et al., 2011). The different systems used to treat livestock manures, such aerobic and anaerobic digesters, anaerobic lagoons, or even the pits used to collect the manures have similar conditions and can act as a reservoir of ARGs (Pei et al., 2007; Resende et al., 2014). Since AD is considered one of the best alternatives to the treatment of these type of biowastes given the possibility of recovering energy, improving nutrient availability and potentially reducing pathogens, there is an increasing interest in how AD affects the fate of ARGs. In one of the first studies on this subject, Ghosh et al. (2009) evaluated the profile of three different tetracycline resistance genes, *tet(X)*, *tet(O)*, and *tet(A)*, during AD of sewage sludge, finding a significant reduction in *tet(X)*, but no significant changes in *tet(O)* and *tet(A)*. They suggested thermophilic AD as a better alternative to reducing levels of these ARGs. Ma et al. (2011) studied the response of nine ARGs encoding resistance to sulfonamide [*sulI*, *sulII*], erythromycin [*erm(B)*, *erm(F)*], and tetracycline [*tet(O)*, *tet(W)*, *tet(C)*, *tet(G)*, *tet(X)*] to various AD conditions. While the sulfonamide ARGs and the *tet(C)*, *tet(G)*, *tet(X)* decreased after 10 and 20 days of mesophilic AD, the erythromycin ARGs and *tet(W)* increased. They also reported better ARG reduction under thermophilic AD. Resende et al. (2014) reported a decline in levels of *ermB*, *aphA2*, and bla_{TEM-1}, ARGs encoding macrolides, aminoglycosides, and beta-lactams resistance, respectively, in AD of cattle manure, although they were persistent during the process. Similarly, Zhang et al. (2015) found different degrees of reduction of some ARGs and a rise of some others.

In summary, there is not a general agreement about the fate of ARGs during AD and it seems to greatly depend on the AD conditions (temperature, HRT, etc.), feed utilized, and the type of ARG. In most of these studies, the presence of residual antibiotic in the feed was not considered, which can put some selective pressure and induce the occurrence of ARGs. More studies are required to

clarify what happens to ARGs during AD and assure that the sludges generated do not bring more ARGs to the environment.

1.8 Ceftiofur in veterinary medicine and its effect on anaerobic digestion

1.8.1 Properties and use of ceftiofur

Ceftiofur (CEF) is a third-generation cephalosporin belonging to the semisynthetic beta-lactams antibiotic class with broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria including some β-lactamase-producing strains (Pubchem Database, 2019). Structurally, it is similar to other beta-lactams/cephalosporins with the characteristic β-lactam ring, but with an oxyimino-aminothiazolyl group attached to the 7-amino cephalosporin nucleus instead of an amino-acyl at position 7-beta (Figure 1.2) (Hornish and Kotarski, 2002; Livermore, 2008). Ceftiofur is exclusively used in veterinary medicine to treat and prevent several infections in cattle, swine, and poultry (Wang et al., 2018; Wittum, 2012). The use of cephalosporins, particularly CEF, to treat mastitis is an extended practice in dairy farms. Mastitis is an inflammation of the utter caused by physical trauma or bacterial infections, that affects a great percentage of dairy cows (10-40%) (Boujenane et al., 2015; Heringstad et al., 2000). Mastitis reduces the milk production and the microorganisms responsible for the infection can change the composition of the milk affecting its quality and causing important economic losses (Heringstad et al., 2000). Ceftiofur is generally administrated via subcutaneous or intramuscular injections and is quickly metabolized to desfuroylceftiofur (DFC) or desfuroylceftiofur dimer (DFC-dimer) which contain the beta-lactam ring and therefore the bactericidal activity (Jaglan et al., 1989; Wang et al., 2018). Similar to other antibiotics, more than 50% of the CEF dose is excreted in urine and feces in biologically active forms, although given their chemical structure, they are expected to manure degrade rapidly in the environment through photolysis, hydrolysis or biological degradation (Ribeiro et al., 2018).

Since it is administrated in doses ranging from a few milligrams per kilogram of body weight (Wang et al., 2018), residual CEF and its metabolites can be found in in concentrations in the order of mg/L.

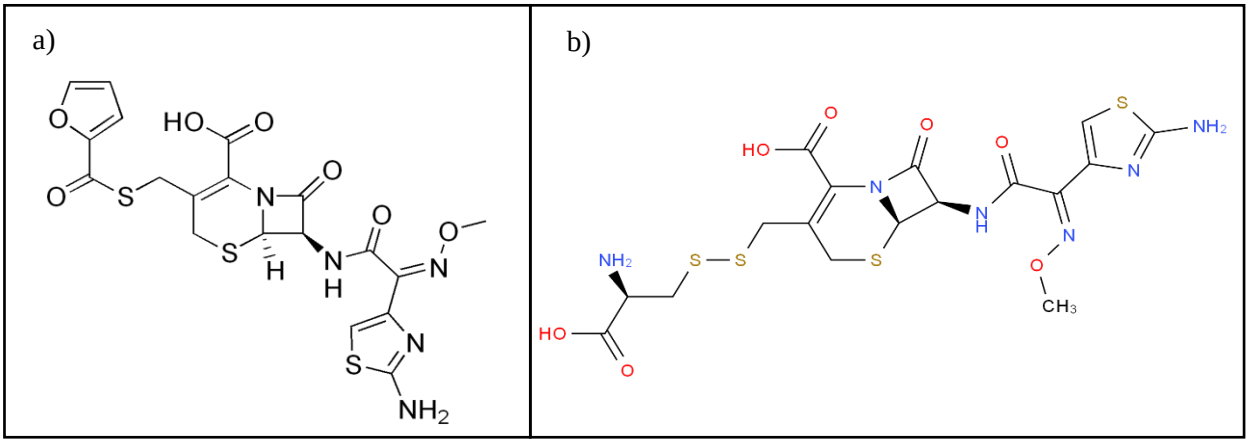


Figure 1.2 Chemical structure of ceftiofur (a) and desfuroylceftiofur (b). (Pubchem Database, 2019)

1.8.2 Cephalosporin resistance

Since beta-lactams were the first antibiotics used to treat infectious diseases many decades ago, the resistance to beta-lactams has been developed and spread all the world, even in microorganisms not exposed to these compounds (Collignon, 2002). The resistance to beta-lactams antibiotics is driven by the production of enzymes known as beta-lactamases capable of hydrolyzing the β-lactam ring and therefore inactivating the antibiotic (Kong et al., 2010). The group of enzymes against cephalosporins are dubbed cephalosporinases and are encoded in genes whose DNA sequences are highly conserved between species (Kong et al., 2010). Nowadays, beta-lactamases that were not originally known to hydrolyze third or fourth generation cephalosporins, like ceftiofur, have developed this ability thanks to random mutations induced by the increasing presence of broad-spectrum beta-lactams (Andersen et al., 2015; Livermore, 2008). The extended-

spectrum cephalosporinase resistance (ESC-R) is emerging as one of the fastest growing forms of resistance and efforts are being made to mitigate its dispersion (Andersen et al., 2015; Public Health Agency Canada, 2017). Some of the typical beta-lactamases with cephalosporinase activity are derivates of enzyme families such as CMY, CTX-M, TEM, SHV, and AmpC (Andersen et al., 2015; Livermore, 2008).

1.8.3 Effect of ceftiofur on anaerobic digestion

Although residual CEF in manures is expected, since it is used to treat one of the most frequent diseases in dairy cattle, its effect on anaerobic digestion has been poorly studied. Panseri et al. (2013) studied how different CEF concentrations (1.7, 6.9, and 13.8 mg/L) affect the final cumulative biogas production of mesophilic (37 °C) AD of swine manure in small batch anaerobic digesters over a period of 60 days. They reported less than 5% reduction in all the digesters treated with CEF, and no differences between the different CEF concentrations were observed. In a similar study, but using synthetic feed, Rodríguez et al. (2017) found 10% CH_4 inhibition with up to 10 mg CEF/L. Unfortunately, neither the changes on the VFAs profile nor the differences on CH_4 during the early stages of AD were evaluated in these studies and very little can be inferred from these results. Besides, in both studies, increasing concentrations of CEF did not cause greater inhibition, so a dose-response relationship could not be established. Howes (2017) studied the effect of 10 mg/L residual ceftiofur on semicontinuous lab-scale anaerobic digesters fed with dairy manure over a period of 50 days under thermophilic conditions (55 °C). During the first 10 days, a slight improvement of the biogas production was observed in the reactors under the influence of CEF but after a few days, this positive effect disappeared. Later on, biogas production rate was inhibited around 15% and tended to increase over time. The organic matter removal was not affected by the presence of CEF in this study and the VFAs profile was not evaluated. In these 3

papers, more than 70% of ceftiofur degradation was reported, although some active metabolites were found in the digestate.

Information about the effects of other cephalosporins on AD is also limited to a few studies (Table 1.2). Cefazolin, a first-generation cephalosporin, did not significantly affect thermophilic or mesophilic AD of dairy manure at concentrations as high as 90 mg/L (Beneragama et al., 2016, 2013; Kitazono et al., 2015). On the other hand, cefalexin, another first-generation cephalosporin, had a varied effect on mesophilic AD of waste activated sludge (Table 1.2). While 600 and 1,000 mg/L caused an increase in methane production, 50 mg/L cefalexin caused almost 40% reduction of this parameter (Lu et al., 2014).

All these results suggest that cephalosporins, like CEF, at concentrations close to levels expected in the environment (1-10 mg/L) would have a minimum impact on the performance of anaerobic digesters and they will be partially degraded in the process, although a significant antibiotic dose will remain active. Nevertheless, the results are not conclusive and are deficient regarding the acute effect these type of antibiotics have during different AD stages.

The effect of cephalosporins on the fate of ARGs in anaerobic digesters is uncertain and very limited information is available. Only recently, Howes (2017) reported higher levels of *cfx(A)*, *mef(A)*, and *tet(Q)*, beta-lactam, macrolide and tetracycline resistance markers, respectively, in semi-continuously fed anaerobic digesters with cattle manure contaminated with ceftiofur compared to non-contaminated manure digesters.

1.9 Summary and Research Objectives

The ability of harvesting energy from carbon-rich biowastes makes anaerobic digestions a great alternative to treat livestock manures. However, its advantages do not end there. Anaerobic digestion has been successfully used to reduce pathogens, mitigate GHG related to livestock management, diminish odors, and concentrate nutrients that later can be utilized as crop fertilizers. Recently, AD has been also suggested as a good option to degrade residual antibiotics and potentially reduce ARGs, preventing them to reach the environment. Nonetheless, there is a constant concern about the effect of these residual antibiotics on the performance of anaerobic digesters since the information available is ambiguous and limited only to the difference on the cumulative biogas production after long digestion times rather than providing details about the biochemical mechanisms during digestion. In that context, the general objectives of this research are:

- To set up and operate a steady-state semi-continuous mesophilic (35°C) lab-scale anaerobic digester fed with dairy manure;
- To evaluate the effect of ceftiofur, one of the most commonly used antibiotics in veterinary medicine, on the performance and stability of mesophilic anaerobic digestion of dairy manure, in terms of biogas production, biochemical intermediates (VFAs), and organic matter removal at different time-points of the process;
- To determine the fate of the cephalosporinase gene *cmy-2* during mesophilic anaerobic digestion in the semi-continuous lab-scale anaerobic digester operating in a steady-state; and
- To evaluate the effect of ceftiofur on levels of the cephalosporinase gen *cmy-2* during mesophilic anaerobic digestion of manure originated from an operational dairy farm.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Bench-scale anaerobic digester set up and operation

A commercial 12 L glass, single-wall, round-bottom bioreactor (New Brunswick Scientific BioFlo 110), from now on referred as bench-scale reactor (BSR), was used in this part of the study. The bioreactor accessories included a gas sampling port, feeding port, stirring apparatus, three baffles, a temperature probe, and a heating jacket. The temperature and agitation rate were controlled with the bioreactor control user interface version 1.20, configuration 1.13. The gas sampling port was connected through a 4.8 mm internal diameter (ID) silicone tubing to a Wet Tip Meter and a liquid trap line to capture condensation. A 1.55 mm ID tube was also adapted and attached to the gas line for biogas sampling. The BSR set-up and components are shown in Figure 2.1.

The BSR was initially inoculated with 4 L of anaerobic sludge and 4 L of cattle manure. The anaerobic sludge was obtained from a mesophilic (30-35 °C) anaerobic digester used to treat biosolids at the municipal wastewater treatment plant (North End Water Pollution Control Centre) in Winnipeg, Manitoba. The manure was collected from the manure pit of a dairy farm (Sweetridge farm) located in Winkler, Manitoba. The BSR was operated with a working volume of 8 L, at mesophilic conditions (35 °C), and fed approximately 623 mL dairy manure 3 days per week to maintain a 30-day HRT. The BSR was continuously stirred at 120 rpm, except just before biogas sampling and during feeding, when stirring was increased to 450 rpm to re-suspend particulate and floating material.

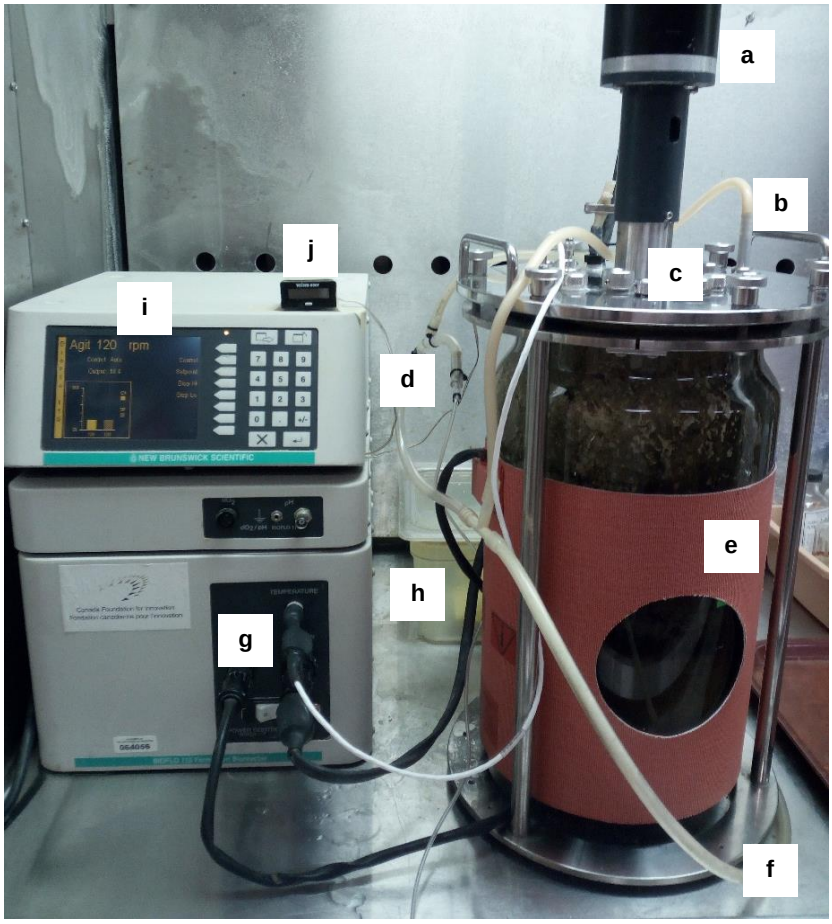


Figure 2.1 Bench-scale reactor set-up

a: Stirring apparatus; **b**: Biogas port; **c**: Feeding port; **d**: Biogas sampling line; **e**: Heater jacket; **f**: Condensation trap; **g**: Temperature probe; **h**: Wet tip meter; **i**: Control user interface; **j**: Tip counter

2.2 Manure and BSR sampling

2.2.1 Manure sampling

Manure was collected periodically from the Sweetridge Farm (~3 months), strained through a ¼" mesh bucket filter to remove large particles (Figure 2.2a), and then individual feeding doses (623 mL) were stored at -20 °C in 800 mL plastic bottles separately until the day before use (Fig. 2.2b). The last manure batch (M9) used in this study was collected from another farm located in Rosser, Manitoba. Each new batch of manure obtained from the farm was labelled successively M1 to M9 and analyzed for total and volatile solids (g/L), total nitrogen (g N/L), total phosphorus (g PO_4^{-3}-

P/L), total and dissolved chemical oxygen demand (g O/L), volatile fatty acids (mg/L), and total alkalinity (g $CaCO_3$/L). Manure samples for DNA isolation were taken and stored in 50 mL tubes at -20 °C. These samples were then used to determine levels of the antibiotic resistance gene (ARG) *cmy-2* through real-time qPCR (details described in Section 2.5.3).

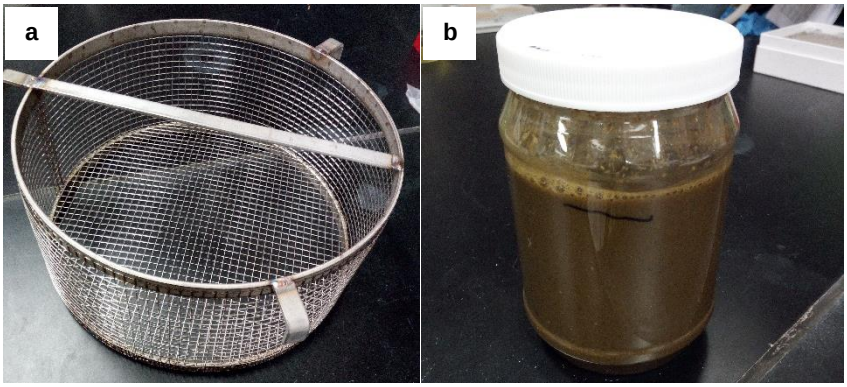


Figure 2.2 Bucket filter (a) and manure dose (b)

2.2.2 BSR sampling

The biogas production was monitored volumetrically with a Wet Tip Meter calibrated to tip every 100 mL of biogas produced. The biogas was normalized to standard conditions (273.15 K and 1 atm) and reported as daily biogas production (L/day). Biogas composition (CH_4, CO_2, H_2, O_2, and N_2) was measured at least 5 days a week. Digestate was analyzed for pH every feeding day and once a week for total (TS) and volatile solids (VS). Monthly digestate samples corresponding to each HRT were analyzed for the same parameters as the manure. Samples for DNA extraction were also taken and stored at -20 °C until further analyses. The DNA was analyzed for the same ARG as the manure.

2.3 Ceftiofur assays: batch anaerobic digestion experiments

2.3.1 Estimation of residual ceftiofur in dairy manure

The residual CEF in dairy manure was predicted following the methodology reported by Panseri et al. (2013) using the following equations:

$$TD = AW\ x\ STD \tag{2.1}$$

$$AAE = \left[TD - \frac{TD}{2K}\right] x\ EF \tag{2.2}$$

Where *TD* represents the total dose of antibiotic administrated (mg), *AW* the animal weight (kg), *STD* the standard therapeutic dose (mg/kg live weight); *AAE* is the total amount of antibiotic excreted (mg), *K* is the ratio between the CEF half-life and period of manure collection, and EF is a factor that accounts for the excretion rate of active metabolites. The parameters considered for the estimation of residual CEF are shown in table 2.1. The CEF concentration in the slurry was then calculated as the ratio of the total antibiotic excreted and the amount of manure produced per cattle in 24h:

$$CCM = \frac{AAE}{MPD} \tag{2.3}$$

Where *CCM* is the CEF concentration in manure (mg/kg) and *MPD* is the manure production per day (kg/day). Assuming that 1 kg of manure is roughly 1 L, the expected CEF ranges from approximately 1 – 19.8 mg/L.

Table 2.1 Parameters for the estimation of residual CEF in cattle manure

Parameter	Value	Source
Animal Weight (AW)	400-450 kg	J. Wang et al. (2018)
Standard Therapeutic Dose (STD)	1.1-2.2 mg/kg	J. Wang et al. (2018)
Half-life	8 days	Gilbertson et al. (1990)
K	1	*
Excretion factor (EF)	0.7	Ribeiro et al. (2018)
Manure produce	30 – 35 kg	Fischer (1998)

*Assuming manure is used the same day than produced

2.3.2 Biochemical Methane Potential (BMP) assays

A biochemical methane potential (BMP) assay is an established method to determine the ultimate methane yield of a feed source under specific anaerobic conditions (Moody et al., 2011). BMP assays are typically used to evaluate methane potential of agricultural residues as co-substrate in anaerobic digestion (Jordaan and Çiçek, 2014; Labatut et al., 2011; Moody et al., 2011). However, they have also been used to assess the impact of anaerobic digestion inhibitors (Lu et al., 2014; Mai et al., 2018), since BMPs provide information about organic matter removal and kinetic parameters such as cumulative biogas/methane production and their respective production rates, which can be associated with hydrolysis and methanogenesis, respectively. Acidogenesis and acetogenesis can also be assessed by determining the VFAs profile during the digestion.

In this study, the effect of CEF on AD was evaluated in batch anaerobic digesters with a set-up similar to BMP assays following the recommendations of Moody et al. (2011). Three sets of BMPs were performed. In the first one, the manure was amended with increasing CEF concentrations (0.2, 2, 10, 50 mg/L) and the biogas production and biogas quality were monitored over a 32-day digestion period. The VFAs profile, TS, VS, total and dissolved COD and pH were measured at the beginning and end of the experiment. Based on the results from the first assay, a second experiment was designed to more closely monitor the VFA profile, VS, TS, COD and dCOD

removal at different time points of the AD process under the presence of 50 mg CEF/L. Levels of the cephalosporinase gene *cmy-2* were also quantified in this second experiment. The third experiment was designed to corroborate if the presence of high CEF concentrations (250 mg/L) would increase the levels of *cmy-2* in the digesters and also evaluate the effect of very high CEF concentration on the performance of AD. More details about the experiments are provided in the following sections

2.3.3 Experimental setup and anaerobic digestion conditions

For the three experiments, the quantity of manure and inoculum were estimated with the aim of producing at least 1,000 mL CH_4 by considering that 1 g COD consumed produces 395 mL CH_4 (Moody et al., 2011). The initial manure volumes were calculated using the following equations (Moody et al., 2011):

$$M_{Sub\ COD} = V_{CH4}\ x\ \frac{1\ g\ COD}{395\ ml\ CH_4}\ x\ Eff \tag{2.4}$$

$$V_{Sub} = \frac{M_{Sub\ COD}}{[COD]_{Sub}} \tag{2.5}$$

Where $M_{Sub\ COD}$ is the mass of substrate COD (g COD) estimated to produce the desired CH_4 volume (V_{CH4}, mL), *Eff* is the expected COD conversion efficiency for the substrate, V_{Sub} is the substrate volume to be added (mL), and $[COD]_{Sub}$ is the substrate COD concentration (g/L).

The digestate from the BSR, with at least 2 weeks of steady biogas production and CH_4/CO_2 ratios, was used as inoculum in the experiments. The volume of inoculum to be utilized in each set of BMP assays was estimated with the following equation:

$$V_{Inoc} = \frac{V_{Sub}\ x\ [VS]_{Sub}}{[VS]_{Inoc}} \tag{2.6}$$

Where V_{Inoc} is the volume of inoculum to be added (mL), $[VS]_{Sub}$ is the substrate VS concentration (g/L), and $[VS]_{Inoc}$ is the inoculum VS concentration (g/L). A nutrient medium with the recommended minerals for the optimal microbial growth was added to obtain a final working volume of 400 mL. The nutrient media was prepared following Moody et al. (2011) recommendations. Its composition is shown in Table 2.2.

The manures used in the three experiments were M5, M6, and M9, respectively, while the inoculum corresponded to the HRT periods of 12 (360 days), 15 (450 days), and 20 (600 days), respectively. The manures and inoculums properties, as well as the VS mass added in each experiment, are shown in Table 3.3 in the results section.

Table 2.2 Composition of the nutrient medium used in the batch experiments.

Compound	Concentration (mg/L)	Compound	Concentration (mg/L)
$NaHCO_3$	6 000	NH_4VO_3	0.50
NH4Cl	400	$CuCl_2*2H_2O$	0.50
$MgSO_4$	250	$Zn(C_2H_3O_2)_2*2H_2O$	0.50
KCl	400	$AlCl_3*6H_2O$	0.50
$CaCl_2*2H_2O$	137	$NaMoO_4*2H_2O$	0.50
$(NH_4)_2HPO_4$	80	H_3BO_3	0.50
$FeCl_3$	33	$NiCl_2*6H_2O$	0.50
$CoCl_2*6H_2O$	10	$NaWO_4*2H_2O$	0.50
KI	10	Na_2SeO_3	0.50
$MnCl_2*4H_2O$	0.50		

2.3.3.1 Ceftiofur preparation

Excenel ®, a commercial ceftiofur sodium powder (Zoetis, Quebec), was re-suspended in its diluent (9 mg/mL benzyl alcohol) to a concentration of 50 mg/mL following the manufacturer's instructions. In experiments one and two, the ceftiofur stock solution was diluted in distilled water

to a final ceftiofur concentration of 5 mg/mL to increase the volumes and make them easier to handle. A benzyl alcohol solution (9 mg/mL) was prepared and diluted similarly and was added to the bottles to offset the difference in volume and organic load caused by the addition of different volumes of the CEF solution.

2.3.3.2 Experiment 1: Increasing CEF concentrations

The first BMP-like assay was designed to evaluate the effect of increasing CEF concentrations on biogas productions and biogas composition over time. The manure, inoculum, and nutrient media were estimated following the procedure stated in the previous section. Briefly, 800 mL glass-bottles (a total of 15) were filled with 114 (±0.5) mL inoculum (HRT 12), 62 (±0.5) mL cattle manure (M5), and 224 (±0.5) mL nutrient media for a final volume of 400 mL. These bottles were supplemented with 0, 16, 160, 800 and 4,000 µL of the 5 mg CEF/mL solution to generate the 0 (Control), 0.2, 2, 10, and 50 mg/L treatments, respectively. Then, the benzyl alcohol solution required to compensate for the difference in volume was added to the different treatment and control bottles. The different treatments were tested in triplicates (three bottles each per treatment and three bottles for controls). The bottles were closed using screw caps with butyl rubber septa and the headspace purged with N_2 for 5 min. Then, they were placed on a stirring base (200 rpm) in a water bath at 35 °C and connected to an automated flow-cell system (Challenge AER-200 Respirometer) to monitor biogas production (Figure 2.3). Headspace samples were taken from each bottle for biogas composition analysis on days 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, and 32. The cumulative and daily biogas, CH_4, CO_2, and H_2 were estimated and normalized per gram of VS added. The average (n= 3) of the cumulative and daily biogas, methane, carbon dioxide, and hydrogen production were reported. The pH, TS, VS, COD, dissolve COD (dCOD), and VFAs

profile were determined at the beginning (day 0) and at the end of the experiment (Day 32) and were reported as the average (n= 3) of each treatment.

2.3.3.3 Experiment 2: VFAs and *cmy-2* profile

The second digestion was carried to analyze changes in the VFA profile and levels of *cmy-2* along the AD process caused by 50 mg CEF/L. Sixteen 800-mL glass bottles were filled with 116 (±0.5) mL inoculum (HRT 15), 114 (±0.5) mL cattle manure (M6), and 170 (±0.5) mL nutrient medium for a final volume of 400 mL. Eight bottles were supplemented with 4 mL of the 5 mg/mL CEF solution to achieve a 50 mg/L concentration. Eight control bottles were supplemented with 4 mL benzyl alcohol solution. The bottles were then incubated and monitored as previously described in *experiment 1*. Based on the results from the first experiment, on days 3, 5, 10 (exponential phase) and 15 (stationary phase), two CEF-treated bottles and two controls bottles were sacrificed to measure TS, VS, pH, VFAs, COD, and dCOD. Each analysis was done in duplicate, which resulted in a total of four measurements per treatment and control for each sampling day. DNA samples from treatment and control bottles were also taken and stored at -20 °C. The DNA isolated was used to measure and compare *cmy-2* levels during the AD process under the influence of CEF. Headspace samples were analyzed for biogas composition (CH_4, CO_2, and H_2) on days 1, 2, 3, 4,

Figure 2.3 BMP assay setup in the water bath and respirometer

5, 6, 7, 8, 9, 10, and 15. The cumulative and daily biogas, CH_4, CO_2, and H_2 were estimated and normalized per gram of VS added.

2.3.3.4 Experiment 3: *cmy-2* profile under high CEF concentration

The third BMP assay was designed to evaluate how the presence of high CEF concentrations (250 mg/L) affect the incidence of *cmy-2* over the AD process. It was carried out similarly to the second experiment. Sixteen 800-mL glass bottles were filled with 106 (±0.5) mL inoculum (HRT 20), 78 (±0.5) mL dairy manure (M9), and 216 (±0.5) mL nutrient medium for a final volume of 400 mL. Eight bottles were supplemented with 2 mL of the CEF stock solution (50 mg/mL) to achieve a 250 mg/L concentration. Eight control bottles were supplemented with 2 mL benzyl alcohol solution (9 mg/mL) similar to the CEF diluent. The bottles were then incubated and monitored as previously described in *experiment 1*. The *cmy-2* levels were measured on days 3, 5, 10, and 15 to match the ones from *experiment 2*, sacrificing two CEF-treatment and two control bottles each time. The TS, VS, pH, VFAs, COD and dCOD profiles were also determined. Each analysis was carried out in duplicates per bottle, with a final $n = 4$ in each sampling day. Headspace samples were analyzed for biogas composition (CH_4, CO_2, and H_2) on days 1, 2, 3, 4, 5 ,6, 7, 8, 9, 10, and 15. The cumulative and daily biogas, CH_4, CO_2, and H_2 were estimated and normalized per gram of VS added.

2.3.4 Methane production modeling and CEF IC_{25} estimation

Different mathematical models can be used to describe the biogas/methane production curves in batch anaerobic digestion and estimate the ultimate theoretical production yields and the maximum rates. In this study, the Reaction Curve-Type (RC) model (Eq. 2.7) was used to fit the cumulative methane production (mL/g VS) of the first experiment using the non-linear regression (*nlstools*) package of the software R.

$$M(t) = P\ \left(1 - \exp\left(-\frac{Rm(t-\lambda)}{P}\right)\right) \quad (2.7)$$

Where *M(t)* represents the cumulative CH_4 production (mL/g VS) at time t (d); *P* the ultimate CH_4 yield (mL/g VS); R_m the maximum CH_4 production rate (mL/d-g VS) and λ the lag phase (d).

The maximum CH_4 production rates (*Rm*) of the treatments in experiment 1 were fitted to a 4 - parameter model using the n- parameter logistic regression (*nplr*) package of the software R. Then, the 20% inhibition concentration (IC_{20}) was determined from the equation obtained using the same R package.

2.4 Analytical methods

2.4.1 Biogas analysis

Methane, CO_2, H_2, O_2 and N_2 concentrations were determined by a gas chromatograph Micro GC 490 (Agilent, USA) equipped with two thermal conductivity detectors (TCD) and two different columns. Methane, H_2, O_2 and N_2 were measured using a Molsieve/5A Plot column with Ar as carrier gas. An HP-PLOT U column was used to measure the CO_2 content using He as carrier gas. The chromatograph was calibrated every sampling day using a 50% CH_4 - 50% CO_2 gas mixture (Praxair, Canada) and air (78% N_2, 22% O_2) and occasionally with natural gas (98% CH_4) and different N_2, CO_2, and H_2 gas standards (Praxair, Canada).

2.4.2 Physicochemical analyses

The pH was measured with a pH 5 Acorn potentiometer (Oaklon, Canada) which was calibrated daily with a pH 7 buffer (Thermo Scientific, USA). Total alkalinity, TS and VS were determined following the Standard Methods 2320B, 2540B, and 2540C, respectively (APHA, 1995). Total and dissolved COD were measured with a closed reflux colorimetric method using HACH TNT 822 vials (Method 8000). The dissolved COD (dCOD) samples were filtered through 0.45 µm

syringe filters (Wyvern Scientific, Canada) before the analysis. Total phosphorous (TP) and total nitrogen (TN) were determined using HACH TNT 844 and TNT 827, respectively. The VFA samples were centrifuged at 3,000 g and the supernatant filtered through 0.2 µm nylon membrane syringe filters (Wyvern Scientific, Canada) and then stored at -20 °C until analysis. The VFA profile entailing formate, acetate, propionate, iso-butyrate, butyrate, iso-valerate and valerate, was determined by high-performance liquid chromatography 1515 (Waters, USA) with a refractive index detector 2414 (Waters, USA). A resin-based column (AMINEX®, HPX-87H) was used for separation using 0.005 M H_2SO_4 as mobile phase. A 10mM VFAs standard solution (Sigma-Aldrich, Canada) was used to generate a 6 point-standard curve for each VFA and then it was used to estimate VFA concentrations.

2.5 Antibiotic resistance genes quantification

2.5.1 DNA isolation

Samples from the different manures, digestate, and the last two CEF assays were centrifuged at maximum speed and the pellets were washed two times with DI water. The DNA was isolated from 350 mg centrifuged pellets using the PowerSoil DNA extraction kit (Omega Bio-tek, USA) following the procedure recommended by the manufacturer. The DNA concentration was determined using a NanoDrop 1000 (Thermo Fisher Scientific, Canada), then the DNA samples were diluted to a final concentration of 20 ng/µL and stored at -20 °C until further analyses.

2.5.2 Primers design

The primers for *cmy-2* used in this study (Table 2.3) were designed by the Dr. Ayush Kumar's team in the Antimicrobial Resistance Laboratory of the Department of Microbiology and Medical Microbiology at University of Manitoba following the methodology described by Fernando et al. (2016).

Table 2.3 Characteristics of the *cmy-2* primers used in this study

Primer name	Sequence	Target gene	Size (bp)	Reference
cmy2_F2	CAGCCACGTTCAGGAGAAA	*cmy-2*	103	This study
cmy2_R2	CAGCATCTCCCAGCCTAATC			

2.5.3 Real-time qPCR

The different manure batches, the monthly BSR samples, and the samples from experiments two and three were analyzed for *cmy-2*. The quantification of the ARG was carried out using the StepOnePlus real-time PCR system (Life Technologies Inc., Canada) following the method described by Fernando et al. (2016). Briefly, each reaction was prepared with 2.68 µL of the 20 ng/µL DNA samples (53.6 ng) and 6.32 µL of a mix containing the primers (9 µM) and 2× SsoFast EvaGreen Supermix (Bio-Rad, Canada). The PCR program consisted of an initial denaturing step at 95 °C for 2 min, followed by 40 amplification cycles at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 10 s in an Eppendorf Mastercycler Pro (Eppendorf, Hamburg, Germany). Reactions were carried out by triplicate. The positive control used in this study was DNA extracted from *Klebsiella pneumoniae* N09-00080, which contains the *cmy-2* gene. A standard curve was generated calculating the copy number (copy No.) of the target gene per ng of DNA in the bacterial strain using the following equation:

$$copy\ No. = \frac{DNA_{Sam} \times N_A}{GS \times 650} \times 10^{-9} \quad (2.8)$$

Where DNA_{sam} is the sample's DNA concentration (ng/µL), N_A is the Avogadro constant (6.022×10^{23} molecules/mole), *GS* is the bacterial genome size (bp), *650* is the base pair molar mass (g/mole), and 10^{-9} is the conversion factor from grams to nanograms. The genome size of *Klebsiella pneumoniae* was assumed to be 5,438,894 bp (Fernando et al. 2016). A 10-fold dilution

series were used to generate the standard curve, then the copy number for each sample was calculated using the slope of the standard curve.

2.6 Data and statistical analyses

All the graphs, data analyses and statistical tests were carried out using the software R version 3.5.2. The values reported are the average of the replicates (n = 2, 3, or 4) and the standard deviations unless specifically stated otherwise. Statistical significance of the differences between the controls and treatments was determined by t-test with a threshold at $p = 0.05$. Difference within treatments was tested by ANOVA and Tukey's Honest Significant Difference test with a threshold at $p = 0.05$.

CHAPTER 3 - RESULTS

3.1. Inoculum development: BSR operation

3.1.1. Manure properties

Nine different manure batches (M1-M9) were used as feed for the BSR. After sieving, each manure batch was analyzed for the parameters listed in Chapter 2, Section 2.2.1. Table 3.1 shows the average of the properties of all the manures. In general, the TN, TP, and dCOD, as well as the alkalinity of the manure, were similar, with very little variation between the different batches. On the other hand, TS, VS and total COD content varied to a larger extent from batch to batch. Nonetheless, the biogas and methane yield per g of VS added were similar with all the manures (Table 3.2).

Table 3.1 Properties of the manures used to feed the BSR

Parameter	Units	Concentration ± Standard Deviation	No. observations
TS	g/L	50.2 ± 12.0	27
VS	g/L	40.7 ± 11.1	27
COD	g/L	54.8 ± 11.8	27
dCOD	g/L	16.0 ± 2.3	27
TN	g/L	2.0 ± 0.8	18
TP	g/L	1.1 ± 0.2	24
Alkalinity	g/L	8.9 ± 2.1	27
pH	-	7.2 ± 0.3	9
VFA profile			
Formate	mg/L	166 ± 218	26
Acetate	mg/L	3,676 ± 1,075	26
Propionate	mg/L	1,078 ± 588	26
Iso-butyrate	mg/L	30 ± 30	26
Butyrate	mg/L	427 ± 246	26
Iso-valerate	mg/L	79 ± 63	26
Valerate	mg/L	240 ± 370	26
Total	mg/L	5,695 ± 1,736	26

3.1.2. BSR performance

The BSR was operated at mesophilic conditions (35 °C) with an HRT of 30 days for over 600 days. It reached a steady-state after day 300 (HRT 10) with an average daily biogas production of 3.43 (± 1.49) L/d and a CH_4/CO_2 ratio of 2.0 (± 0.4). The organic parameters such as TS, VS and total COD were always lower in the effluent (BSR samples) than the influent (manures). An overview of the BSR performance during the whole operation period is shown in Appendix 1. A summary of the parameters monitored and the VFA profile of the BSR during steady-state operation is shown in Table 3.2. The BSR presented low levels of total VFA under steady-state conditions (< 200 mg/L). The pH was also stable with values around 7.5.

Table 3.2 BSR properties during steady-state operation

Parameter	Units	Average ± SD	No. observations
Biogas production	L/d	3.43 ± 1.5	188
CH_4 production rate	L/d	2.25 ± 1	184
Biogas yield	L/kg VS added	292 ± 108	188
CH_4 yield	L/kg VS added	194 ± 72	184
CH4/CO2 ratio	-	2.0 ± 0.4	185
pH	-	7.5 ± 0.1	97
TS	g/L	37.2 ± 8.0	66
VS	g/L	27.4 ± 7.2	66
COD	g/L	32.6 ± 8.9	66
dCOD	g/L	7.3 ± 3.6	66
TN	g/L	2.5 ± 1.2	66
TP	g/L	1.1 ± 0.1	66
Alkalinity	g/L	11 ± 3.6	60
VFA profile			
Formate	mg/L	17 ± 18	51
Acetate	mg/L	123 ± 134	51
Propionate	mg/L	2.3 ± 6.3	51
Iso-butyrate	mg/L	2.8 ± 5.7	51
Butyrate	mg/L	3.6 ± 6.9	51
Iso-valerate	mg/L	0.2 ± 0.4	51
Valerate	mg/L	0.1 ± 0.2	51
Total	mg/L	149 ± 144	51

3.2. Effect of ceftiofur on anerobic digestion

In each respirometer experiment evaluating the impact of Ceftiofur, a 1:1 manure/inoculum VS ratio was used to initiate the digestion. Therefore, since three different manures and inoculums were used for different experiments, the quantities required were varied. The properties and quantities of the manures and inoculums used in each CEF experiment are presented in Table 3.3. The manure VS mass added in experiments 1, 2, and 3 were 2.87, 3.28, and 3.64 g of VS, respectively. These values were used to normalize the biogas production in each experiment because differences between experiments were expected given their different manure VS content.

Table 3.3 Characteristics of the manures and inoculums used in the BMP-like assays

		Experiment 1		**Experiment 2**		**Experiment 3**	
Parameter	*Units*	*Manure (M5)*	*Inoculum (HRT 12)*	*Manure (M6)*	*Inoculum (HRT 15)*	*Manure (M9)*	*Inoculum (HRT 20)*
Volume	mL	62 ± 0.5	114 ± 0.5	114 ± 0.5	116 ± 0.5	78 ± 0.5	106 ± 0.5
TS	g/L	55.8 ± 0.2	46.0 ± 4.0	38.5 ± 1.1	44.6 ± 2.3	54.1 ± 0.5	56.2 ± 1.0
VS	g/L	46.4 ± 0.2	35.4 ± 3.9	28.8 ± 0.9	25.0 ± 0.4	44.4 ± 0.5	46.7 ± 0.8
COD	g/L	57.4 ± 2.0	39.9 ± 3.3	44.5 ± 5.1	35.9 ± 1.0	65.3 ± 4.0	62.6 ± 3.5
dCOD	g/L	17.6 ± 0.4	7.5 ± 0.3	14.6 ± 0.2	25.5 ± 1.4	19.5 ± 0.2	19.6 ± 1.1
pH		7.4	7.5	7.5	7.5	7.2	7.4
VFA profile							
Formate	mg/L	0	18 ± 5	0	28 ± 12	90 ± 7	30 ± 38
Acetate	mg/L	5,148 ± 146	249 ± 34	5,391 ± 121	183 ± 15	3,605 ± 67	235 ± 19
Propionate	mg/L	1,106 ± 100	25 ± 1	1,058 ± 17	1 ± 1	1,410 ± 12	0
Iso-butyrate	mg/L	73 ± 37	0	0	0	0	0
Butyrate	mg/L	589 ± 59	16 ± 4	488 ± 38	5 ± 4	753	0
Iso-valerate	mg/L	119 ± 24	0	95 ± 23	0	154	0
Valerate	mg/L	85 ± 18	0	85 ± 67	0	0	0
Total	mg/L	7,120 ± 230	307 ± 44	7,118 ± 177	217 ± 32	6015	266 ± 32

3.2.1. Experiment 1: Effect of CEF dosing concentrations on anaerobic digestion

3.2.1.1 Effect on biogas production

A detailed summary of the CH_4 and CO_2 cumulative production (mL/gVS), their respective production rates (mL/d/gVS) for each treatment, and the results of the statistical tests, is shown in Appendix 2.

3.2.1.1.1 Methane

Figure 3.1 compares the cumulative methane production and the daily methane production rate of the control and CEF treatments (0.2, 2, 10, 50 mg/L). The 0.2 mg CEF/L treatment had no impact on either the cumulative or the methane production rate during the digestion (p-value >0.05). There was a small reduction (19%) of the daily methane production rate on day 5 under the presence of 2 mg/L of CEF, although the final cumulative production was comparable to the control (p-value >0.05). Under the presence of 10 mg CEF/L, the cumulative CH_4 production was significantly reduced up to 17% during the first three days. After day 8 and until day 20, a similar effect was observed. However, after day 15, a second biogas production peak was observed, making the final cumulative methane production (day 32) similar to the control (p-value > 0.05). Although the maximum methane production rate was comparable to the control, it was achieved one day later (day 4). The presence of 50 mg CEF/L caused a significant decline (~20%) in the cumulative biogas production during the whole digestion period, and the maximum daily production rate (which was achieved on day 2) decreased by 29% compared to the control.

Figure 3.1 also shows the CH4/CO2 ratios in the biogas based on daily production rate (Fig 3.1c) and cumulative production (Fig. 3.1d). Higher CH_4/CO_2 ratios were observed in the bottles treated with ceftiofur, although only under 50 mg CEF/L the difference was significant (p-value <0.05) during the whole digestion period.

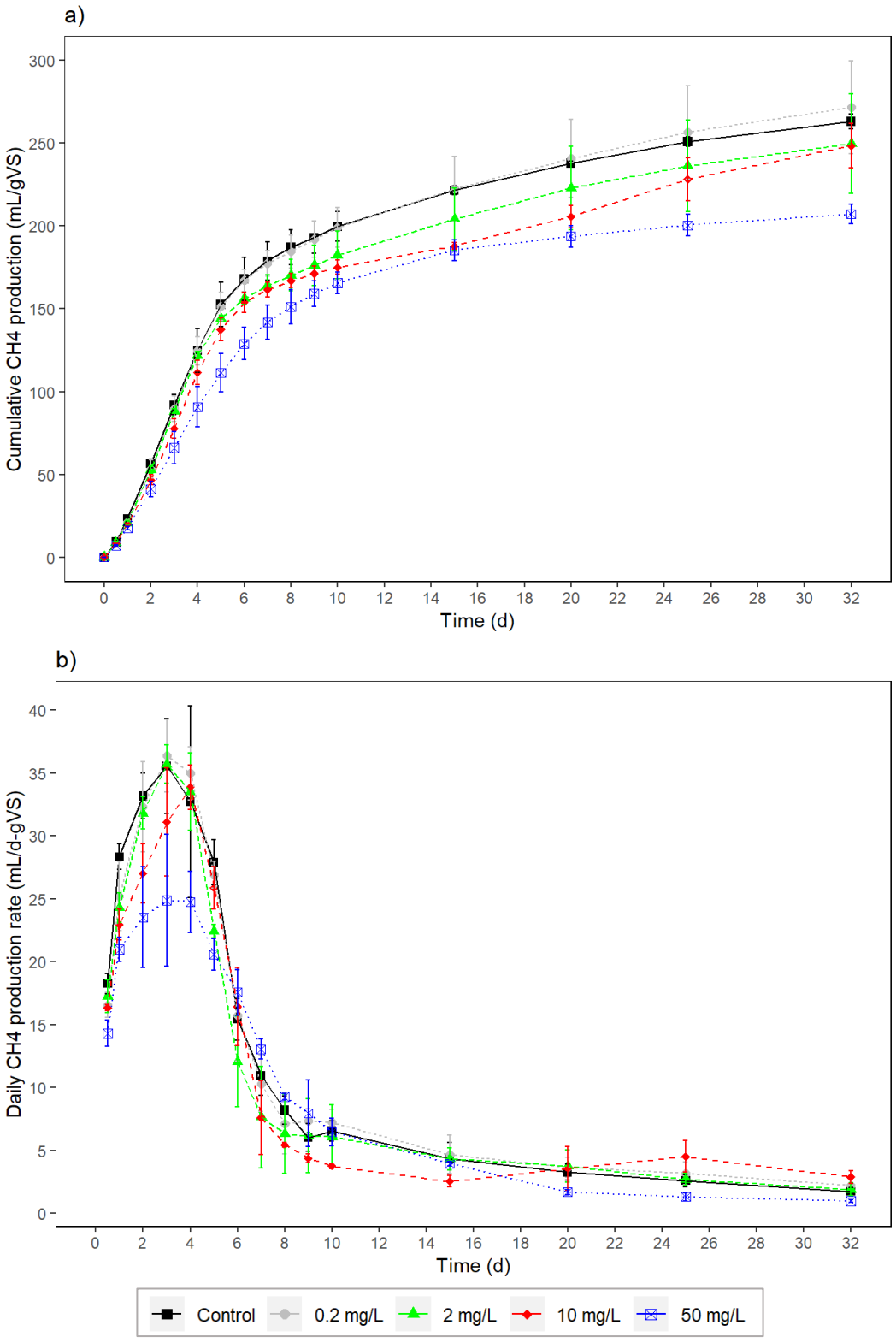


Figure 3.1 Effect of increasing CEF concentrations on cumulative (a) and daily methane production rate (b).

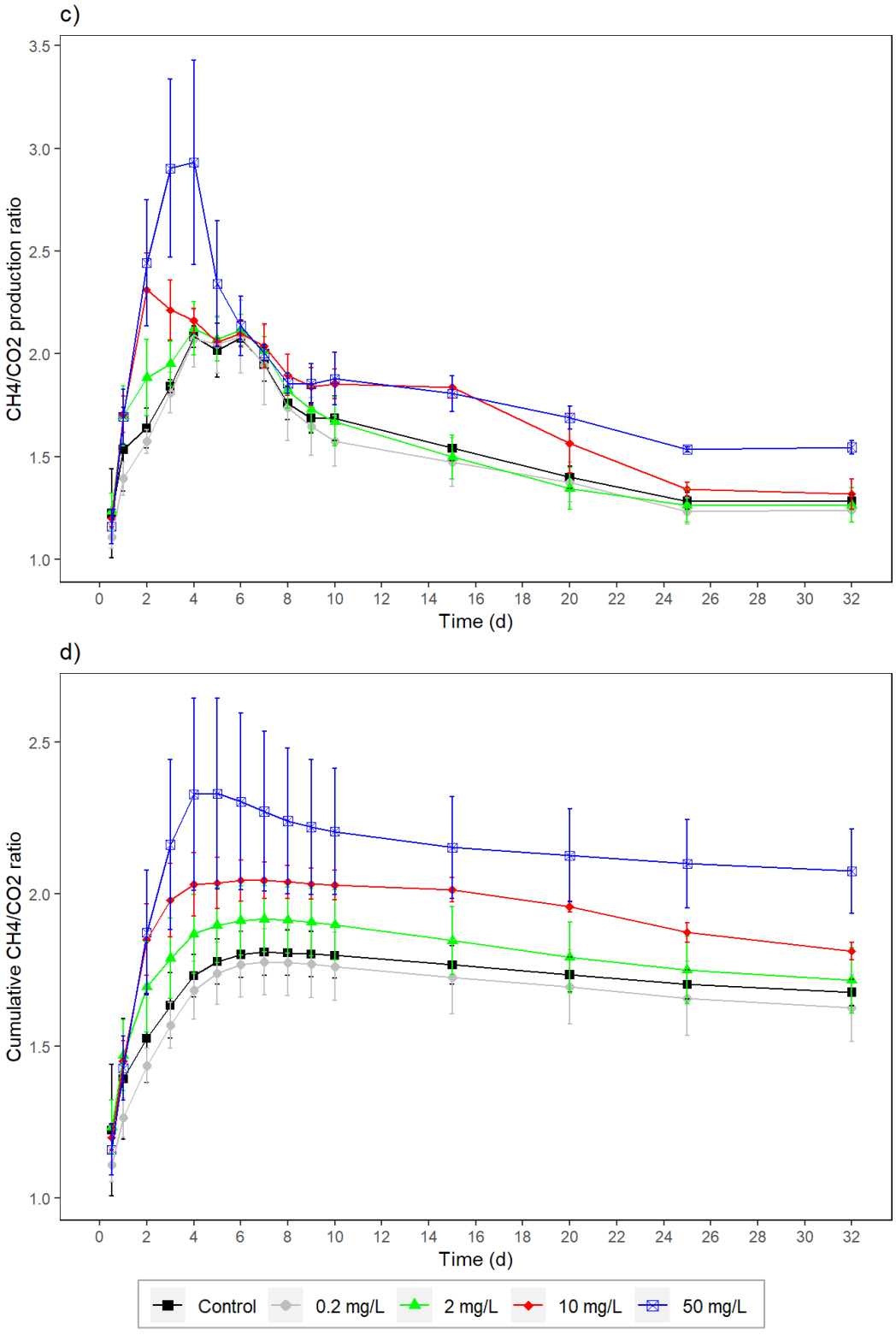


Figure 3.1 Effect of increasing CEF concentration on the CH_4/CO_2 ratios in daily biogas production (c) cumulative production (d) in experiment 1.

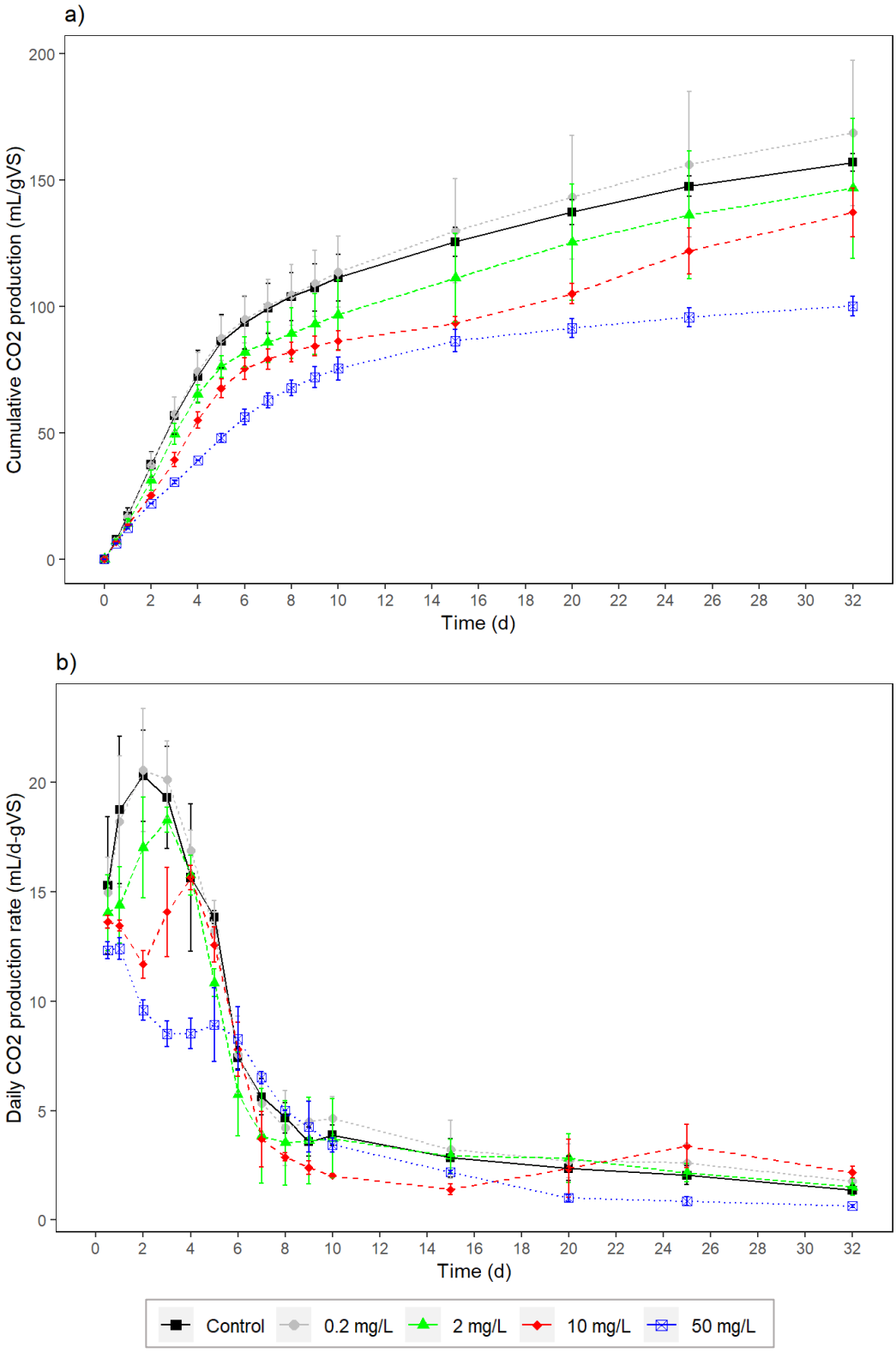


Figure 3.2 Effect of increasing CEF concentrations on cumulative CO_2 (a) and daily CO_2 production rate (b) in experiment 1.

3.2.1.1.2 Carbon dioxide

The carbon dioxide production of the different treatments and control is shown in Figure 3.2. A drop in the CO_2 production rate for the 10 and 50 mg CEF/L treatments was observed during the first 3 days (p-value <0.05), therefore lower cumulative CO_2 yields were observed in both treatments. However, after the second biogas production peak, the 10 mg/L treatment reached similar values to that of the control. There was no significant effect (p-value > 0.05) on CO_2 production caused by 0.2 and 2 mg CEF/L at any point of the digestion.

3.2.1.2 Methane production modeling and CEF IC_{25} estimation

The methane kinetic parameters estimated with the reaction curve-type (RC) model (Eq. 2.7) are shown in Table 3.4. The fit of the reaction curve-type model was good (R^2 > 0.97) in all the treatments. The ultimate methane production (P) and the maximum production rate (R_m) were significantly reduced only under concentrations of 10 and 50 mg CEF/L (p-value <0.05), whereas no difference was observed on the lag phase (λ) in any treatment.

Table 3.4 Estimated kinetic constants from the three-parameter reaction curve-type model

Treatment	P (mL/g VS)	R_m (mL/d-g VS)	λ (d)	R^2
Control	253.0 ± 7.4	43.5 ± 2.9	0.2 ± 0.2	0.99
0.2 mg/L	248.5 ± 11.2	41.7 ± 4.0	0.2 ± 0.2	0.97
2 mg/L	237.4 ± 10.8	39.6 ± 4.1	0.2 ± 0.3	0.99
10 mg/L	228.8 ± 9.3 *	37.7 ± 3.5	0.2 ± 0.2	0.98
50 mg/L	206.5 ± 6.1 *	33.5 ± 2.2 *	0.3 ± 0.2	0.99

*Statistically different to the control based on their 95% confidence intervals.

The effect of the different CEF concentrations on the theoretical maximum methane production rate is shown in Figure 3.2. The 10%, 20% and 25% inhibitory concentration are shown in Table 3.5. The parameters of the model used to estimate the IC concentrations are provided in Appendix 3.

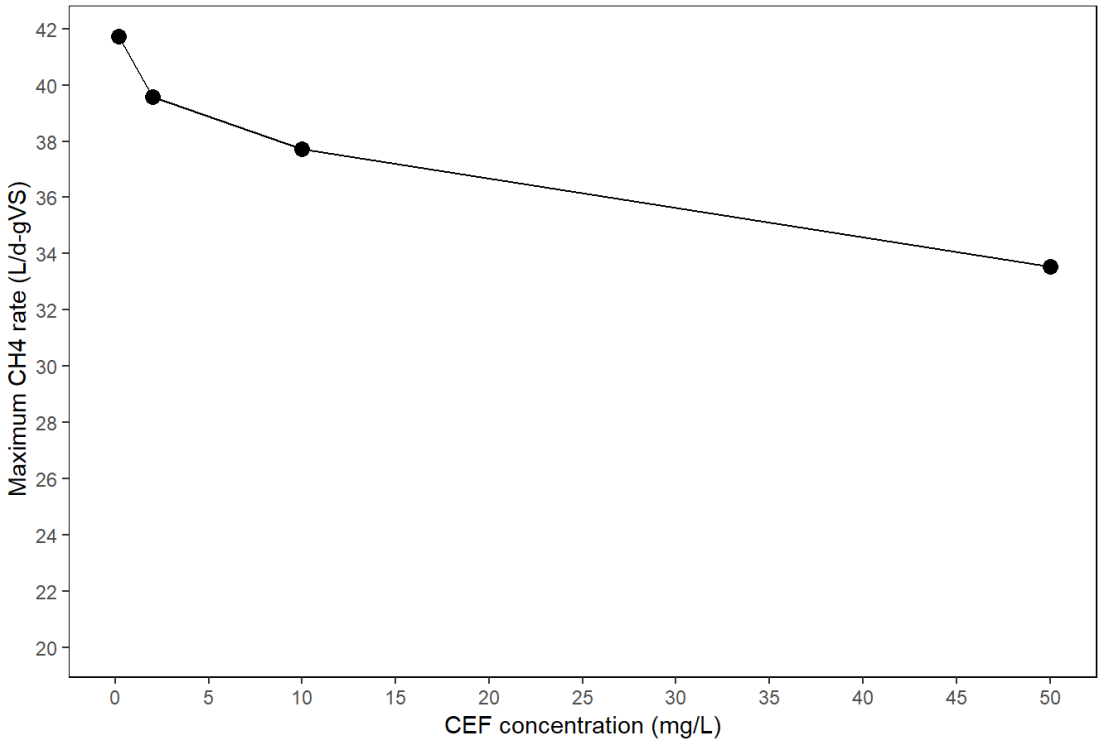


Figure 3.3 Effect of CEF on the theoretical maximum daily methane production rate

Table 3.5 CEF inhibitory concentration with 95% confidence intervals

Inhibition	**CEF concentration (mg /L)**		
	Lower limit	Value	Upper limit
10%	2.6	3.7	5.1
20%	28	33	39
25%	59	67	77

3.2.1.3 Effect of CEF on organic matter removal

Table 3.5 presents the organic matter removal expressed in TS, VS, COD, and dCOD (g/L) in each CEF treatment and the pH before and after the digestion. The pH at the end of the experiment (Day 32) was similar in all treatments and control. Although the TS, VS, and COD removal were slightly lower in the treatments, no statistical difference was observed other than the COD removal for the

50 mg CEF/L treatment. The dCOD consumed was slightly higher in the treatments than the control.

Table 3.6 Summary of the pH and the TS, VS, COD, and dCOD removal in experiment 1

Treatment	TS	VS	COD	dCOD	pH	
	g	*g*	*g*	*g*	*Initial*	*Final*
Control	2.52 ± 0.25	2.86 ± 0.27	1.38 ± 0.09	0.63 ± 0.15	7.82	7.27
0.2 mg/L	2.31 ± 0.06	2.64 ± 0.02	1.96 ± 0.40	0.95 ± 0.11*	7.85	7.28
2 mg/L	2.96 ± 0.15	3.28 ± 0.15	0.87 ±0.40	0.84 ± 0.23	7.86	7.25
10 mg/L	2.34 ± 0.28	2.83 ± 0.11	1.16 ± 0.39	0.65 ± 0.04	7.87	7.27
50 mg/L	2.37 ± 0.19	2.48 ± 0.10	1.08 ± 0.05 *	1.04 ± 0.03*	7.92	7.30

* Statistically different to the control with 95% confidence

The VFA profile before and after the digestion is shown in Figure 3.4. The control and the treatments started with a similar VFA profile. None of the different CEF concentrations tested in this experiment caused a significant VFA accumulation after 32 days of AD.

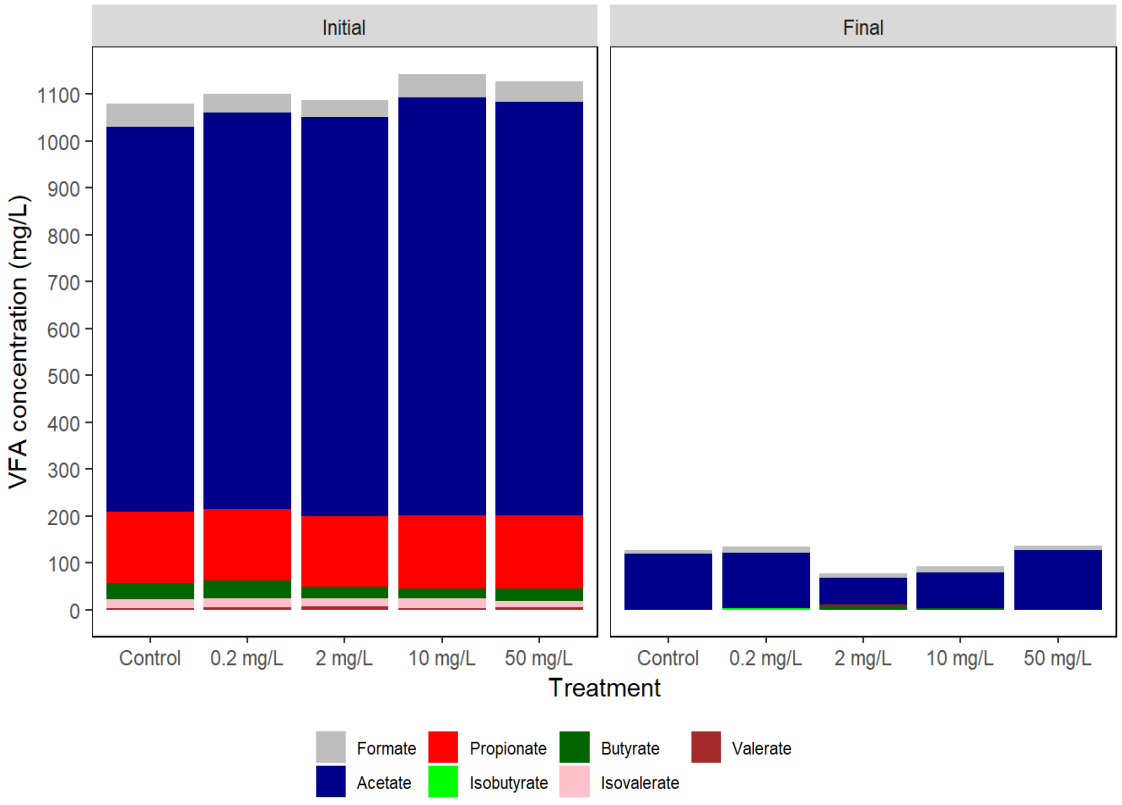


Figure 3.4 VFA profile before and after a 32-days AD period in experiment 1

3.2.2. Experiment 2: Effect of ceftiofur on different time-points of the anaerobic digestion process

3.2.2.1 Effect on biogas production

A detailed summary of the cumulative CH_4 and CO_2 production (mL/gVS), their respective production rates (mL/d-gVS), and the results of the statistical tests can be found in Appendix 4. The significance of the differences between control and treatment are also shown in Appendix 4. The cumulative methane production curve and the daily production rates of the controls and the 50 mg CEF/L treatment bottles are shown in Figure 3.5. The average cumulative methane production was lower in CEF-treated bottles during the whole digestion period, however, no statistical significance was found. The daily methane production rate (Figure 3.5b) was always lower in CEF treated bottles, although only on day 2, the 15% reduction was statistically significant (p-value < 0.008). The maximum daily methane production rates were observed between day 4 and 7 in both treatments and controls. Figure 3.6 compares the cumulative and the daily CO_2 production rate of control and treatment bottles. The CEF-treated bottles produced significantly less CO_2 (mL/gVS) than the controls during most of the digestion period (p-value <0.05). A drop greater than 30% of the daily CO_2 production rate was observed on treatment bottles on days 2 and 3 (p-values < 0.05).

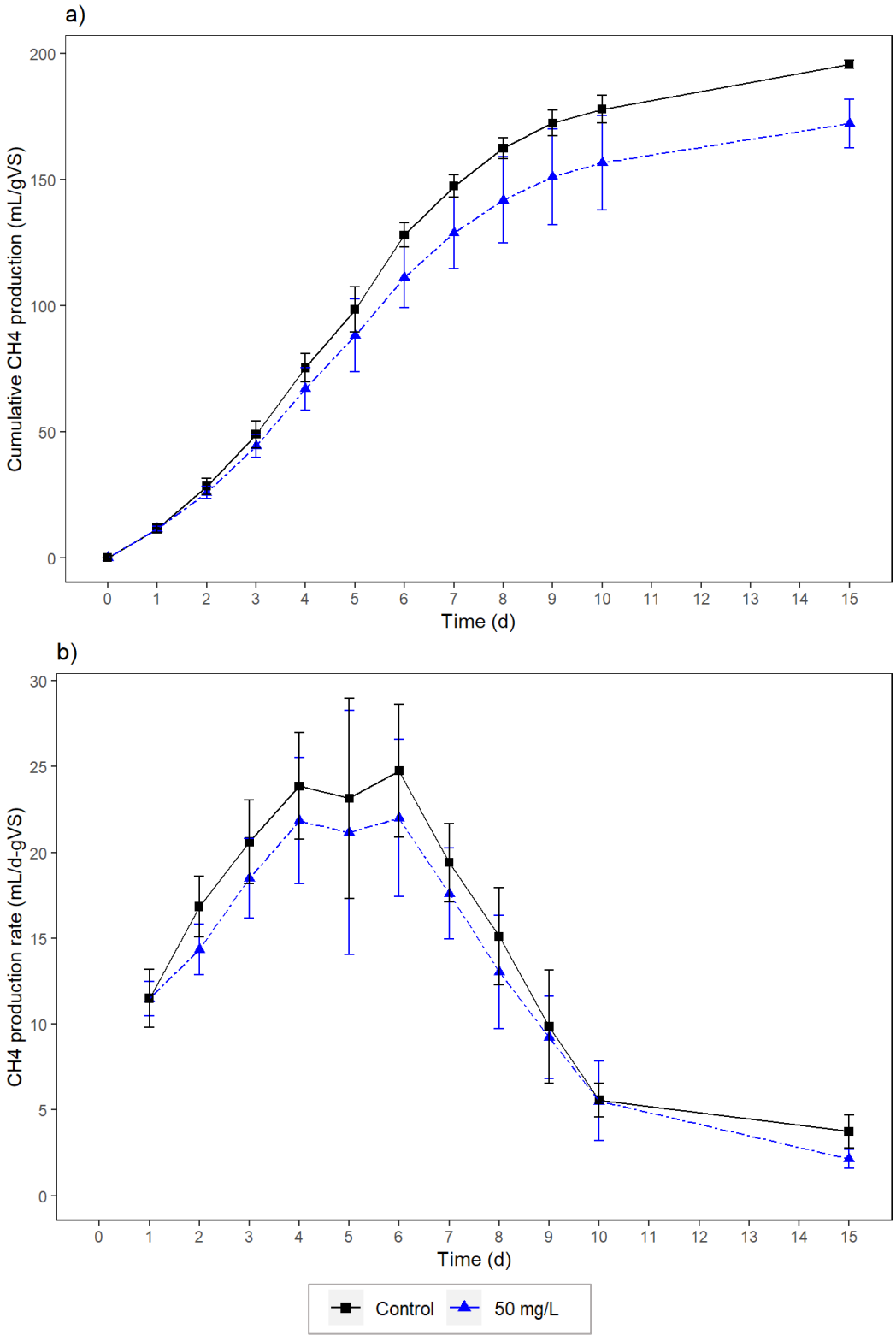


Figure 3.5 Effect of 50 mg CEF/L on cumulative (a) and daily methane production rate (b).

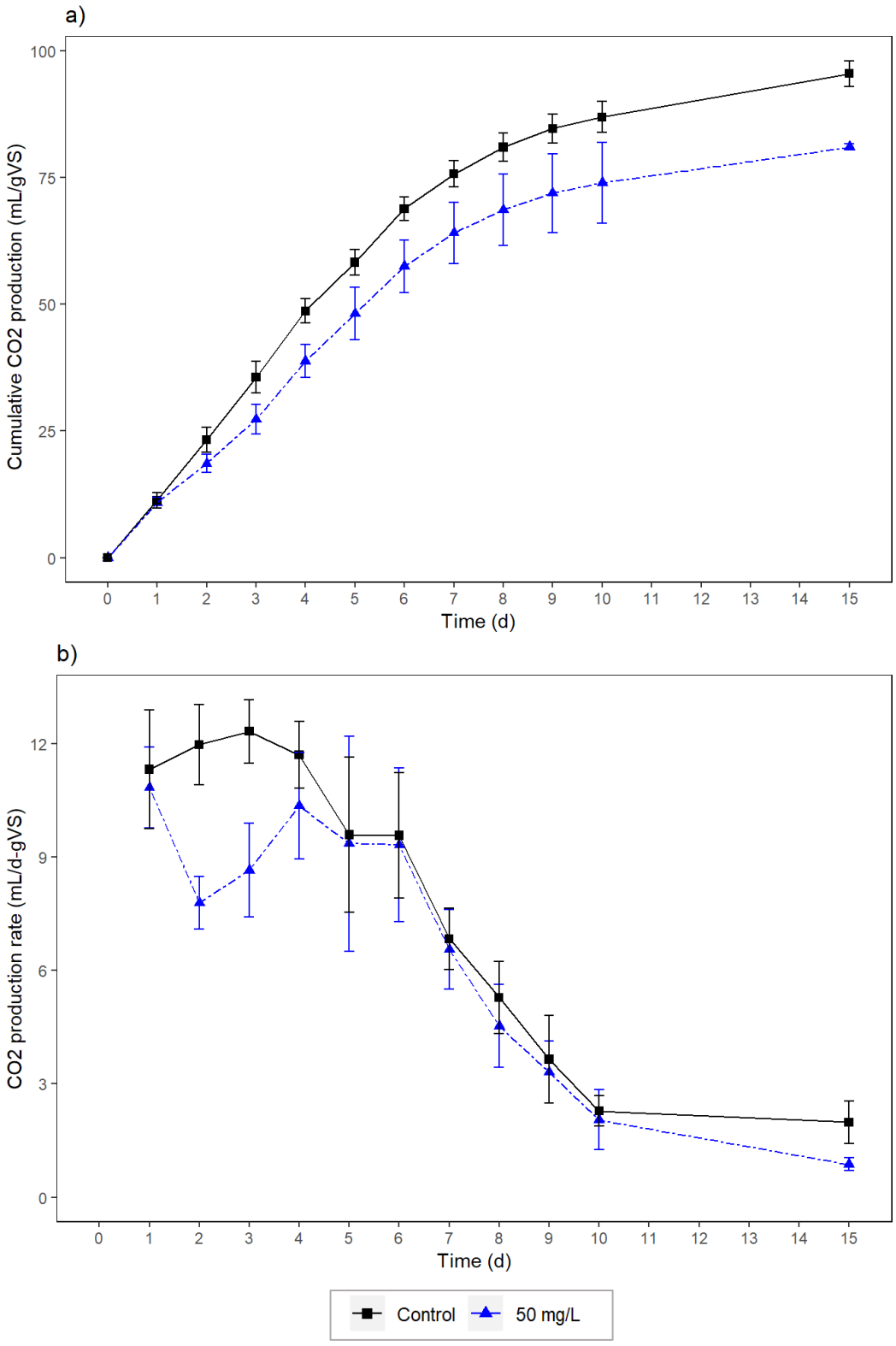


Figure 3.6 Effect of 50 mg CEF/L on cumulative (a) and daily CO_2 production rate (b).

3.2.2.2 Effect of 50 mg CEF/L on hydrogen levels in Experiment 2

Figure 3.7 contrasts the average H_2 concentration (ppm) in the headspace of the bottles with and without 50 mg CEF/L. A summary of the H_2 concentrations on the sampling days and the statistical tests is shown in Appendix 5. Significantly lower levels of H_2 were found in CEF-treated bottles during the first 2 days of digestion (p-value <0.05). After that, slightly higher H_2 levels were consistent in treatment bottles. On day 15, both control and treatment ended up with similar H_2 levels (67 and 76 ppm, respectively).

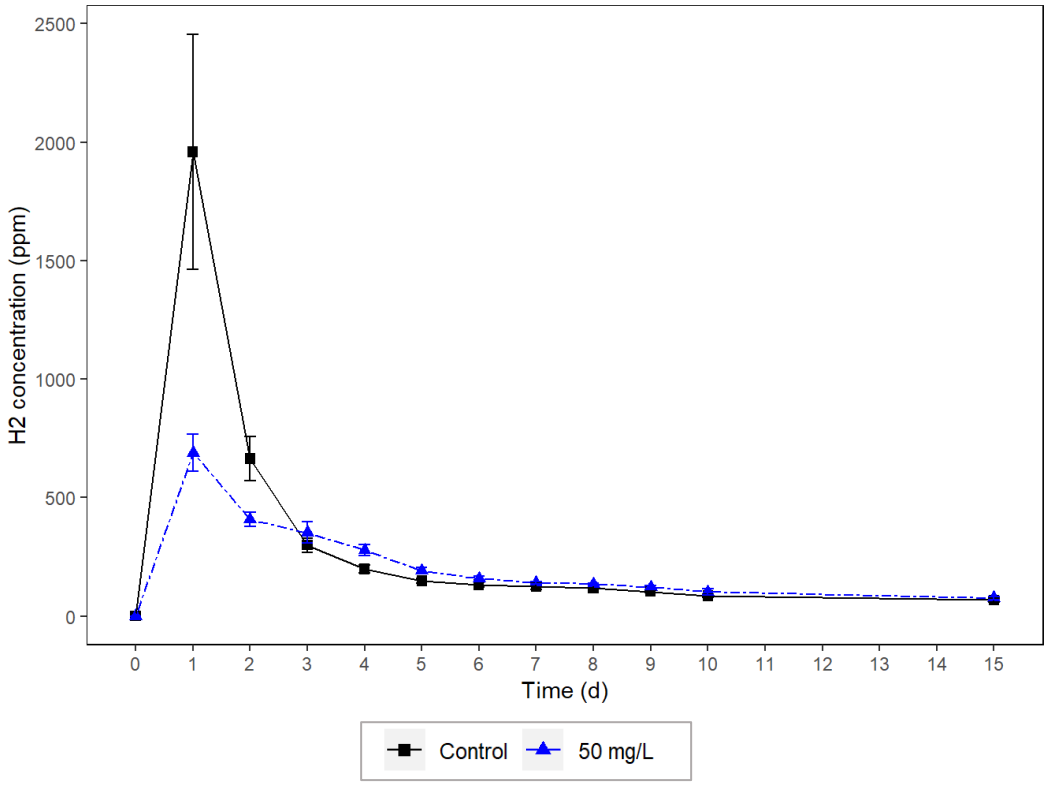


Figure 3.7 Effect of 50 mg CEF/L on hydrogen concentration over time.

3.2.2.3 Effect of 50 mg/L of CEF on the VFA profile during AD

Figure 3.8 shows the total VFA concentration of the treated and no-treated bottles on days 0, 3, 5, 10, and 15. A summary with the average total VFA concentration, the differences between control and treatment, and their respective statistical test are shown in Appendix 6. The treatment and the control bottles started with the same amount of total VFA (2,141 mg/L). On day 3, the CEF-treated bottles had 18% less total VFA concentration compared to the controls (p-value < 0.05). On day 10, slightly higher levels were found on treated bottles (p-value < 0.005). At the end of the experiment (day 15), very low VFA levels (~100 mg/L) were found in both treatment and control bottles.

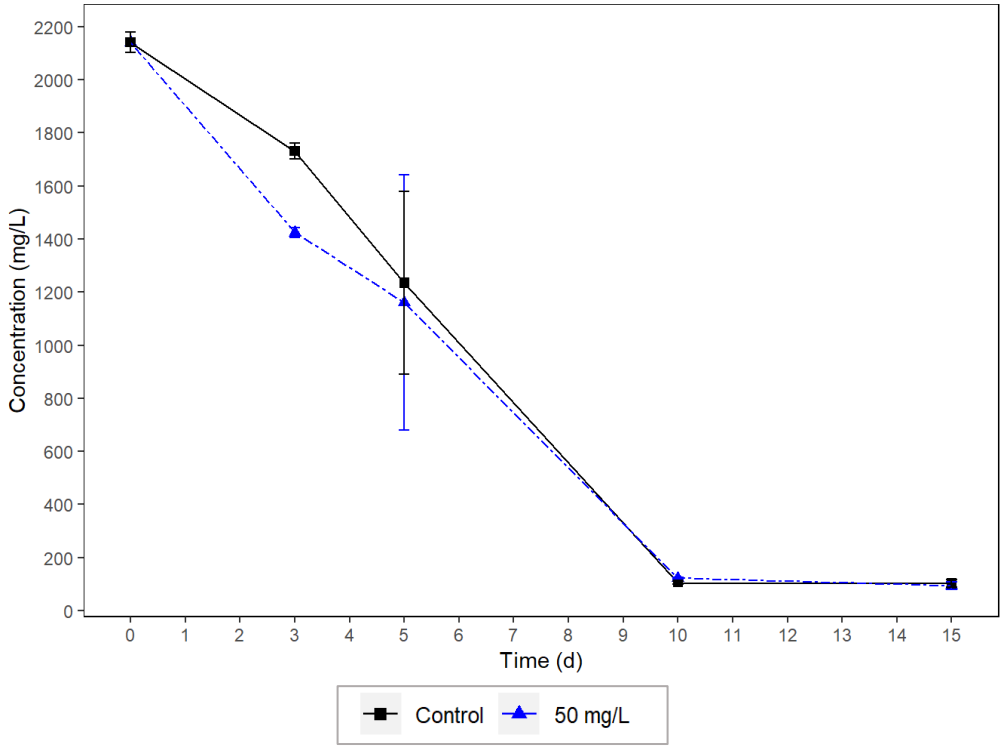


Figure 3.8 Effect of 50 mg CEF/L on total VFA concentration over time

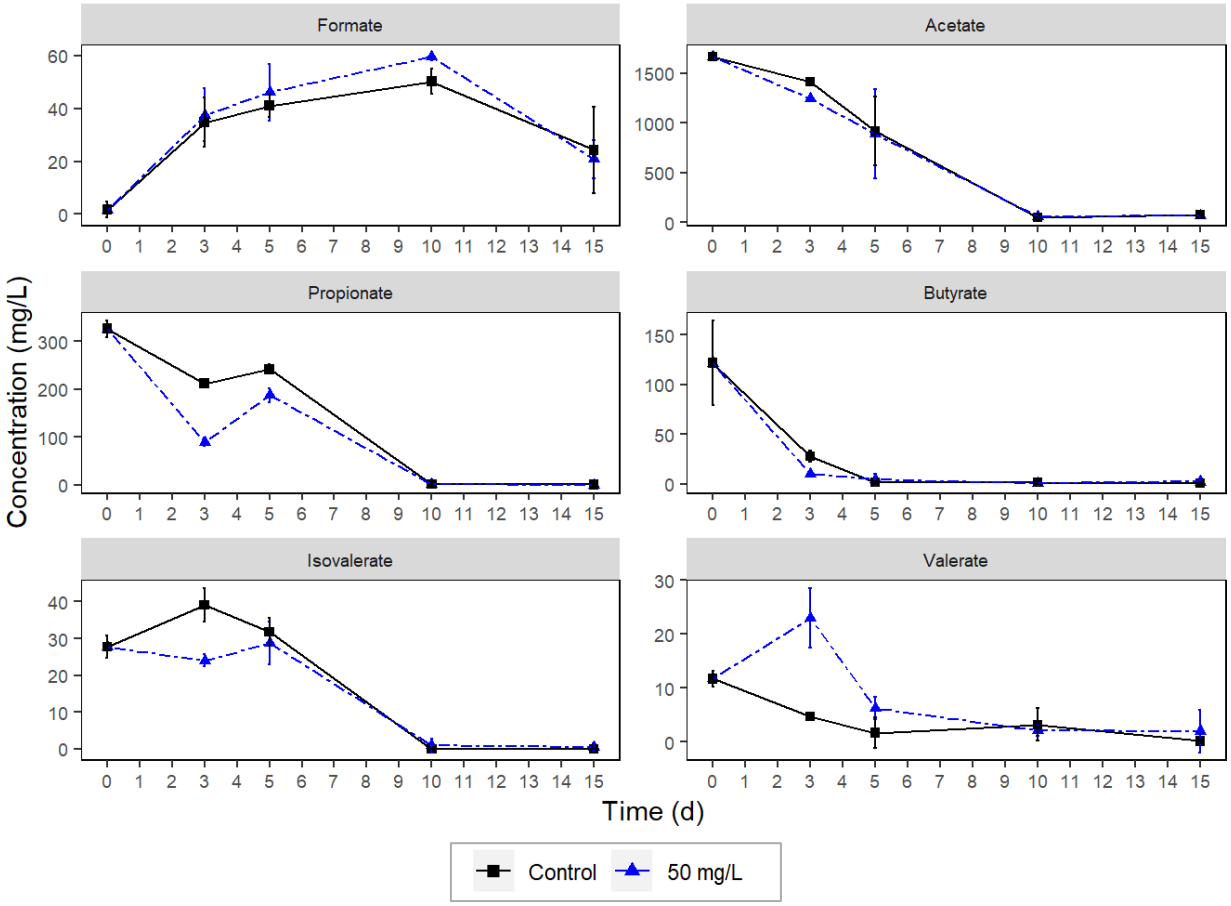


Figure 3.9 Effect of 50 mg CEF/L on the VFA profile over time

The VFA profile, including formate, acetate, propionate, butyrate, isovalerate, and valerate, during the sampling days are shown in Figure 3.9. Isobutyrate was not detected in this experiment. A summary with the average concentrations, the difference between the control and the treatment, and the statistical test results are shown in Appendix 7. On day 3, the VFA profile of controls and CEF-treated bottles was significantly different (p-value <0.05). Lower acetate, propionate, butyrate, and isovalerate levels were found on bottles treated with 50 mg CEF/L, while valerate was slightly higher; formate was similar for both, control and treatment. On day 5, only propionate was significantly lower in treatment bottles. At day 10, most of the VFAs were already consumed

and the leftovers were fairly similar between control and treatment, although formate and acetate on day 10, and butyrate on day 15, were statistically different.

3.2.2.4 Effect of 50 mg CEF/L on pH

The pH of CEF-treated and control bottles are shown in Figure 3.10. Both conditions started with similar pH (7.80) and on day 3, it decreased to 7.35 and 7.52 in control and treatment bottles (p-value <0.05), respectively. After day 5 and until the end of the experiment (day 15), the pH was stable and around 7.40 in both conditions.

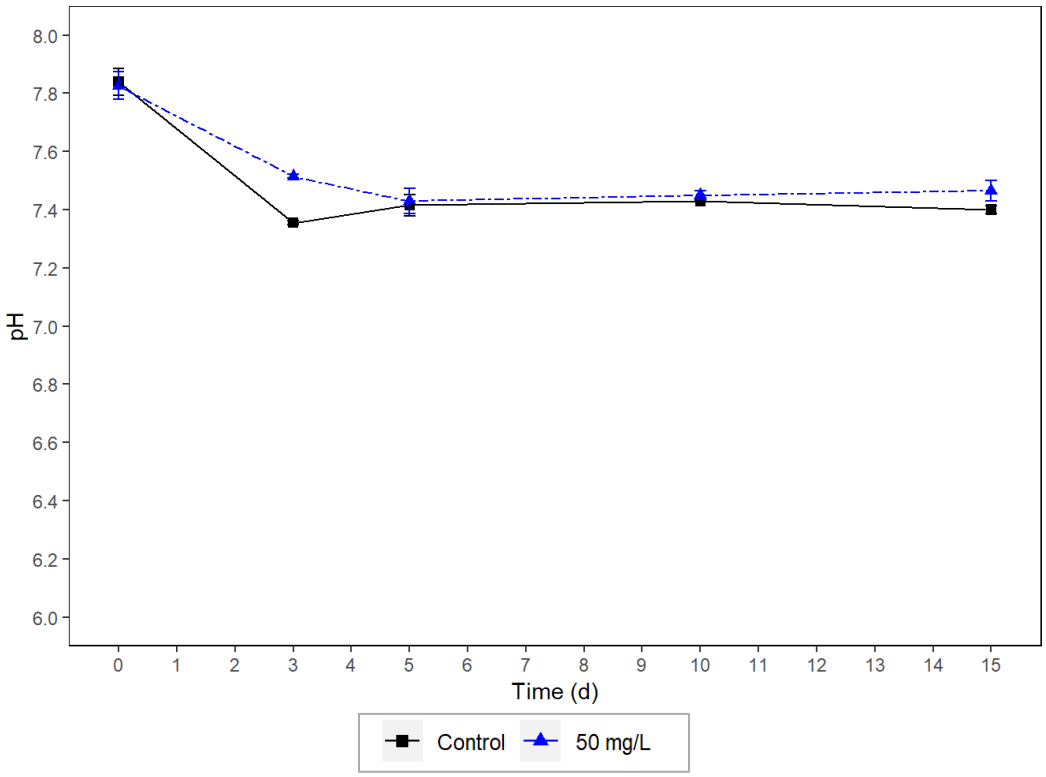


Figure 3.10 Effect of 50 mg CEF/L on pH over time

3.2.2.5 Effect of 50 mg/L on organic matter removal

A summary contrasting the organic matter concentration (COD, dCOD, TS, and VS) of controls and CEF-treated bottles over time and their statistical test are presented in Appendix 8.

3.2.2.5.1 Total and dissolved COD

Figure 3.11 presents the total and dissolved COD concentrations at the different sampling days (0, 3, 5, 10, and 15). The presence of 50 mg CEF/L did not have a significant effect on the total COD removal during the digestion. In both, the treatment and the control, more than 42% of the initial COD was consumed at day 15. On day 3, bottles treated with 50 mg/L of CEF presented slightly higher dCOD (7.4 g O/L) than controls (6.0 g O/L) (p-value <0.05). However, on the following days (day 5, 10, and 15) similar dCOD levels were measured (p-value >0.05). By the end of the experiment, around 48% of the initial dCOD was removed in both conditions.

3.2.2.5.2 Total and volatile solids

The total (TS) and volatile (VS) solids of the control and treated bottles during the different sampling days (0, 3, 5, 10, and 15) are shown in Figure 3.12. Whereas in the control bottles a stable TS removal rate was observed from day 3 until day 10, the CEF-treated bottles showed significant TS removal after day 5. On the other hand, most of the VS removal occurred between days 3 and 10 in both CEF-treated and non-treated bottles. Nevertheless, CEF-dosed bottles had significantly higher VS level on day 5 and 15 (p-value <0.05). By the end of the experiment, control bottles achieved 16% and 11% of TS and VS removal, whereas the treatments achieved 11% and 8%, respectively.

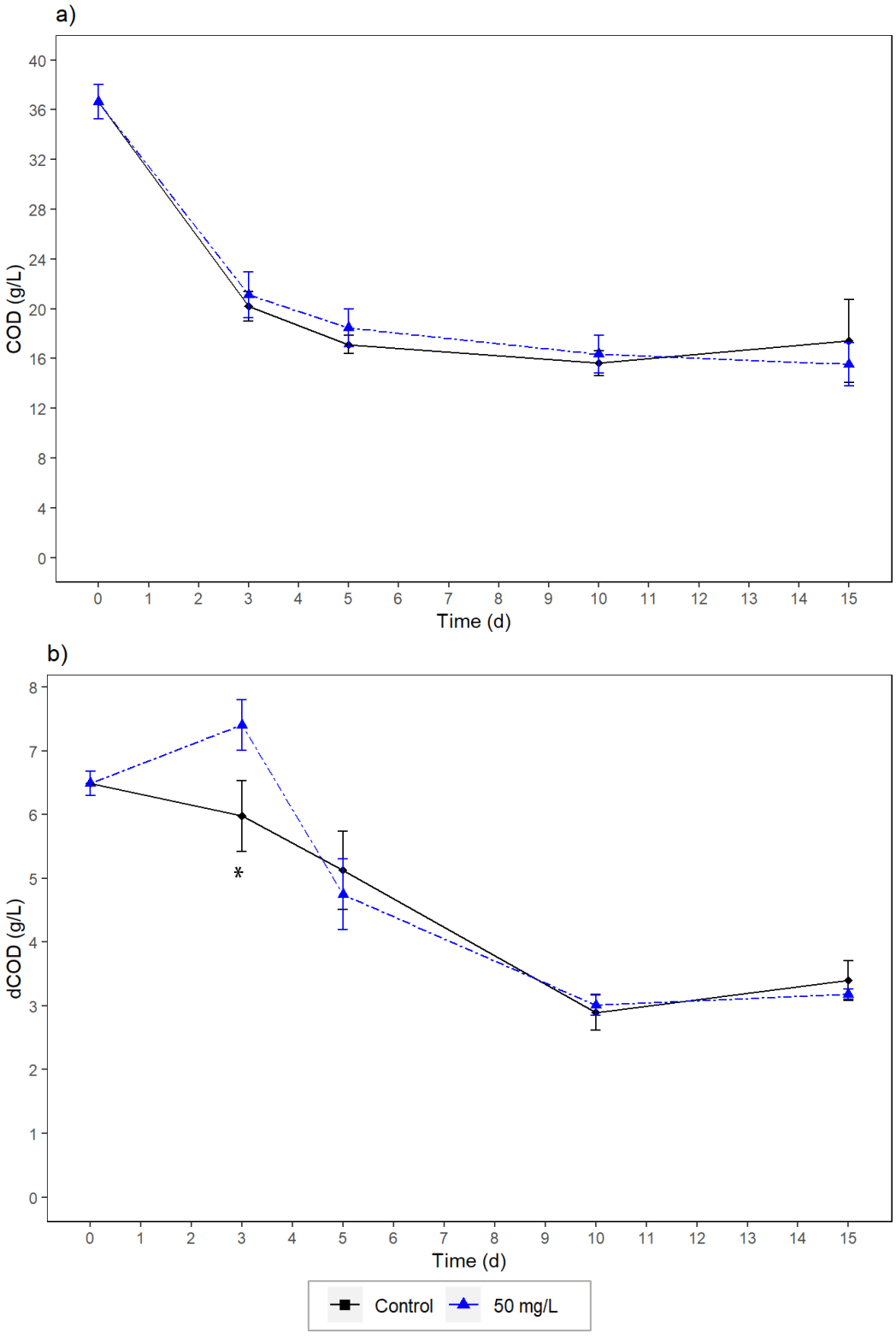


Figure 3.11 Effect of 50 mg CEF/L on COD (a) and dCOD (b) concentration over time.

*Statistically significant difference with 95% confidence.

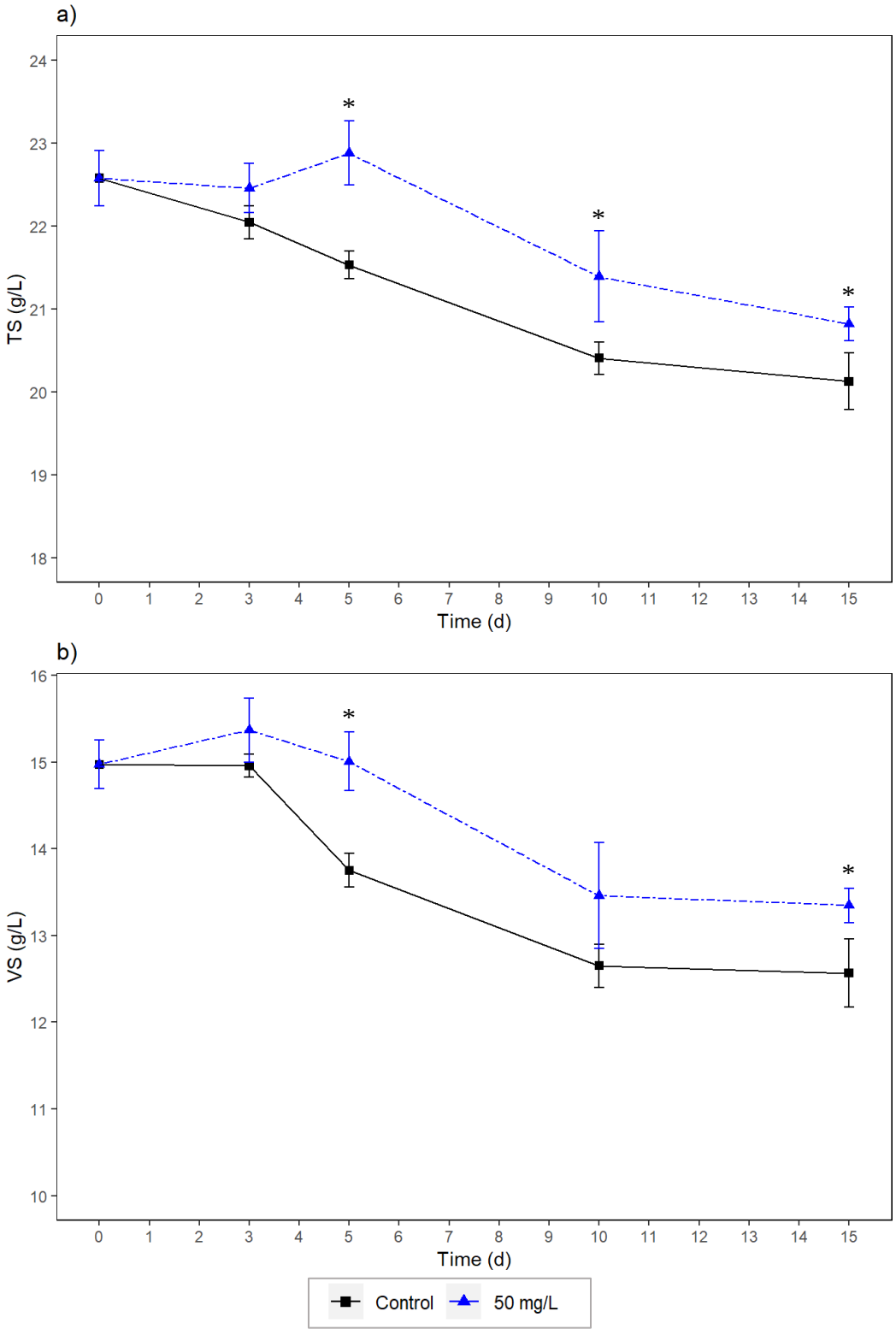


Figure 3.12 Effect of 50 mg CEF/L on total (a) and volatile (b) solids over time.

*Statistically significant difference with 95% confidence.

3.2.3. Experiment 3: Effect of a ceftiofur shock-load on the stability of mesophilic anaerobic digestion

3.2.3.1 Effect on biogas production

A detailed summary with the average of the CH_4 and CO_2 cumulative production (mL/g VS), their daily production rate (mL/d-gVS) and the statistical test are presented in Appendix 8. Figure 3.13a describes the cumulative methane production over time. The cumulative CH_4 of the bottles containing 250 mg CEF/L was dramatically reduced by 25% on the first day, and more than 40% after the second day (p-value <0.05). The daily methane production rate (Fig. 3.13b) was also reduced in similar proportion during the first 8 days of the digestion (p-value <0.05).

The cumulative CO_2 production (Figure 3.14a) was also greatly affected by addition of 250 mg/L of CEF. During the whole digestion period, levels of CO_2 below 50% of the controls were observed in the CEF-treated bottles (p-value < 0.005). The daily CO_2 production rate (Figure 3.14b) was reduced by more than 35% on day 1 and it declined on the following days reaching more than 80% reduction on day 5 (p-value < 0.005).

Hydrogen levels in the headspace (Figure 3.15) were also significantly lower in treatment bottles during the first 2 days (p-value < 0.05), although similar values were measured in the following days (p-value > 0.05). The higher hydrogen concentration was measured on day 1 in both CEF-dosed and control bottles, 784 (±114) and 416 (±94) ppm, respectively. Nevertheless, the hydrogen was consumed in the following days, and methane continued to be evolved.

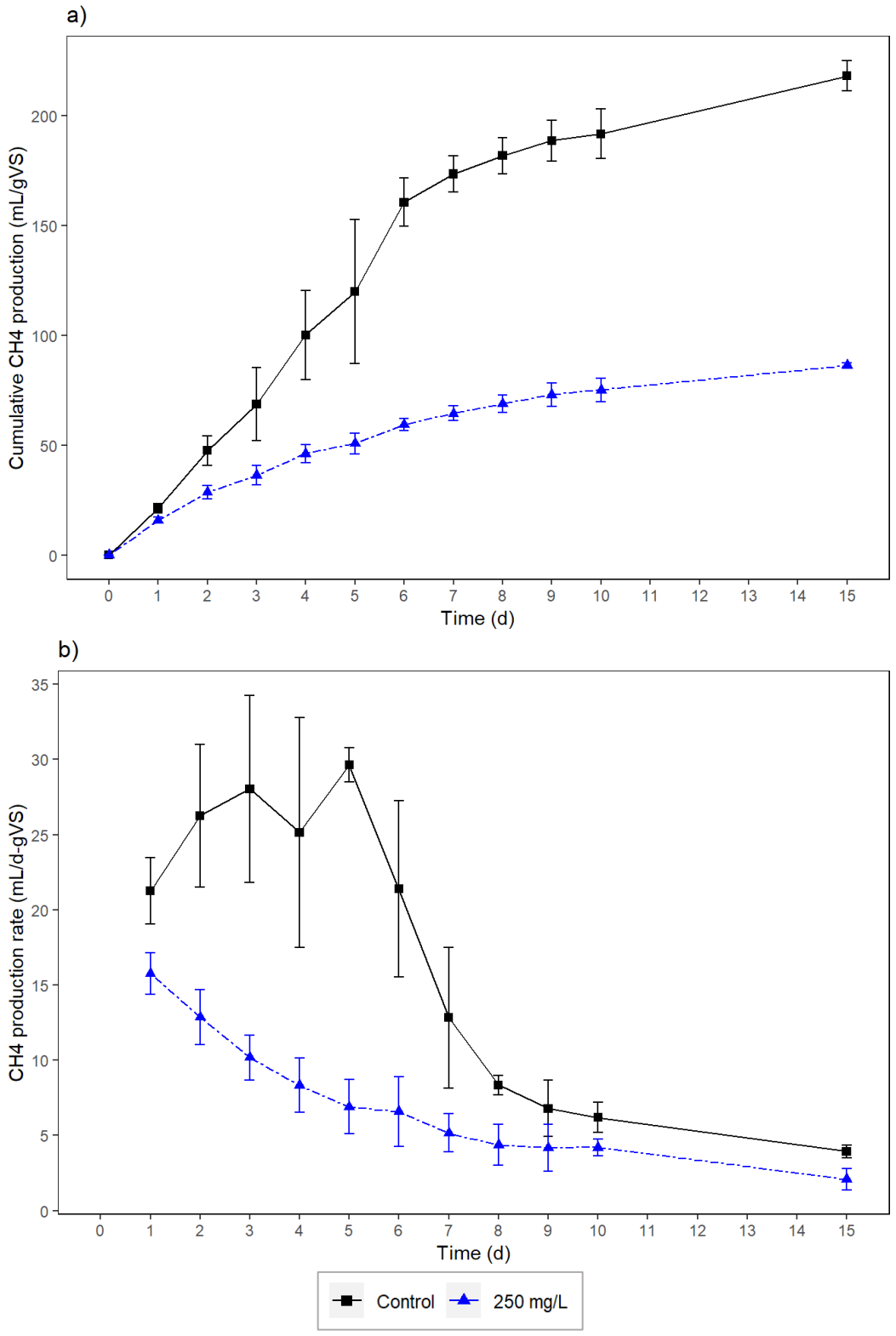


Figure 3.13 Effect of 250 mg CEF/L on cumulative (a) and daily methane production rate (b)

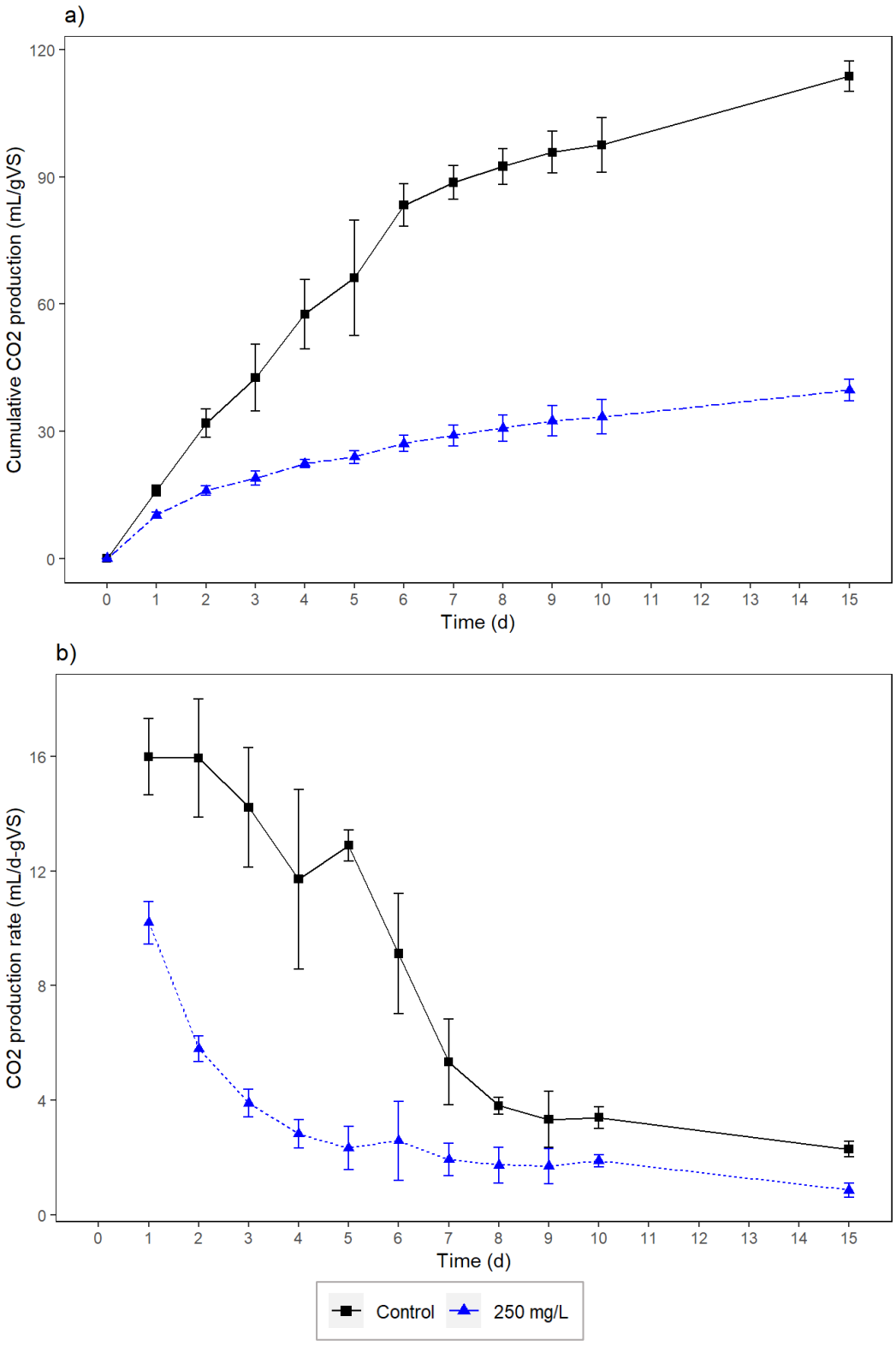


Figure 3.14 Effect of 250 mg CEF/L on cumulative (a) and daily CO_2 production rate (b)

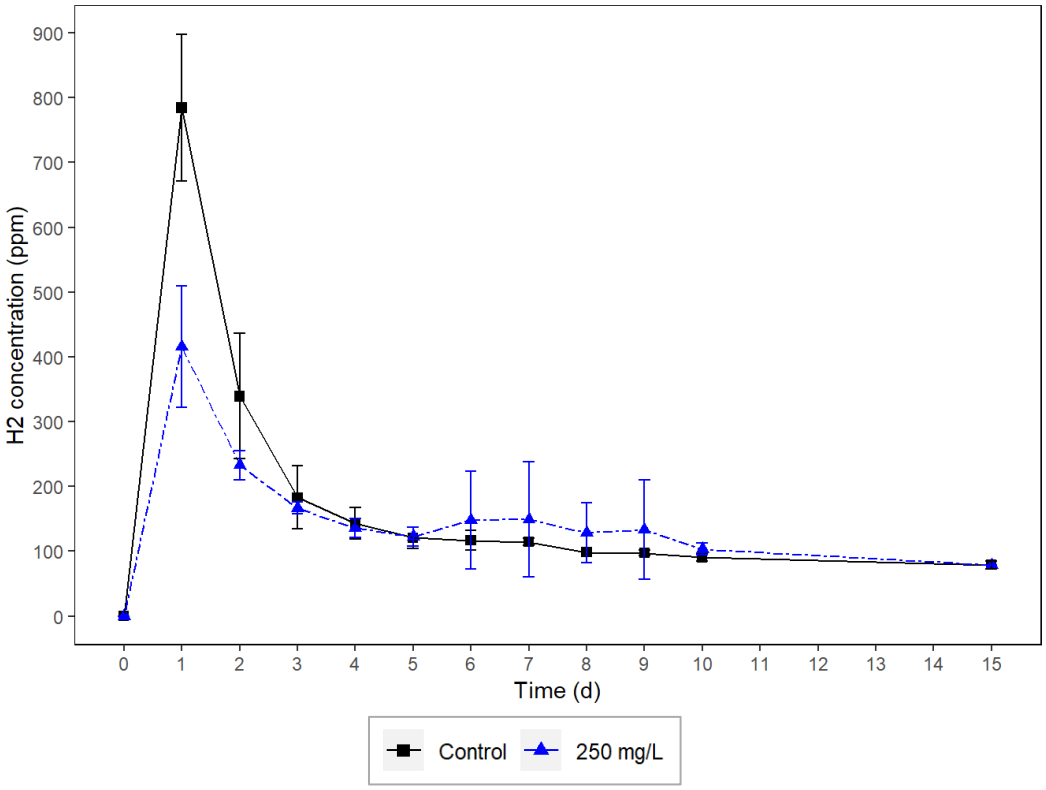


Figure 3.15 Effect of 250 mg CEF/L on hydrogen concentration over time

3.2.3.2 Effect of 250 mg/L of CEF on the VFA profile during AD

Figure 3.16 shows the total VFA concentration (mg/L) during AD of dairy manure with and without 250 mg CEF/L. A summary with the total VFA average (mg/L), the difference between control and treatment, and their respective statistical test are listed in Appendix 11. Whereas the total VFA concentration increased on day 3 in the control bottles, it decreased in the CEF-dosed treatment bottles. Between day 3 and 5, no changes in total VFA was observed in treatment bottles, while control bottles showed a constant VFA consumption until day 10. On day 10, slightly higher VFA concentrations were found in CEF-treated bottles. At the end of the experiment (day 15), very low VFA concentrations were measured in both conditions.

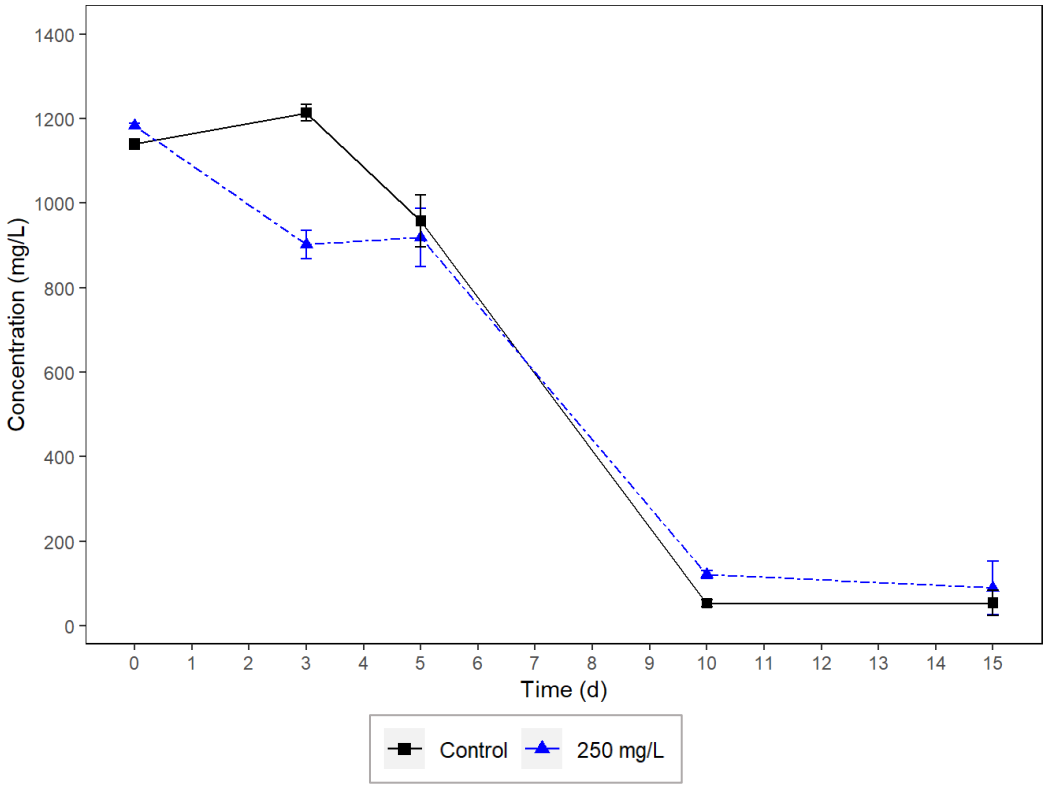


Figure 3.16 Effect of 250 mg CEF/L on total VFA concentration over time

Figure 3.17 shows the different VFA profile found in CEF-treated and control bottles during the sampling days (0, 3, 5, 10, and 15). A detailed summary of the average, the difference and the statistical test of each VFA is shown in Appendix 12. While formate, butyrate, and valerate were found in significantly higher concentrations in the bottles amended with 250 mg CEF/L during days 3 and 5 (p-values < 0.05), acetate, propionate, and isovalerate were lower (p-value < 0.05). Nonetheless, at the end of the experiment, similar VFA levels were measured in control and treatment bottles.

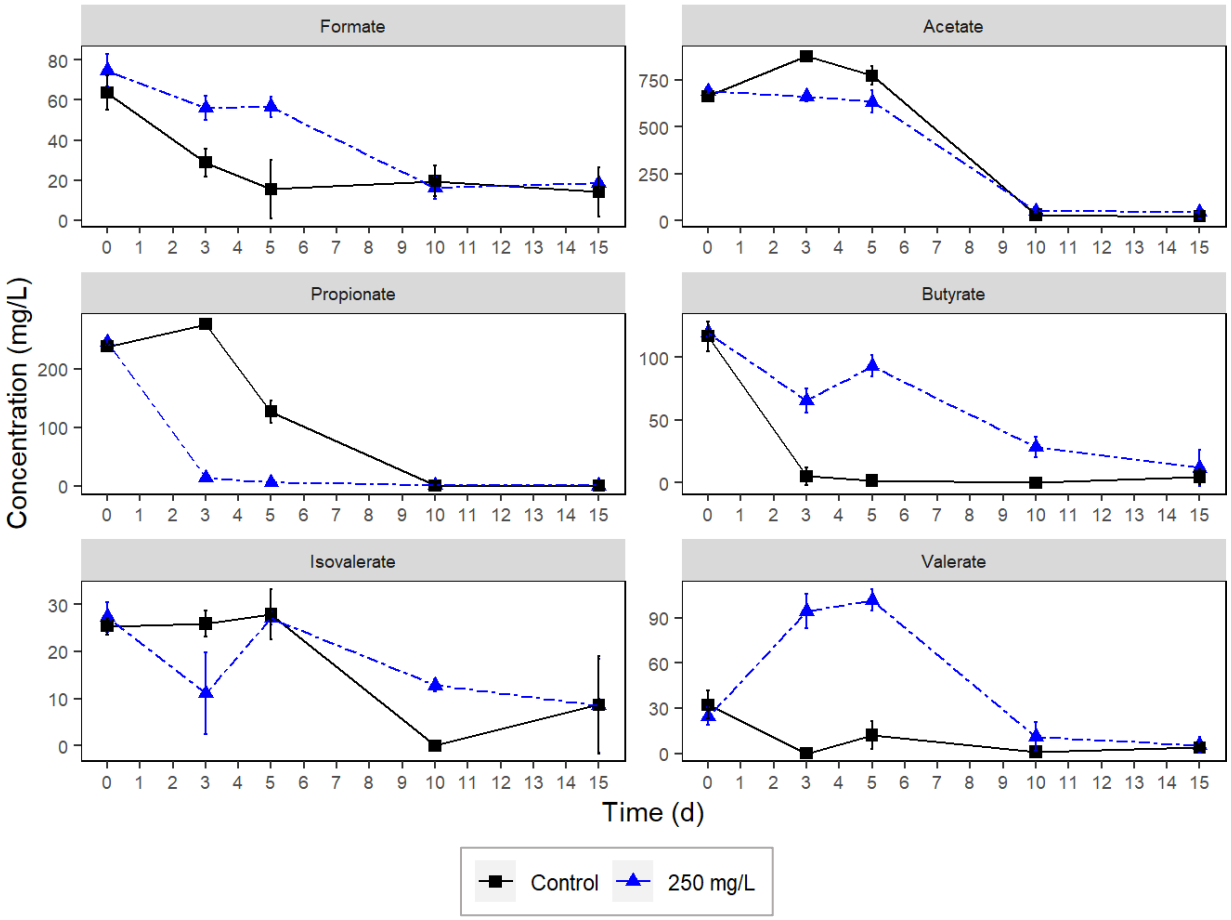


Figure 3.17 Effect of 250 mg CEF/L on the VFA profile over time

3.2.3.3 Effect of 250 mg/L on pH

The AD reactions started with a similar pH of 7.70 on day 0. From day 3 and ahead, the pH of the bottles treated with 250 mg CEF/L had slightly higher pH levels than the control. On day 15, the pH of treatment and control were 7.29 and 7.41, respectively. Figure 3.18 shows the pH profile during the anaerobic digestion process.

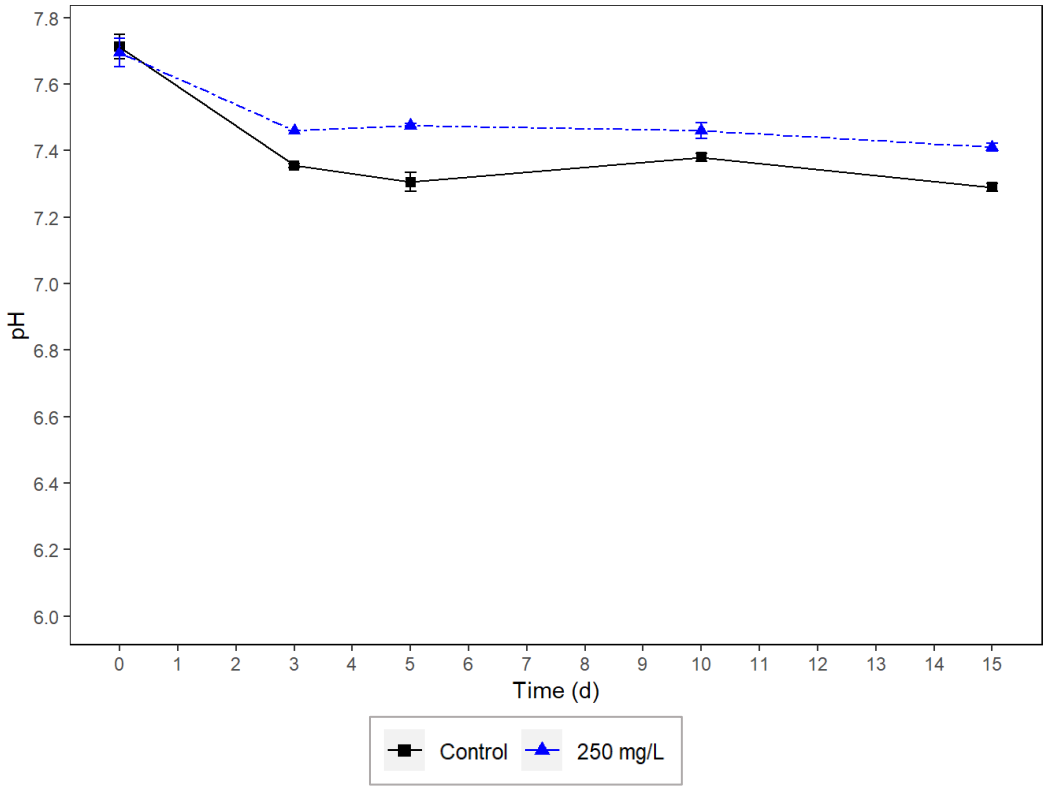


Figure 3.18 Effect of 250 mg CEF/L on pH over time

3.2.3.4 Effect of 250 mg CEF/L on organic matter removal

A summary with the average of the total and dissolved COD (g O/L), total and volatile solids (g/L), as well as their respective removal rates and statistical tests, are listed in Appendix 13.

3.2.3.4.1 Effect on total and dissolved COD removal

The COD and dCOD concentrations on CEF-treated and control bottles on days 0, 3, 5, 10, and 15 are shown in Figure 3.19. The AD reaction amended with 250 mg CEF/L started with slightly higher COD concentration than the control, 23.7 and 22.1 g/L, respectively (p-value < 0.05). During the whole digestion, higher total COD levels were observed in CEF-treated bottles. At the end of the experiment (day 15), treatment bottles achieved only 23% COD removal, whereas the controls achieved 32%. On the other hand, both conditions started with similar dCOD content (6.8 g O/L) and similar concentrations were found on day 3 and 5. However, the 250 mg CEF/L treated bottles maintained slightly higher levels on days 10 and 15 (p-value <0.05). At the end of the experiment, the average dCOD removal was 23% in treatment bottles, while more than 30% was observed in non-treated bottles (p-value < 0.05).

3.2.3.4.2 Effect on TS and VS removal

The 250 mg CEF/L treated bottles started with significantly higher TS and VS levels than the controls (p-value <0.05), and they were higher during the whole digestion period (Figure 3.20). Most of the TS removal took place during the first 3 days in the bottles dosed with CEF, whereas in control bottles this period was longer, including day 5. The removal rate was slightly higher in treated bottles on day 3 (p-value < 0.05). However, no differences between control and treatment removal rates were observed in the following days. On the other hand, the VS removal rates were similar on days 3, 5, and 10, whereas on day 15, greater VS removal were observed in control bottles.

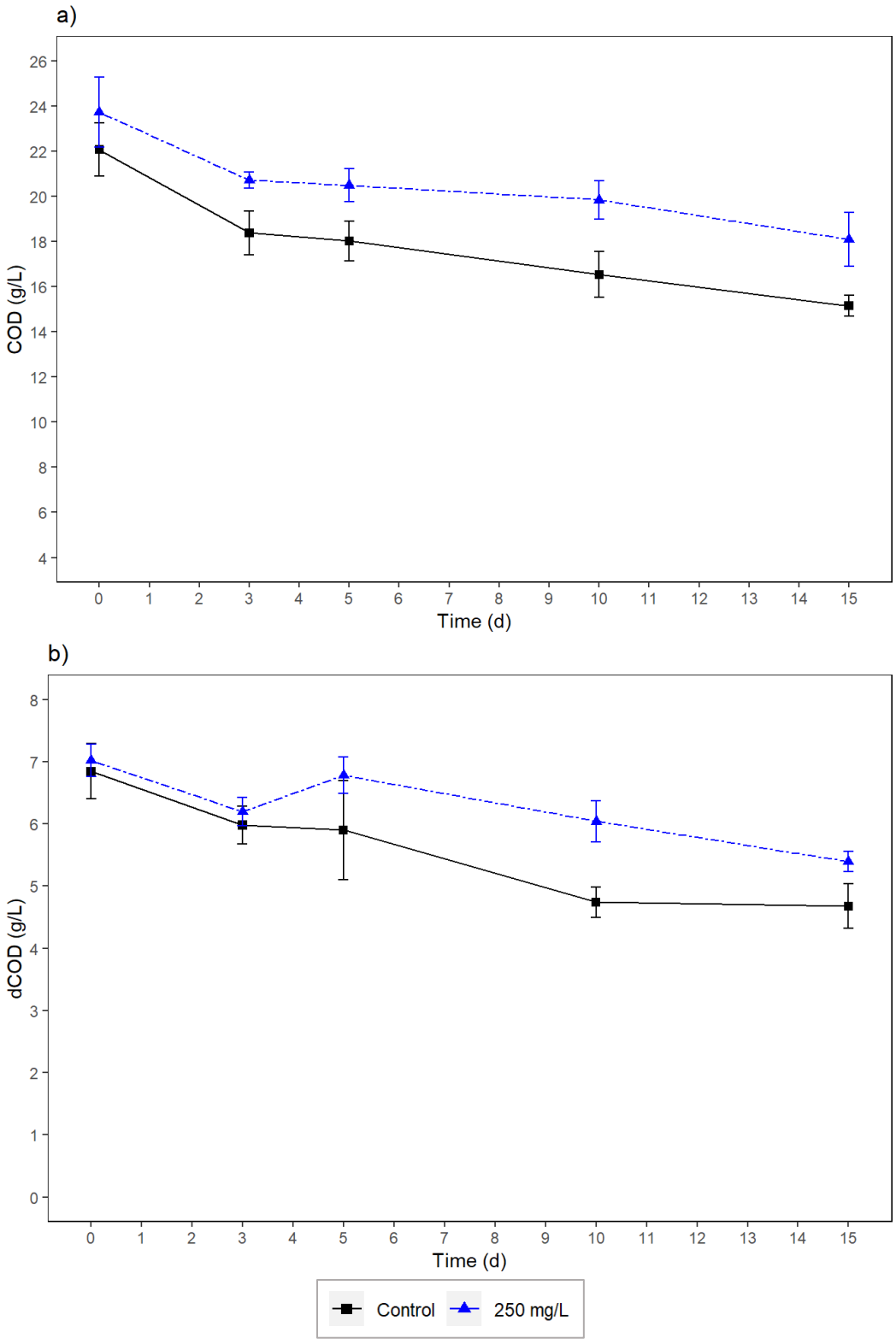


Figure 3.19 Effect of 250 mg CEF/L on total (a) and dissolved (b) COD over time

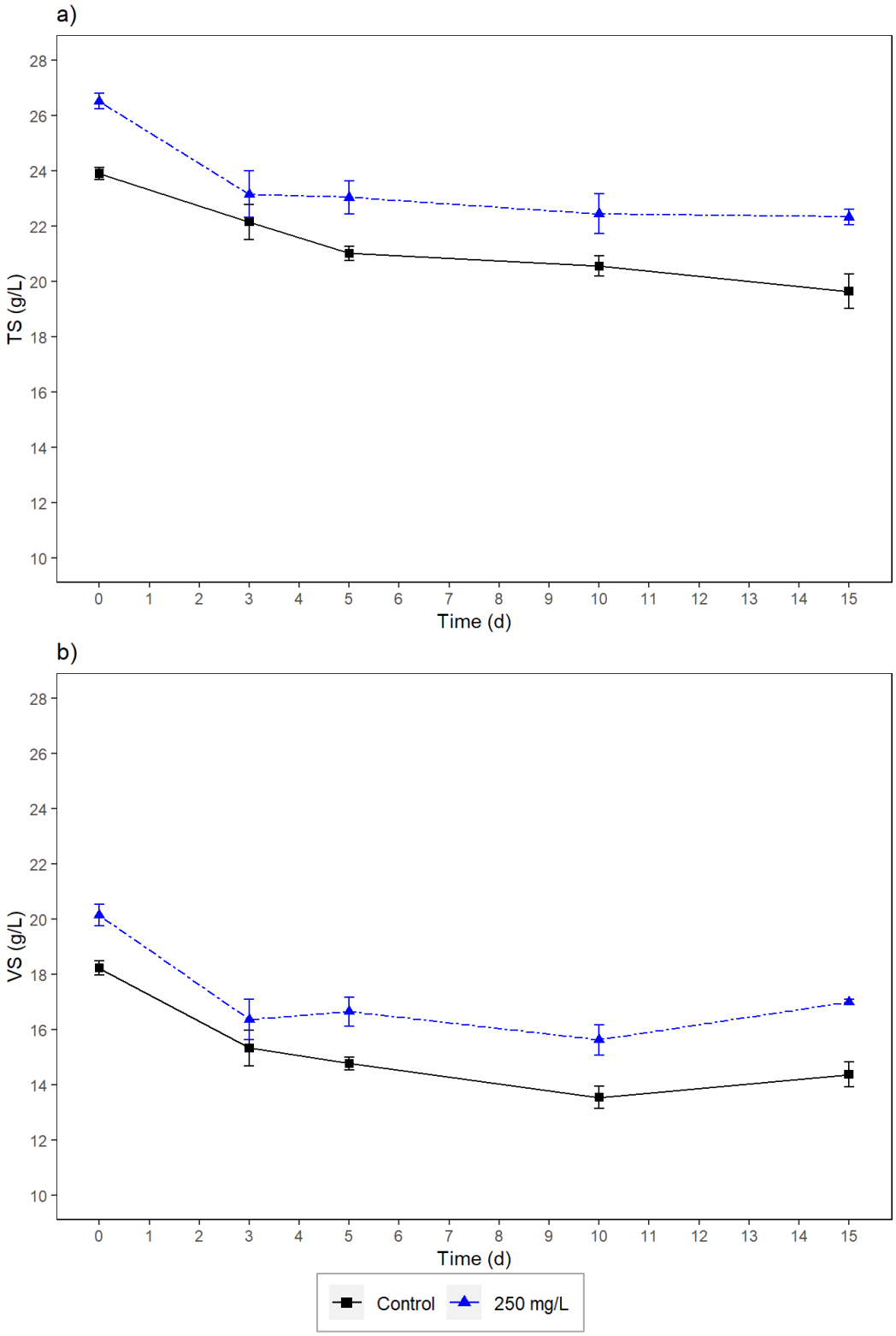


Figure 3.20 Effect of 250 mg CEF/L on total (a) and volatile (b) solids over time i

3.3. Quantification of the ARG *cmy-2*

3.3.1. Standard curve

Figure 3.21 presents the 6-point standard curve generated to calculate the *cmy-2* copy number using the DNA extracted from *Klebsiella pneumoniae* N09-00080. The goodness of fit (R^2) was 0.98, while the slope and the intercept were -0.67 and 22.1, respectively. Both, the slope and the intercept were used to calculate the copy numbers of the samples.

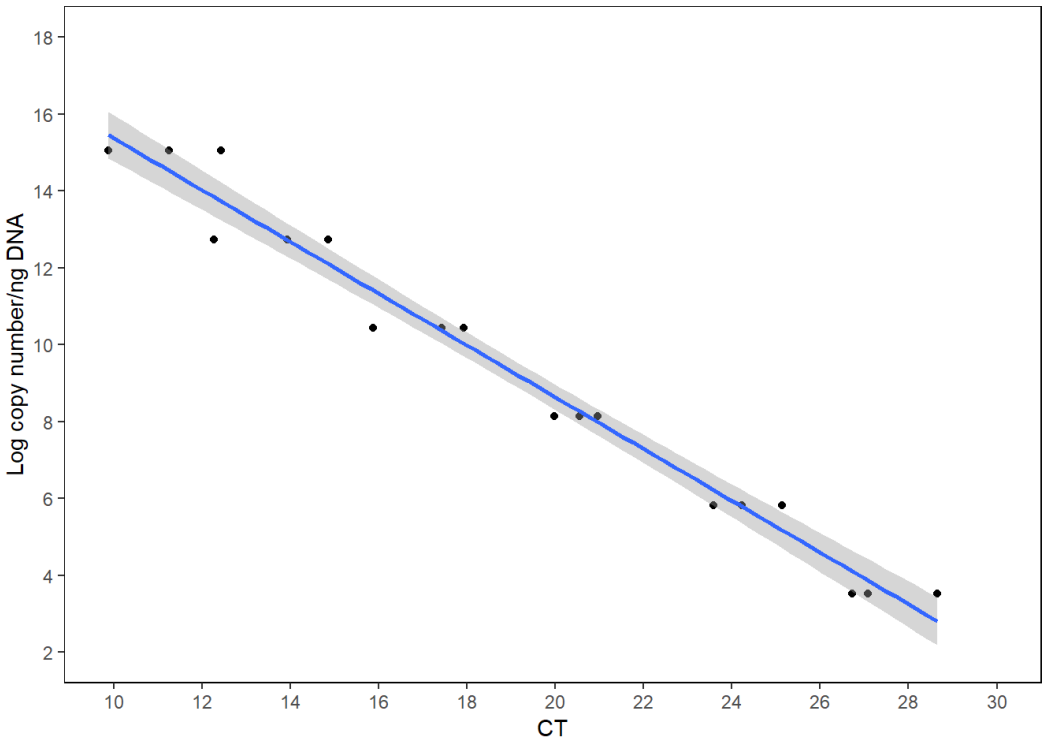


Figure 3.21 Standard curve for quantification of *cmy-2*

3.3.2. Level of *cmy-2* in manure and BSR samples

3.3.2.1 Manure

Figure 3.22 shows the copy numbers of the cephalosporinase *cmy-2* gene per ng of DNA present in 350 mg of centrifuged manure samples. The different manures batches used to feed the BSR were all positive for *cmy-2* with levels ranging from 60 to 160 copies/ng DNA. No DNA was recovered from the first manure (M1), while manure 8 (M8) was not analyzed since it was only used for a couple of weeks and then M9 was used instead. The manures used in the different BMP-like experiments, M5, M6, and M9 had 64 (± 8), 141 (± 14), and 102 (± 26) copies of *cmy-2*/ng DNA, respectively.

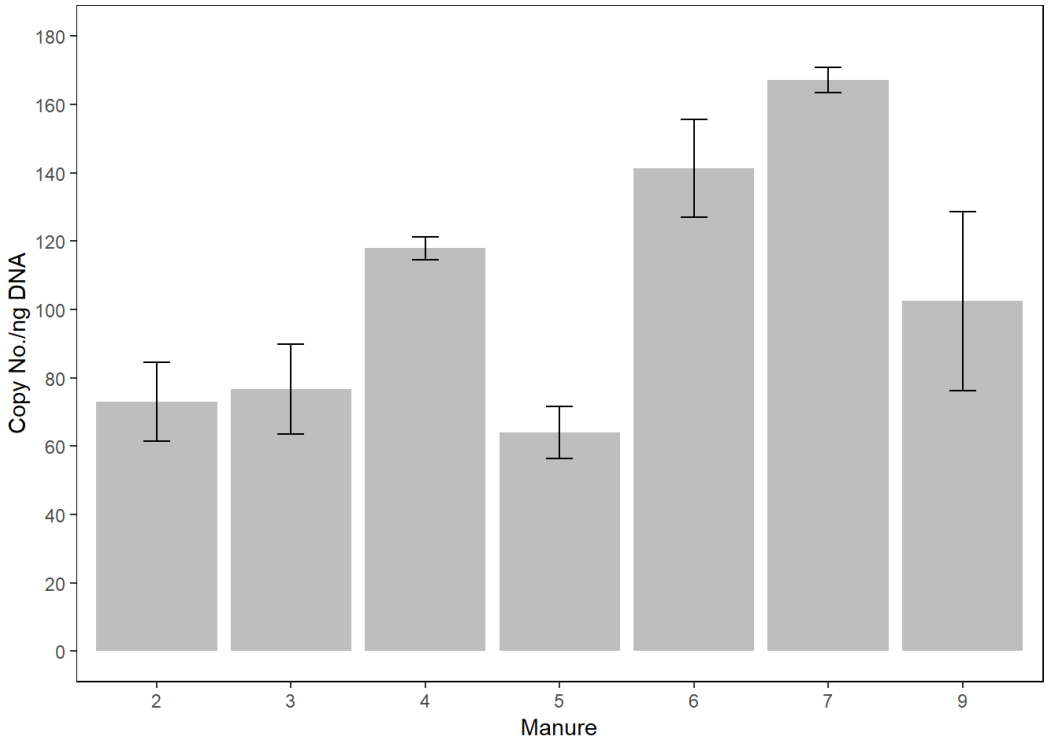


Figure 3.22 Level of *cmy-2* in the different manure batches

3.3.2.2 Digestate

Figure 3.23 shows the copy numbers of the cephalosporinase *cmy-2* gene measured per nanogram of DNA extracted from 350 mg of the centrifuged pellets of the BSR effluent samples during the whole operation period. The gene was detected in all monthly BSR samples (HRT) during the whole period of operation. During the first 9 months, *cmy-2* levels were slightly higher than the following samples. However, despite the high variability of the results, levels in digestate samples represented less than 10% of those found in the raw manure batches. The BSR samples used as inoculum for the different BMP-like experiments, HRT-12, HRT-15, and HRT-20, contained 0.10 (± 0.04), 0.31 (± 0.15), and 1.61 (±0.52) copies of *cmy-2*/ng DNA, respectively.

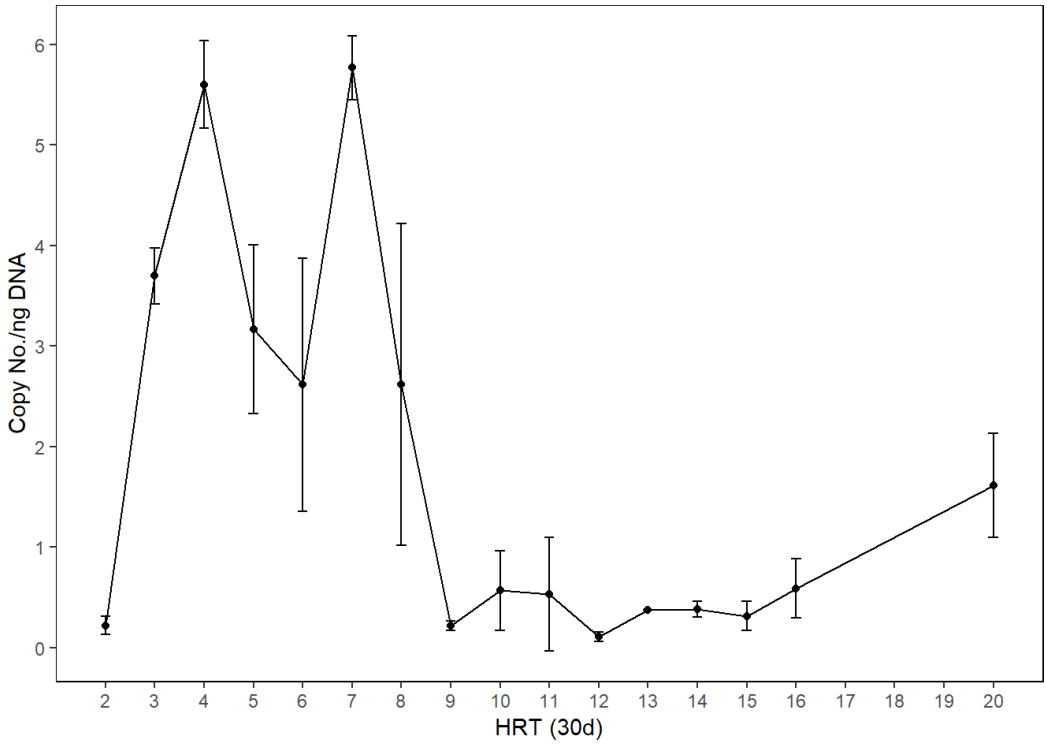


Figure 3.23 Levels of *cmy-2* in the monthly BSR samples

3.3.3. Levels of *cmy-2* in AD amended with 50 mg CEF/L

Figure 3.24 contrasts the *cmy-2* levels in batch AD with and without 50 mg CEF/L during a 15-day digestion period. A summary with the average of the copy No. of control and CEF-treated bottles, as well as the results of the statistical test comparing control and treatment on sampling days is shown in Appendix 14. On days 3, 5 and 10, slightly higher levels were detected in CEF bottles, although only on day 10, the difference was statistically significant (p-value <0.05). The CEF-treated and non-treated batch AD at day 15 resulted in similar *cmy-2* levels (1-2 copies/ng DNA), which means more than 55% *cmy-2* removal was observed.

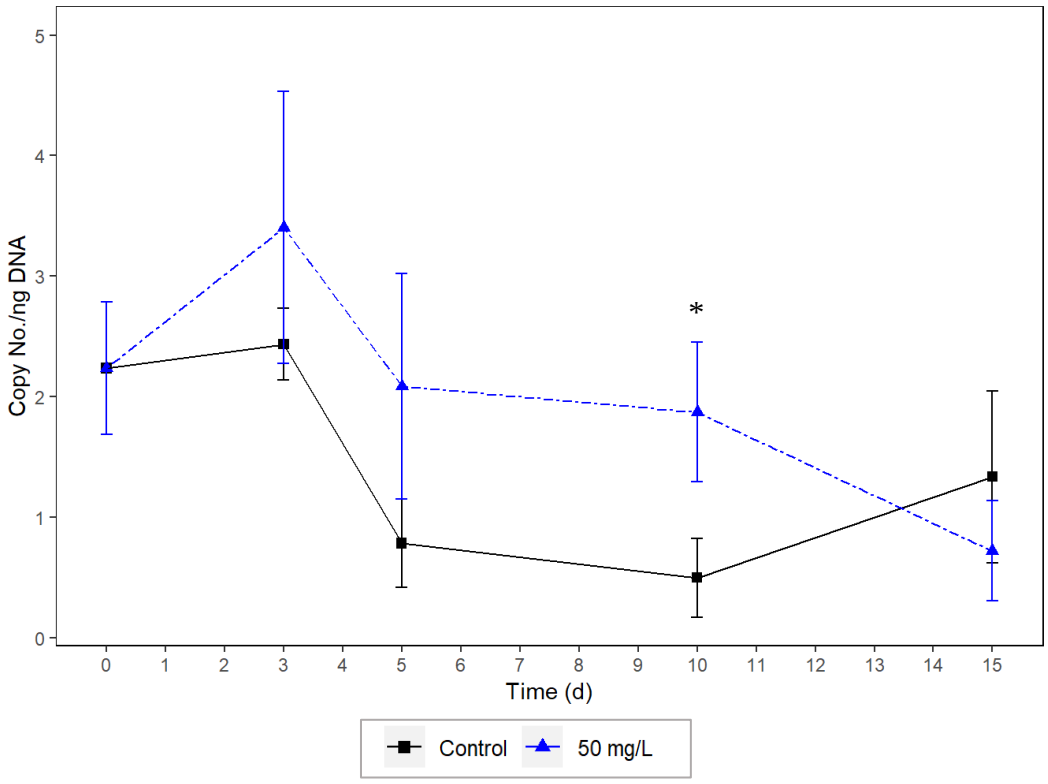


Figure 3.24 Effect of 50 mg CEF/L on *cmy-2* levels.

*Indicates statistically significant difference between control and treatment with 95% confidence.

The results of the Tukey's Honest Significant test are shown in Table 3.7. This test allows to compare whether there is a difference in *cmy-2* levels on the different days of the same conditions (control or treatment). A significant reduction of the copy No. of the gene *cmy-2* was observed in control bottles on days 3, 5, and 10 (p-value <0.05). At the end of the experiment (day 15) there was a reduction although it was not statistically significant (p-value >0.05). Under the presence of 50 mg CEF/L, *cmy-2* levels were not statistically different (p-value >0.05) on the different days, although the average values declined over time.

Table 3.7 Tukey's Honest Significant Difference test for *cmy-2* levels under the presence of 50 mg CEF/L.

	Control				**50 mg CEF/L**			
Sample Day	Diff.	Lower limit copy No. ng/DNA	Upper limit	p-value	Diff.	Lower limit copy No. ng/DNA	Upper limit	p-value
3-0	0.20	-0.85	1.24	0.98	1.17	-0.51	2.85	0.25
5-0	-1.45	-2.50	-0.41	0.005	-0.15	-1.83	1.53	0.99
10-0	-1.74	-2.78	-0.70	0.001	-0.36	-2.04	1.32	0.96
15-0	-0.90	-1.95	0.14	0.11	-1.52	-3.20	0.16	0.09
5-3	-1.65	-2.69	-0.61	0.001	-1.32	-3.00	0.36	0.16
10-3	-1.94	-2.98	-0.89	0.0003	-1.53	-3.21	0.15	0.08
15-3	-1.10	-2.14	-0.06	0.04	-2.68	-4.36	-1.00	0.001
10-5	-0.29	-1.33	0.76	0.91	-0.21	-1.89	1.47	0.99
15-5	0.55	-0.49	1.59	0.50	-1.37	-3.05	0.31	0.14
15-10	0.84	-0.21	1.88	0.15	-1.15	-2.83	0.53	0.26

3.3.4. Levels of *cmy-2* in AD amended with 250 mg CEF/L

The effect of a very high CEF dose (250 mg/L) on *cmy-2* levels during batch AD is shown in Figure 3.25. A summary with the average of the copy No. of control and CEF-treated bottles, as well as the results of the statistical test comparing control and treatment on sampling days is shown in Appendix 15. The bottles dosed with 250 mg CEF/L had significantly higher *cmy-2* copy No. than those CEF-free on days 3, 5 and 15 (p-value < 0.05). At the end of the digestion (day 15), the presence of 250 mg CEF/L reduced the removal of *cmy-2* from 92% in control bottles to 65%.

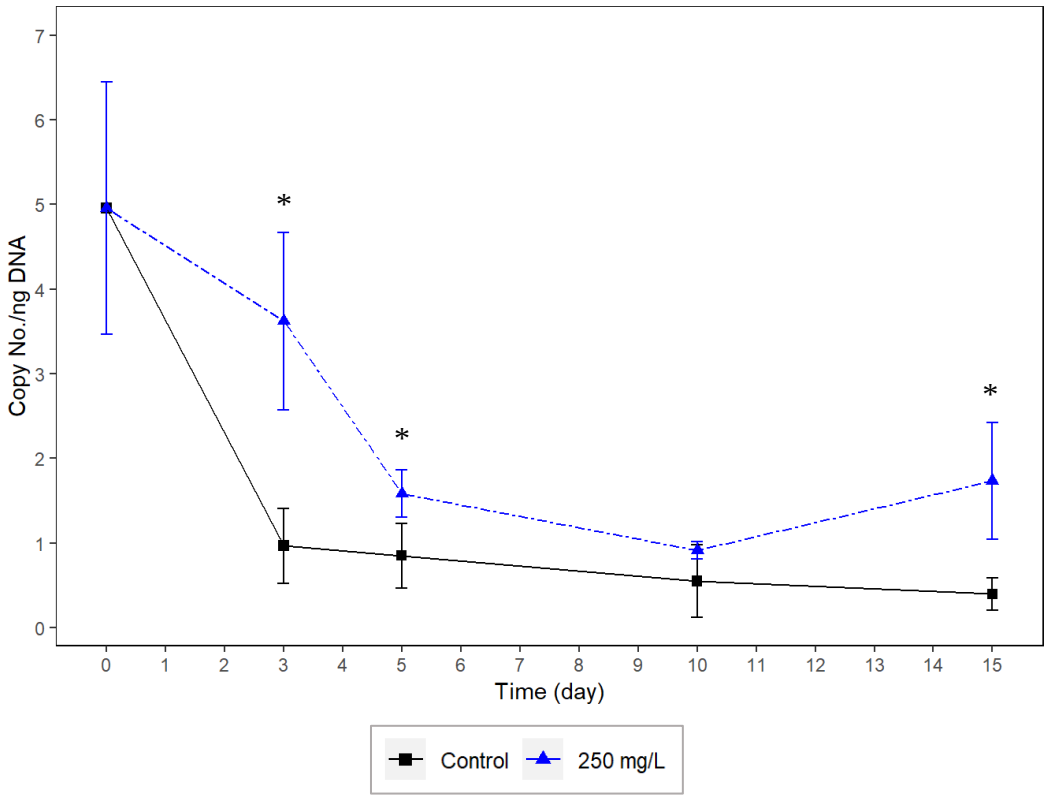


Figure 3.25 Effect of 250 mg CEF/L on levels of *cmy-2*.

*Indicates statistically significant difference between control and treatment with 95% confidence.

The results of the Tukey's Honest Significant test of Experiment 3 are shown in Table 3.8. In this experiment, the controls exhibited higher *cmy-2* removal rates, showing significantly lower levels since day 3 and until the end of the experiment (p-value < 0.05). However, no significant difference was observed within days 3, 5, 10, and 15. On CEF-treated bottles, *cmy-2* levels significantly declined after day 5. Moreover, day 5, 10, and 15 were also different than day 3.

Table 3.8 Tukey's Honest Significant Difference test for *cmy-2* levels under the presence of 250 mg CEF/L.

	Control				**250 mg CEF/L**			
Samples Day	Diff.	Lower limit copy No. ng/DNA	Upper limit	p-value	Diff.	Lower limit copy No. ng/DNA	Upper limit	p-value
3-0	-3.99	-5.62	-2.36	1.4E-05	-1.33	-3.26	0.59	0.25
5-0	-4.10	-5.73	-2.48	1.0E-05	-3.37	-5.30	-1.45	6.1E-05
10-0	-4.41	-6.03	-2.78	4.3E-06	-4.04	-5.96	-2.12	8.7E-05
15-0	-4.56	-6.19	-2.93	2.8E-06	-3.22	-5.14	-1.30	9.0E-05
5-3	-0.12	-1.74	1.51	0.99	-2.04	-3.96	-0.12	0.04
10-3	-0.42	-2.05	1.21	0.93	-2.71	-4.63	-0.78	0.005
15-3	-0.57	-2.20	1.06	0.81	-1.89	-3.81	0.04	0.06
10-5	-0.30	-1.93	1.33	0.98	-0.67	-2.59	1.26	0.82
15-5	-0.46	-2.08	1.17	0.91	0.15	-1.77	2.08	0.99
15-10	-0.15	-1.78	1.47	0.99	0.82	-1.10	2.74	0.69

CHAPTER 4 - DISCUSSION

The main objective of this research was to determine the impact of different CEF concentrations on the performance of mesophilic anaerobic digestion of dairy manure and evaluate the fate of the antibiotic resistance gene *cmy-2* during the process. To this end, three different experiments were carried out. In the first one, increasing CEF concentrations (0.2, 2, 10, and 50 mg/L) were tested to evaluate their effect on methane production and determine the CEF IC_{20}. Also, experiment 1 provided insights into organic matter removal and VFA consumption under the presence of the antibiotic. In the second experiment, time-dependent changes on organic matter removal and the VFA profile caused by the dosing of 50 mg CEF/L during AD digestion were evaluated and linked to the biogas production profile to analyze which stages of AD were affected. Experiment 2 was also used to measure how levels of *cmy-2* were affected by the presence of the antibiotic. The last experiment was performed with the objective of amplifying the effect of CEF on all these parameters by increasing the dose to 250 mg/L, to better understand the processes that were affected.

4.1. Effect of ceftiofur on anaerobic digestion

4.1.1. Effect on methane production

Results from the CEF dosing experiment (Figure 3.1) indicate that only CEF concentrations higher than 10 mg/L might have a significant effect on the final cumulative methane production after a 32-day digestion period. Similar results have been reported by Panseri et al. (2013) and Rodríguez et al. (2017) that observed insignificant effects of comparable CEF concentrations in batch anaerobic mesophilic digesters fed with swine manure and synthetic feed, respectively. Although the feed used in these studies were different, they agree that CEF at the expected concentrations in the manure (~10 mg/L) had a minor impact in methane production in AD. The results from

experiment 1 and 2 suggest that even concentrations as high as 50 mg CEF/L do not cause a major failure of the AD process other than 20% reduction in the final methane production yield. On the other hand, as seen in experiment 3 (Figure 3.13), the methane production was reduced by more than 60% under the presence of 250 mg CEF/L, although this concentration is very unlikely to be found in manure. No other study dealing with such high CEF concentrations (50-250 mg/L) in AD is available in the literature to compare these observations. Moreover, the reported effect of similar concentrations of other cephalosporins on AD is very variable. For example, whereas Beneragama et al. (2013) found that 90 mg/L of cefazolin increased methane production by 9% in thermophilic AD of dairy manure, Lu et al. (2014) reported inconsistent results (increases and reductions of methane production) while using cefalexin in concentrations from 50-2,000 mg/L in mesophilic AD of waste activated sludge (WAS). Nevertheless, these results demonstrate that methanogenesis still occurs even at very high cephalosporin concentrations.

Comparing the effect of different antibiotics on methane production during AD is difficult, since the effects vary considerably and depend on many factors such as the type of feed (swine manure, dairy manure, WAS, or synthetic feed), temperature, retention time, AD system (batch, fed-batch, or continuous), the type and state of the inoculum, the antibiotic and its mode of action. However, within the mesophilic AD of dairy manure assays, CEF seems to have a similar effect on methane production to some tetracyclines (Beneragama et al., 2013; Ke et al., 2014b), and a greater effect than other penicillins and cephalosporins (Kitazono et al., 2015; Lallai et al., 2002; Mitchell et al., 2013; Panseri et al., 2013). However, its effect is low compared to ionophores, which have shown more than 20% inhibition at concentrations below 1 mg/L (Arikan et al., 2018). It is evident that antibiotics known to harm methanogens, such as ionophores, will have a greater impact on methane production than those that do not (such as CEF).

Nevertheless, determining the effect of an antibiotic based only on the final cumulative methane production after a long digestion period might lead to incomplete conclusions, since its effect can be stronger at the beginning of the AD process and then decline over time. This was clearly observed in those bottles treated with 10 mg CEF/L in experiment 1, which were affected to a greater extent during the first 20 days, reaching cumulative methane production reductions as high as 17%, and after a second biogas production peak, reached final values comparable to the control (Figure 3.1). Also, the daily methane production rates support this observation. Although the 10 mg/L treatment and the control had a similar maximum methane production rate, the CEF-treated bottles achieved it one day later, indicating a slow-down of the process, but an eventual recovery. A similar observation was reported by Beneragama et al. (2013) when amending thermophilic anaerobic digesters with 60 and 90 mg/L of cefazolin, a cephalosporin similar to CEF. This phenomenon could be caused by antibiotic degradation over time. The CEF half-life has been reported between 8-22 days (Gilbertson et al., 1990) and some studies have documented more than 70% of CEF degradation during the first 2 weeks of AD (Howes, 2017; Kitazono et al., 2015; Panseri et al., 2013; Rodríguez et al., 2017). Based on this, it can be assumed that after the first few days of operation, part of the CEF dose is degraded, and consequently, the antibiotic loses part of its potency. However, the 50 mg CEF/L treatment did not have the same behavior during either the 15-day or 32-day digestion periods. This could have two explanations. The first one is that even though CEF was been degraded during the process, the initial concentration was high enough to maintain part of the biological activity after partial degradation during the AD period. However, if the digestion time is extended, the antibiotic could further lose potency leading to a second biogas production peak and achieving final yields similar to those of controls. This was observed in other studies looking at long digestions periods (Lu et al., 2014; Zhi and Zhang, 2019). The

second hypothesis is that high antibiotic concentrations harm irreversible key microbial communities in the digester, limiting the amount or the type of organic matter that can be degraded. Nevertheless, this seems improbable since several studies have demonstrated that the microbial population in AD has high resilience and is capable of adapting and recovering after different types of perturbations (Amha et al., 2018). These observations suggest that anaerobic digesters operating with long retention times (> 30 days) could tolerate CEF doses as high as 10 mg/L with minor effects on methane production, while those operating with short retention times (< 20 days) might show more pronounced effects. Unfortunately, no other study has reported similar behavior, thus, more evidence must be collected to confirm this hypothesis.

The inhibitory concentration IC_{10}, IC_{20}, and IC_{25} (Table 3.5) were estimated based on the maximum daily production rates of the reaction curve type model (Eq. 2.7) rather than the ultimate cumulative methane production, since the former could better represent the microbial dynamics of the AD process under the stress of a toxic compound. A similar approach was used by Mai et al., (2018) when determining the IC_{50} of ciprofloxacin in AD. The CEF IC values estimated in these studies were very low compared to other antibiotics. For example, Mai et al. (2018) reported an IC_{20} of 0.11 mg/L of ciprofloxacin while Zitomer et al. (2013) found an IC_{50} below 0.1 mg/L of the ionophore commercially known as *Rumesin*. It also corroborates that CEF does not have a major impact on methane production.

The relatively small effect of even very high cephalosporins concentrations on methane production during AD has been documented in some studies (Beneragama et al., 2016, 2013; Kitazono et al., 2015; Panseri et al., 2013). Beneragama et al. (2013) attributed this attenuated effect to the adaptation of the microbial population to the presence of these type of antibiotics. Cephalosporins have been used to treat animal disease for decades, therefore, resistant bacteria could be part of the

microbial consortia of the manure and related environments. In this study, the consistent presence of the cephalosporinase gene *cmy-2* in several manure samples and in the anaerobic digester, confirms that the antibiotic-resistance carrying bacteria are extensively present in the environment. Whether or not they help to diminish the effect of cephalosporins in AD requires a deeper study.

4.1.2. Effect on the different stages of anaerobic digestion

Since beta-lactams antibiotics like CEF act by interrupting the cell wall synthesis (Ghooi and Thatte, 1995), they do not directly affect methanogens. Hence, the difference in methane production observed in AD containing this type of antibiotic is caused by the disturbance of some microorganisms involved in other stages of AD. In this section, the effect of CEF on the different stages of AD is discussed.

4.1.2.1 Hydrolysis

Results from experiment 1 (Table 3.6) indicate that CEF affected hydrolytic bacteria since the total COD removal was slightly lower in the 50 mg CEF/L-treated bottles. This difference in COD consumption (0.3 ± 0.14 g) matches well with the difference in methane production between the 50 mg CEF/L and control bottles at the end of experiment 1 (Figure 3.1 and calculations shown in Appendix 2). Additionally, CEF-treated bottles, on average, showed lower dCOD levels (higher dCOD net removal) at the end of the 32-day digestion period. This suggests that the microbial population was able to take and consume the soluble COD (dCOD) but it failed to hydrolyze more complex COD. During the AD process, the complex organic matter (COD) needs to be degraded to simpler compounds which are then converted into simpler organic molecules (VFAs) and then into acetate (Amani et al., 2010). These latter stages constitute the soluble COD, which in the end, represents the organic matter that can be converted into methane. Although the effect of 50 mg CEF/L in experiment 2 was not as prominent as in experiment 1, and the COD and dCOD

measurements had more variation (Figure 3.11 and Appendix 8), less hydrolytic activity is suspected during the whole digestion period, since average COD removal levels were lower in CEF-treated bottles. This is supported by the lower TS and VS removal levels in the CEF-treated bottles. The total solids (TS) content includes the total COD and other inorganic material, therefore, COD removal implies a reduction of the TS content, while the VS accounts for the dCOD in a similar way. Additionally, the significantly lower COD removal levels under the presence of 250 mg CEF/L in experiment 3 (Figure 3.19 and Appendix 13), provides evidence of the negative effect of CEF on hydrolysis. The gap of methane production between treated and control reactions can also be attributed to the difference in COD removal. Lower organic matter consumption, in terms of total COD removal, was linked to a decline in biogas production, which is a typical observation in studies about antibiotics and AD (Mai et al., 2018; Rodríguez et al., 2017; Zhi and Zhang, 2019). Third generations cephalosporins act against both, gram-positive and gram-negative bacteria (Devansh and Kumar, 2015), therefore, it is very likely that the hydrolytic community involved in AD is sensitive to CEF. The lower CO_2 production rates observed in CEF-treated bottles (Figures 3.6 and 3.14) could suggest that some of the most affected microorganisms were responsible for the degradations of more oxidized polymers. Poly-saccharides are usually considered more oxidized than proteins, thus, inhibition of their degradations stands a probable option. Unfortunately, the composition of the residual carbon was not analyzed in greater detail in this study, which could help to corroborate this hypothesis.

4.1.2.2 Acidogenesis and acetogenesis

Changes in the VFA profile during different time-points of AD indicate that CEF could disturb both acidogenesis and acetogenesis (Figures 3.8, 3.9, 3.16, and 3.17). Taking a closer look, higher formate, butyrate, and valerate levels, linked to lower acetate concentrations during the first 5 days

of digestion, implies that acetogens were slowed down by the antibiotic, reducing the conversion of these VFAs into acetate. The conversion of VFAs to acetate is not thermodynamically feasible ($\Delta G > 0$) and it can only proceed with the help of microbial syntrophic relationships (Cirne, 2006). Therefore, disturbance to the syntrophic community leads to a decline of acetogenesis. Nonetheless, this effect was temporal in CEF-treated bottles, because by day 10 it was no longer observed. A short term effect on acetogenesis was also observed by Lins et al. (2015) when studying the effect of gentamicin, chloramphenicol, and neomycin on AD. On the other hand, the dramatic drop of propionate levels in digesters containing CEF could be related to the lower H_2 levels (Figures 3.7 and 3.15). When H_2 exceeds tolerable levels (400-500 ppm), it can alter the metabolic pathway and induce the production of propionate via acetate oxidation (Cheng, 2010; Thiele and Zeikus, 1988) through the following reaction:

$$CH_3COOH + CO_2 + 3H_2 \rightarrow CH_3CH_2COOH + 2H_2O \quad (3.1)$$

Lins et al. (2015) observed this propionate production mechanism when H_2 levels increased in AD disturbed with gentamicin, chloramphenicol, and neomycin. In this study, since the control reactions reached higher H_2 levels on day 1 (Figures 3.7 and 3.15), propionate production was induced in the following days, and once H_2 levels returned to normal, propionate levels started to decrease. H_2 levels were always below the limit in CEF-treated reactions, thus, this propionate generation mechanism was never activated. The lower H_2 levels can be associated with the lower butyrate and valerate reduction rates, which produce H_2:

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad (3.2)$$

Therefore, less acetate production from butyrate and valerate caused a decline in H_2 levels, which prevented the propionate generation in the CEF-treated reactions. Although some differences in the VFA profile was observed in the early stages of the process, the effect of CEF on acidogenesis

and acetogenesis was minor, and it did not compromise the stability of the AD process. This can also be observed in the pH of the media, which shows almost no change compared to the control in all the experiments (Figures 3.10 and 3.18).

4.1.2.3 Methanogenesis

This study demonstrates that methanogenesis still occurs even at very high CEF concentrations, as seen in Figures 3.1, 3.5, and 3.13. It is evident that as the CEF dose increases, less methane is produced, but this is attributed to disturbance to the upstream reactions rather than harm to methanogens. When a toxic compound affects methanogens in AD, a rapid increase of acetate and H_2 is seen. This has been extensively observed when antibiotic known to inhibit methanogens are dosed in anaerobic digesters (Arikan et al., 2018; Lins et al., 2015; Mai et al., 2018; Zhi and Zhang, 2019). In this study, even at the highest CEF dose (250 mg/L), no acetate accumulation was observed at any point of the AD process (Figure 3.16) and, consequently, no dramatic changes in the pH occurred (Figure 3.18). Beneragama et al. (2013) observed a similar behavior while studying the effect of cefazolin, a first-generation cephalosporin, on AD of dairy manure. This agrees with what is expected from a beta-lactam antibiotic. This type of antibiotic acts by binding to the penicillin-binding proteins (PBP) responsible for the peptidoglycan cross-linking, preventing cell wall synthesis. Hence, since Archaea lack any form of peptidoglycan in the outside of the cell membrane (Khelaifia and Drancourt, 2012), they are not affected.

Although overall methanogenesis seems to be unaffected, the pathway generating the precursors (H_2, CO_2, and acetate) could have changed due to the presence of CEF. This can be inferred evaluating the biogas quality, measured as the CH_4/CO_2 ratio which was higher in the digesters containing CEF (Figure 3.1c). The difference in the CO_2 productions rates (Figures 3.2, 3.6, and 3.14) and the hydrogen levels in the digester's headspace (Figures 3.7 and 3.15) make this even

more evident. The reduction of hydrogen levels and the drop of the CO_2 production in the CEF-treated bottles suggests that hydrogenotrophic methanogenesis was slightly more prominent. In the literature, some studies have shown an enrichment of hydrogenotrophic methanogens after an antibiotic shock (Lins et al., 2015; Mai et al., 2018). However, in this study, the reduction in CO_2 and H_2 levels are better associated with a reduction of net production rather than higher consumption rates, and the higher CH_4/CO_2 rates are observed only because there was less acetate available for acetotrophic methanogens.

4.2. Fate of *cmy-2* in anaerobic digestion

4.2.1. Levels of *cmy-2* in manure

Considerably high levels of the cephalosporinase gene *cmy-2* were found in all the different manure batches used to feed the BSR (Figure 3.22). Except for M9, all the feed manure batches came from the same farm. This implies that *cmy-2* was consistently present in the microbiome of the herd, since the manures were collected every few months during the nearly 2-year period of this study. Interestingly, ARG levels also seem to be increasing over time (M2-M7), although M5 levels are an exception to this trend. M5 was higher in solids content compared to the other batches and lower DNA yields were achieved, something that could have caused some interference in the quantification of *cmy-2*. Densely populated microbial environments such as manures are known to have the perfect conditions for the development and transference of ARGs (Karkman et al., 2018). Thus, an increase of *cmy-2* levels in the farm over time seems reasonable. Unfortunately, no information about the antibiotic usage regime in the farm was available to evaluate if the presence of antibiotics was adding some selective pressure that could have driven increase in the ARG level or corroborate that these changes were due to natural variation. The presence of several ARGs in animal manures have been extensively documented in the last few years (Howes, 2017; Ma et al.,

2011; Munir and Xagoraraki, 2011). This represents a serious problem because manures are usually applied on agricultural lands as soil fertilizer and this practice can potentially contribute to the spread and accumulation of ARGs in the environment (Munir and Xagoraraki, 2011; Pérez-Valera et al., 2019). Unfortunately, a long-term study on the fate of ARGs in a farm could not be found. Future works should address the development of ARGs and how manure management affects their spread into the environment.

4.2.2. Levels of *cmy-2* after anaerobic digestion

The quantification of *cmy-2* levels of the BSR samples (Figure 3.23) confirmed that AD is a good alternative for ARG removal (> 90%), as some studies have previously suggested (Ghosh et al., 2009; Ma et al., 2011; Sun et al., 2016). ARG removal during AD could be related to the microbial community evolution. Some studies have suggested that the ecological niche is taken by different functional microorganisms during the different stages of AD, and under steady conditions, the functional microorganisms do not allow the growth of some ARGs-carrying bacteria (R. Wang et al., 2018). Although the mechanism is not completely clear, it is well-established that AD is very effective at eliminating pathogens. Thus, assuming that pathogens are more likely to carry ARGs, they should not proliferate under these conditions. The potential for significant reduction of ARGs after an AD process has important implications for agricultural practices. Considered a fairly conventional manure management and biogas generation process, AD can also be suggested as a means for mitigating the proliferation of antibiotic resistant bacteria. Further investigations on this subject are required to assess the fate of other antibiotic-resistance markers to be able to further generalize these observations.

On the other hand, the fact that detectable levels of the ARG are observed in samples from a stable anaerobic digester (BSR) indicates that a group of microorganisms present in a healthy AD process

contains this gene. Ghosh et al. (2009) observed that tetracycline-resistance genes diminish after thermophilic AD but in a second mesophilic AD stage, they increase again. The authors attributed this phenomenon to horizontal gene transfer. However, it is also likely that the gene-carrier microorganisms are able to proliferate when changes in environmental conditions force shifts in microbial community structure and reshape populations in order to be able to perform new biochemical reactions. Sun et al. (2016) observed less abundance of some species of the phylum Bacteroidetes and Proteobacteria during thermophilic AD compared to mesophilic AD. Microorganisms from these phyla are often associated with the hydrolytic stage in AD and are believed to be responsible for carrying most of the ARGs (Sun et al., 2016).

4.2.3. Effect of CEF on *cmy-2* levels during anaerobic digestion

Once the presence of the cephalosporinase gene was confirmed in both the raw manure and the digestate, the next step was to evaluate how CEF would affect its fate during the AD process. Unfortunately, the nutrient media composition used in the batch experiments altered the DNA isolation process reducing the DNA yields by more than 60% in some cases (data not shown). Therefore, the copy numbers in these experiments are likely to be diminished and represent only a fraction of the total. Nonetheless, these results provide good insights about the changes caused by the antibiotic. The CEF dosing experiments show that even under the presence of very high CEF concentrations (50 and 250 mg CEF/L), a significant reduction of *cmy-2* levels can be achieved in a 15-day AD process (Figures 3.24 and 3.25). Howes (2017) documented a similar effect of CEF on another cephalosporinase gene (*cfxA*) during a semi-continuous thermophilic AD process with an HRT of 10 days. These observations indicate that the removal of cephalosporin-resistance genes in AD is not considerably affected by the presence of CEF. However, CEF seems to alter *cmy-2* levels in different points of the AD process. In fact, between days 3 and 5, when the

highest biogas production rates were observed and the highest microbiological activity is presumed, *cmy-2* levels were significantly higher in CEF treatments than controls. This appeared to be related to the type of microorganisms that were active during this time rather than the horizontal transfer of the gene because these levels decreased later on. A community structure analysis is recommended to provide more evidence of the microorganisms carrying the *cmy-2* gene and correlate them to the AD progress. Moreover, a single shock-dose study can only provide information about the ultimate behavior of the ARG already present in AD. However, it does not allow to follow the evolutionary process of development of a novel antibiotic resistance gene.

4.3. Engineering significance of this study

This study provides a better understanding of the effect of one of the most commonly used antibiotics in dairy farms on the performance of mesophilic anaerobic digestion, a well-established technology that has been used for decades to stabilize biowastes such as municipal wastewaters and animal manures and the production of energy-rich biogas. The main contributions can be summarized as follow:

Results from this study indicate that the presence of residual ceftiofur concentrations below 10 mg/L would have a minor impact on the performance and stability of mesophilic anaerobic digesters operating at HRTs between 15 and 30 days. These CEF concentrations represent what could be expected in the manure of herds treated with ceftiofur. With residual ceftiofur concentrations of 10 mg/L or higher, a reduction of more than 10% in methane production is expected, especially in anaerobic digesters working with shorter HRTs (< 20 days). In addition, higher concentrations (≥ 50 mg/L) can reduce methane production rates by more than 20%. Very high concentrations are unlikely to happen on a regular basis, but in case they occur, the results suggest that the stability of the process is not irreversibly affected and only a decline in methane

production can be anticipated. Nevertheless, reductions of 10-20% in biogas production in on-farm anaerobic digesters represent a risk that can compromise the economic sustainability of the process.

The other main contribution of this research is related to the spread of antibiotic resistance genes to the environment. A common practice all over the world is the application of manures as crop fertilizer on land after some stabilization treatment. Our results indicate that a considerable amount of ARGs are present in animal manures and they could be released into the environment, driving the accumulation and development of more resistant microorganisms. These findings serve as evidence of the spread of antibiotic-resistant bacteria due to agricultural activities. Our results highlight that anaerobic digestion stands as a very good alternative for the reduction of these ARGs from animal manures, alleviating some of the risks in the greater environment. This also suggests that even under the presence of antibiotics, the removal of ARG can be achieved in anaerobic digestion. These findings deserve further attention, as the proliferation and spread of ARGs represents a serious public health risk.

CHAPTER 5 -

LIMITATIONS AND RECOMMENDATIONS FOR FUTURE WORK

Several limitations were identified for this study that can be taken into consideration when conducting future research projects on antibiotics and anaerobic digestion. The interpretation of the results of this project is limited to mesophilic (35 °C) anaerobic digestion of dairy manure, with digestion periods between 15 to 32 days and considering that the inoculum source was a continuously-stirred anaerobic dairy manure digester operating under mesophilic conditions at an HRT of 30 days. Any other conditions could not be represented by this study. Also, this research project only used one type of antibiotic, a cephalosporin from the family of the beta-lactams antibiotics, which are well known to have little to no effect on methanogens. Future studies on this topic should evaluate a variety of antibiotic including those known to have significant effects on methanogens. This will provide more data and broader insights on the type and magnitude of impact antibiotics can have on AD processes.

The effect of ceftiofur was evaluated using biochemical signals such as biogas composition, organic matter removal (COD, dCOD, TS, and VS), VFAs, and pH. However, other aspects could not be quantified during the study due to lack of appropriated equipment, such as the ceftiofur degradation over time, microbial biomass quantification/identification, and microbial community structure. Future studies should attempt to evaluate these parameters since they could provide a better understanding of the digesters affected by the presence of antibiotics.

The number of biological replicates was limited to two or three for each of the treatments by the capacity of the respirometers, which made it difficult to find statistical significance in the results. Increasing the sample size (biological replicates) is recommended to improve the statistical rigour of the research. In addition, this study was designed to measure some biochemical and biological

markers during specific days of the batch anaerobic digestion experiments. Although these time-points are thought to represent the main stages of the AD process, more and better information can be collected with additional samples during the digestion.

Future works should consider that animal manure rather than synthetic feeds introduces higher variation levels on the measurements due to high heterogeneity. For example, COD and dCOD measurements were considerably affected by the high insoluble content in the samples analyzed, increasing the variation and experimental errors. Another example is the DNA extraction yields that were affected by the high salt content of the nutrient media used in the batch experiments, which could have diminished the *cmy-2* quantification. Another issue that should be considered when using raw manure is the presence of undesirable or toxic compounds that can affect the performance of the anaerobic digester. During this study, 2 batches of manure, M7 and M8, fed to the inoculum source (BSR), were suspected to contain a toxic compound, probably copper sulfate, derived from sanitization practices and manure management at the farm.

The absolute *cmy-2* copy number was calculated using a calibration curve generated from bacteria used as an antibiotic-resistant reference and then normalized against the total DNA. There are other known methods to estimate levels of ARG, like the relative quantification of the 16S rRNA gene. However, the quantification using a standard curve was recommended by Dr. Ayush Kumar's team in the Antimicrobial Resistance Laboratory of the Department of Microbiology and Medical Microbiology at the University of Manitoba because this method is less affected by the presence of multicopy plasmids or uncharacterized bacteria. Nevertheless, in order to investigate the ARGs hosts and the reason why the gene is removed during AD, future studies should consider determining the 16S gene which would provide insights about the microbial processes involved.

CHAPTER 6 - CONCLUSIONS

This study evaluated the effect of ceftiofur, one of the most commonly used antibiotics in dairy farms, on the performance of mesophilic anaerobic digestion of dairy manure. It also determined the fate of the ARG *cmy-2*, a ceftiofur resistance marker, in a mesophilic anaerobic digester and how the presence of ceftiofur alters the ARG removal in 15-day batch anaerobic digestions. The evidence collected during the study led to the following conclusions:

Low ceftiofur concentrations (0.2-2 mg/L) do not affect either the methane production or the stability of mesophilic anaerobic digestion of dairy manure in 32-day digestion periods. Ceftiofur concentrations of 10 mg/L can significantly reduce methane productivity by more than 10% especially in anaerobic digesters working with shorter retention times (HRT < 20 days), while higher ceftiofur concentrations (> 50 mg/L) can dramatically reduce methane production by up to 60%. Nevertheless, even with those high concentrations, the stability of the anaerobic digestion process is not compromised.

The mechanism by which ceftiofur exerts its effect on anaerobic digestion is through the inhibition of hydrolytic bacteria that are responsible for solubilizing complex organic matter. This is reflected in a drop in organic matter removal during the process. In addition, ceftiofur seems to slow the acetogenic consortia down which is observed through lower VFA conversion into acetate, although this effect is temporal and does not affect the overall stability of the process. This study also corroborates that ceftiofur does not have an effect on methanogens even at very high concentrations, which is supported by the observation that neither acetate nor hydrogen accumulated during the process.

The absolute *cmy-2* quantification indicates that high amounts of this cephalosporinase gene are being released into the environment since high levels of the gene were consistently found in dairy

manure of an operating commercial farm. Nevertheless, the results strongly suggest that levels of this ARG can be successfully reduced in mesophilic anaerobic digestion, although it cannot be completely removed. Also, the presence of even very high ceftiofur concentrations (50-250 mg/L) does not seem to considerably increase levels of *cmy-2* in 15-day mesophilic anaerobic digestion trials. However, a long-term study is required to determine if the antibiotic can influence an incremental change of the abundance of this and other ARGs.

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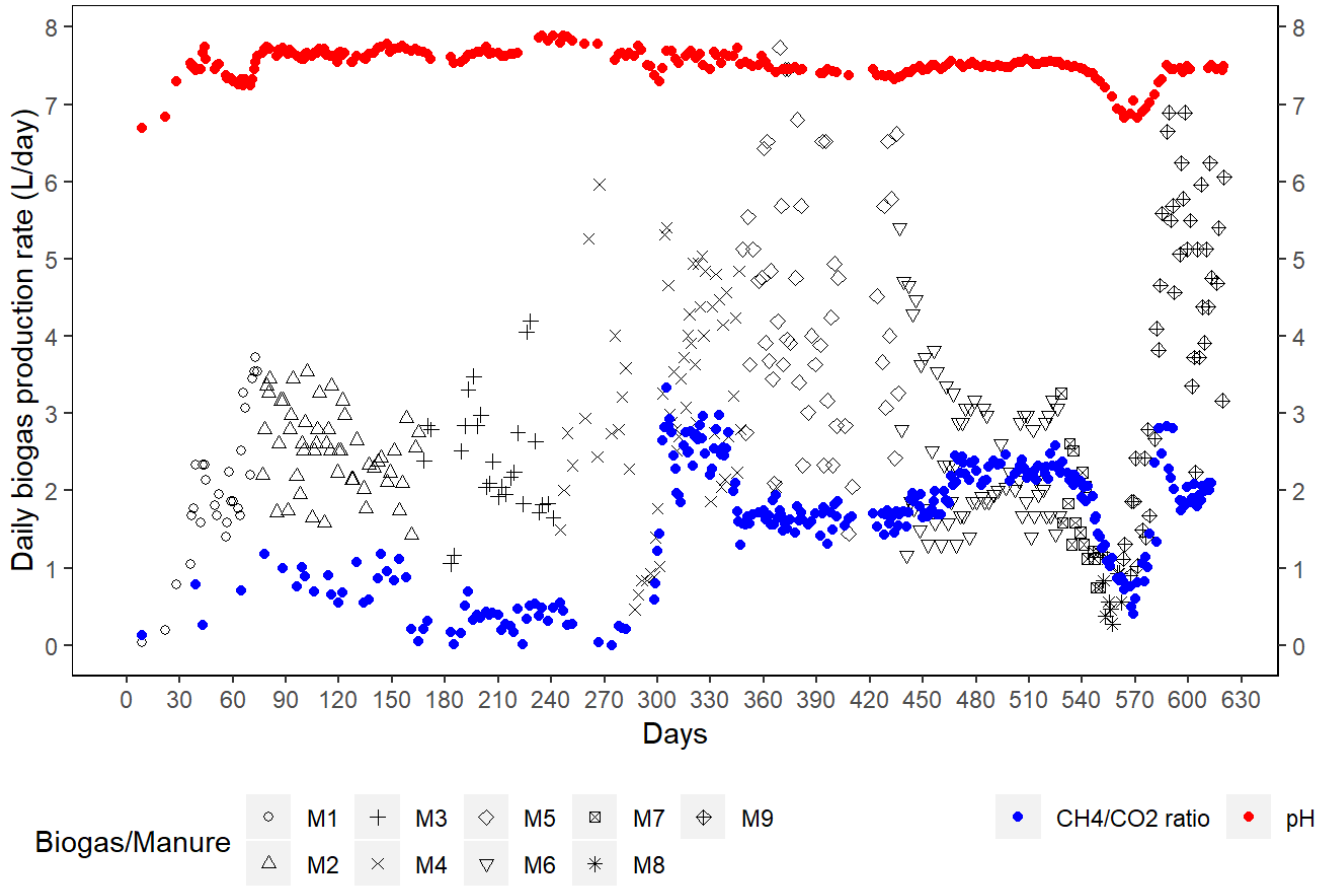
APPENDICES

Appendix 1 BSR performance during operation period



Appendix 2. Summary of the effect of different CEF concentrations on CH_4 and CO_2 production

		CH4								CO2							
Day	Treatment	Cumulative production mL/g VS	SD	Reduction	p-value	Daily rate mL/d-g Vs	SD	Reduction	p-value	Cumulative production mL/g VS	SD	Reduction	p-value	CO2 rate mL/d-g Vs	SD	Reduction	p-value
0.5	Control	9.13	0.40			18.26	0.81			7.65	1.58			15.30	3.15		
	0.2 mg/L	8.28	0.50	9%	0.09	16.55	1.00	9%	0.09	7.49	0.80	2%	0.88	14.97	1.60	2%	0.88
	2 mg/L	8.62	0.65	6%	0.33	17.25	1.30	6%	0.33	7.02	0.86	8%	0.59	14.05	1.72	8%	0.59
	10 mg/L	8.17	0.11	11%	0.04	16.34	0.23	11%	0.04	6.82	0.14	11%	0.46	13.64	0.29	11%	0.46
	50 mg/L	7.15	0.52	22%	0.008	14.30	1.04	22%	0.008	6.17	0.19	19%	0.24	12.34	0.38	19%	0.24
1	Control	23.30	0.82			28.33	1.01			17.03	3.08			18.76	3.37		
	0.2 mg/L	20.89	1.88	10%	0.14	25.22	2.75	11%	0.18	16.60	2.30	3%	0.86	18.22	3.01	3%	0.85
	2 mg/L	20.77	1.24	11%	0.05	24.29	1.19	14%	0.01	14.22	1.73	16%	0.26	14.40	1.74	23%	0.14
	10 mg/L	19.64	0.71	16%	0.005	22.94	1.23	19%	0.005	13.54	0.20	20%	0.19	13.45	0.25	28%	0.11
	50 mg/L	17.63	0.99	24%	0.002	20.97	0.98	26%	0.0008	12.37	0.42	27%	0.12	12.41	0.50	34%	0.08
2	Control	56.46	2.61			33.16	1.80			37.34	5.16			20.32	2.09		
	0.2 mg/L	53.18	5.37	6%	0.41	32.29	3.57	3%	0.73	37.18	5.10	0%	0.97	20.58	2.81	-1%	0.90
	2 mg/L	52.57	2.50	7%	0.14	31.81	1.29	4%	0.35	31.25	3.95	16%	0.18	17.03	2.30	16%	0.14
	10 mg/L	46.66	2.99	17%	0.01	27.02	2.37	19%	0.03	25.23	0.82	32%	0.05	11.69	0.64	42%	0.01
	50 mg/L	41.17	4.64	27%	0.01	23.53	4.02	29%	0.04	21.97	0.10	41%	0.03	9.60	0.48	53%	0.009
3	Control	92.00	6.06			35.54	3.79			56.65	7.38			19.31	2.33		
	0.2 mg/L	89.56	7.62	3%	0.69	36.39	2.90	-2%	0.76	57.32	6.79	-1%	0.91	20.14	1.76	-4%	0.65
	2 mg/L	88.25	1.46	4%	0.40	35.68	1.53	0%	0.96	49.55	4.16	13%	0.24	18.30	0.58	5%	0.53
	10 mg/L	77.75	5.96	15%	0.04	31.09	4.30	13%	0.25	39.31	2.80	31%	0.04	14.08	2.04	27%	0.04
	50 mg/L	66.04	9.84	28%	0.02	24.87	5.24	30%	0.05	30.48	0.65	46%	0.02	8.51	0.58	56%	0.01
4	Control	124.72	13.14			32.72	7.63			72.31	10.35			15.66	3.37		
	0.2 mg/L	124.52	8.61	0%	0.98	34.95	2.11	-7%	0.67	74.19	7.68	-3%	0.81	16.88	0.93	-8%	0.60
	2 mg/L	121.76	2.55	2%	0.74	33.50	3.05	-2%	0.88	65.32	3.58	10%	0.37	15.77	0.91	-1%	0.96
	10 mg/L	111.62	7.48	11%	0.23	33.87	1.75	-4%	0.82	54.97	3.16	24%	0.09	15.66	0.56	0%	0.99
	50 mg/L	90.78	12.27	27%	0.03	24.74	2.44	24%	0.21	38.99	0.16	46%	0.03	8.52	0.69	46%	0.06

Day	Treatment	CH4								CO2							
		Cumulative production mL/g VS	SD	Reduction	p-value	Daily rate mL/d-g Vs	SD	Reduction	p-value	Cumulative production mL/g VS	SD	Reduction	p-value	CO2 rate mL/d-g Vs	SD	Reduction	p-value
5	Control	152.62	13.25			27.89	1.77			86.14	10.65			13.84	0.29		
	0.2 mg/L	151.42	8.71	1%	0.90	26.91	1.06	4%	0.46	87.40	8.84	-1%	0.88	13.21	1.41	5%	0.52
	2 mg/L	144.17	2.01	6%	0.38	22.41	0.56	19%	0.02	76.16	4.20	12%	0.24	10.85	0.62	22%	0.01
	10 mg/L	137.49	6.99	10%	0.18	25.88	1.67	7%	0.22	67.56	3.65	22%	0.08	12.59	0.80	9%	0.10
	50 mg/L	111.37	11.48	27%	0.02	20.58	1.25	26%	0.01	47.93	1.74	44%	0.02	8.93	1.68	35%	0.03
6	Control	168.04	13.10			15.43	1.65			93.56	10.38			7.42	0.51		
	0.2 mg/L	167.13	6.82	1%	0.92	15.71	3.62	-2%	0.91	94.96	9.35	-1%	0.87	7.56	1.77	-2%	0.91
	2 mg/L	156.24	3.50	7%	0.26	12.06	3.62	22%	0.25	81.91	5.96	12%	0.18	5.75	1.92	23%	0.27
	10 mg/L	153.93	6.09	8%	0.19	16.43	3.10	-7%	0.65	75.36	4.23	19%	0.08	7.80	1.23	-5%	0.66
	50 mg/L	128.92	9.71	23%	0.02	17.56	1.80	-14%	0.20	56.22	3.17	40%	0.02	8.29	1.46	-12%	0.41
7	Control	179.00	11.60			10.95	1.57			99.19	9.85			5.63	0.83		
	0.2 mg/L	177.42	7.65	1%	0.86	10.29	2.22	6%	0.70	100.28	10.46	-1%	0.90	5.33	1.36	5%	0.76
	2 mg/L	163.89	6.60	8%	0.14	7.66	4.03	30%	0.29	85.75	8.11	14%	0.14	3.84	2.16	32%	0.29
	10 mg/L	161.54	4.44	10%	0.11	7.61	2.93	31%	0.18	79.06	4.07	20%	0.06	3.69	1.27	34%	0.10
	50 mg/L	141.97	10.38	21%	0.02	13.04	0.80	-19%	0.13	62.72	3.03	37%	0.02	6.51	0.28	-16%	0.20
8	Control	187.19	10.59			8.20	1.10			103.86	9.48			4.67	0.70		
	0.2 mg/L	184.56	10.05	1%	0.77	7.14	2.43	13%	0.55	104.49	12.12	-1%	0.95	4.21	1.71	10%	0.70
	2 mg/L	170.21	9.52	9%	0.11	6.32	3.14	23%	0.41	89.28	10.04	14%	0.14	3.53	1.93	25%	0.42
	10 mg/L	166.98	4.23	11%	0.07	5.45	0.21	34%	0.05	81.93	3.91	21%	0.04	2.88	0.19	38%	0.04
	50 mg/L	151.23	10.18	19%	0.01	9.26	0.27	-13%	0.23	67.71	3.04	35%	0.02	4.99	0.01	-7%	0.51
9	Control	193.22	9.73			6.02	1.10			107.44	9.28			3.58	0.65		
	0.2 mg/L	191.89	10.98	1%	0.88	7.33	0.99	-22%	0.20	109.00	13.07	-1%	0.88	4.50	0.95	-26%	0.25
	2 mg/L	176.36	12.19	9%	0.14	6.15	2.95	-2%	0.95	92.91	11.99	14%	0.18	3.63	1.98	-2%	0.97
	10 mg/L	171.37	4.39	11%	0.04	4.39	0.35	27%	0.11	84.32	3.88	22%	0.03	2.39	0.31	33%	0.07
	50 mg/L	159.19	7.58	18%	0.01	7.96	2.63	-32%	0.33	71.97	4.17	33%	0.01	4.25	1.17	-19%	0.44

		CH4								CO2							
Day	Treatment	Cumulative production mL/g VS	SD	Reduction	p-value	Daily rate mL/d-g Vs	SD	Reduction	p-value	Cumulative production mL/g VS	SD	Reduction	p-value	CO2 rate mL/d-g Vs	SD	Reduction	p-value
10	Control	199.75	8.96			6.53	0.81			111.32	9.13			3.88	0.47		
	0.2 mg/L	199.14	11.94	0%	0.95	7.25	1.03	-11%	0.40	113.64	14.04	-2%	0.82	4.65	0.98	-20%	0.31
	2 mg/L	182.45	14.45	9%	0.17	6.09	2.52	7%	0.79	96.63	13.75	13%	0.21	3.72	1.81	4%	0.89
	10 mg/L	175.14	4.24	12%	0.03	3.77	0.20	42%	0.02	86.36	3.82	22%	0.03	2.03	0.07	48%	0.02
	50 mg/L	165.65	6.58	17%	0.01	6.47	1.09	1%	0.94	75.39	4.51	32%	0.01	3.43	0.34	12%	0.25
15	Control	221.54	2.68			4.36	1.26			125.51	5.74			2.84	0.87		
	0.2 mg/L	222.43	19.61	0%	0.94	4.66	1.58	-7%	0.81	129.80	20.61	-3%	0.76	3.23	1.33	-14%	0.69
	2 mg/L	204.13	18.85	8%	0.25	4.34	0.88	1%	0.98	111.25	17.56	11%	0.29	2.92	0.77	-3%	0.90
	10 mg/L	187.90	2.07	15%	0.0001	2.55	0.44	41%	0.12	93.31	2.59	26%	0.004	1.39	0.25	51%	0.09
	50 mg/L	185.39	6.19	16%	0.004	3.95	0.18	9%	0.63	86.32	4.41	31%	0.001	2.19	0.07	23%	0.32
20	Control	237.96	1.12			3.28	0.66			137.30	4.96			2.36	0.56		
	0.2 mg/L	240.68	23.55	-1%	0.86	3.65	0.79	-11%	0.57	143.26	24.46	-4%	0.72	2.69	0.77	-14%	0.58
	2 mg/L	222.85	25.29	6%	0.41	3.74	1.29	-14%	0.62	125.36	23.13	9%	0.47	2.82	1.12	-20%	0.57
	10 mg/L	205.70	6.75	14%	0.01	3.56	1.73	-8%	0.82	105.05	4.12	23%	0.001	2.35	1.33	0%	0.99
	50 mg/L	193.85	6.49	19%	0.01	1.69	0.21	48%	0.04	91.34	3.81	33%	0.0003	1.00	0.15	57%	0.04
25	Control	250.97	2.69			2.60	0.48			147.48	3.99			2.04	0.43		
	0.2 mg/L	256.53	28.15	-2%	0.77	3.17	0.92	-22%	0.42	156.23	28.79	-6%	0.65	2.60	0.87	-28%	0.39
	2 mg/L	236.36	27.49	6%	0.45	2.70	0.47	-4%	0.81	136.10	25.25	8%	0.52	2.15	0.42	-6%	0.76
	10 mg/L	228.33	12.99	9%	0.09	4.52	1.27	-74%	0.11	121.96	9.07	17%	0.003	3.38	0.99	-66%	0.13
	50 mg/L	200.43	6.50	20%	0.002	1.32	0.31	49%	0.02	95.62	3.70	35%	0.0001	0.86	0.20	58%	0.03
32	Control	263.19	4.46			1.75	0.30			157.01	3.50			1.36	0.23		
	0.2 mg/L	271.83	28.11	-3%	0.65	2.18	0.03	-25%	0.12	168.57	28.86	-7%	0.56	1.76	0.02	-29%	0.09
	2 mg/L	249.72	30.04	5%	0.52	1.91	0.50	-9%	0.66	146.67	27.64	7%	0.58	1.51	0.40	-11%	0.61
	10 mg/L	248.50	13.45	6%	0.19	2.88	0.50	-65%	0.04	137.22	9.65	13%	0.06	2.18	0.27	-60%	0.02
	50 mg/L	207.31	5.95	21%	0.0003	0.98	0.09	44%	0.04	100.08	4.00	36%	0.0001	0.64	0.06	53%	0.03

p-values were calculated with a t-test. Green color highlights the significant difference between treatment and control with a p-value <0.005.

Appendix 3 4-P logistic model summary for CEF IC_{20} estimation

Parameter	Value
Bottom asymptote:	-1.65
Top asymptote:	0.99
Inflexion point at (x, y):	4.68, -0.33
Goodness of fit:	0.99
Weighted Goodness of fit:	0.99
Standard error:	0.006, 0.003

Appendix 4 Summary of the effect of 50 mg CEF/L on CH_4 and CO_2 production

		CH_4								CO_2							
Day	Treatment	Cumulative production	SD	Reduction	p-value	Daily rate	SD	Reduction	p-value	Cumulative production	SD	Reduction	p-value	Daily rate	SD	Reduction	p-value
		mL/g VS				mL/d-g Vs		mL/g VS		mL/g VS				mL/d-g VS			
1	Control	11.5	1.7			11.5	1.7			11.3	1.6			11.3	1.6		
	50 mg/L	11.5	1.0	0%	0.99	11.5	1.0	0%	0.99	10.8	1.1	4%	0.49	10.8	1.1	4%	0.49
2	Control	28.3	3.2			16.8	1.8			23.3	2.5			12.0	1.1		
	50 mg/L	25.8	2.4	9%	0.10	14.3	1.5	15%	0.01	18.6	1.8	20%	0.0008	7.8	0.7	35%	0.000001
3	Control	49.0	5.3			20.6	2.4			35.6	3.1			12.3	0.8		
	50 mg/L	44.3	4.6	9%	0.08	18.5	2.3	10%	0.10	27.3	2.9	23%	0.0001	8.7	1.2	30%	0.0001
4	Control	75.3	5.7			23.9	3.1			48.7	2.4			11.7	0.9		
	50 mg/L	66.9	8.4	11%	0.07	21.8	3.7	8%	0.33	38.8	3.2	20%	0.0002	10.4	1.4	11%	0.08
5	Control	98.4	9.0			23.2	5.8			58.3	2.5			9.6	2.1		
	50 mg/L	88.1	14.5	11%	0.17	21.2	7.1	9%	0.61	48.2	5.2	17%	0.003	9.4	2.8	2%	0.87
6	Control	128.0	4.9			24.8	3.9			68.9	2.3			9.6	1.7		
	50 mg/L	111.2	12.1	13%	0.06	22.0	4.6	11%	0.39	57.6	5.2	16%	0.01	9.3	2.0	3%	0.85
7	Control	147.4	4.4			19.4	2.3			75.7	2.6			6.8	0.8		
	50 mg/L	128.8	14.2	13%	0.07	17.6	2.6	9%	0.34	64.1	6.1	15%	0.02	6.6	1.0	4%	0.69
8	Control	162.5	4.1			15.1	2.8			81.0	2.8			5.3	1.0		
	50 mg/L	141.8	17.1	13%	0.09	13.0	3.3	14%	0.38	68.6	7.1	15%	0.03	4.5	1.1	14%	0.34
9	Control	172.3	5.1			9.8	3.3			84.7	2.9			3.6	1.2		
	50 mg/L	151.1	18.9	12%	0.11	9.2	2.4	6%	0.77	72.0	7.8	15%	0.04	3.3	0.8	9%	0.65
10	Control	177.9	5.5			5.6	1.0			87.0	3.1			2.3	0.4		
	50 mg/L	156.6	18.7	12%	0.10	5.5	2.3	1%	0.96	74.0	8.0	15%	0.04	2.1	0.8	10%	0.63
15	Control	195.7	1.7			3.7	1.0			95.5	2.5			2.0	0.6		
	50 mg/L	172.1	9.7	12%	0.17	2.1	0.5	43%	0.21	81.1	0.6	15%	0.06	0.9	0.2	56%	0.20

p-values were calculated with a t-test. Green color indicates significant difference between treatment and control with a p-value <0.005.

Appendix 5 Summary of the effect of 50 mg CEF/L on H_2 levels

Day	Treatment	H_2	SD	Difference	p-value
		ppm		ppm	
1	Control	1959	497		
	50 mg/L	690	79	1269	0.0001
2	Control	664	94		
	50 mg/L	408	31	257	0.0001
3	Control	299	31		
	50 mg/L	352	46	-54	0.02
4	Control	198	18		
	50 mg/L	278	22	-80	0.0001
5	Control	148	9		
	50 mg/L	191	13	-42	0.0001
6	Control	132	10		
	50 mg/L	157	12	-26	0.02
7	Control	125	14		
	50 mg/L	140	5	-15	0.13
8	Control	118	9		
	50 mg/L	134	5	-16	0.03
9	Control	103	5		
	50 mg/L	121	9	-18	0.02
10	Control	85	5		
	50 mg/L	102	14	-17	0.08
15	Control	67	8		
	50 mg/L	76	7	-8	0.38

p-values were calculated with a t-test. Green color means significant difference between treatment and control with a p-value < 0.05.

Appendix 6 Summary of the effect of 50 mg CEF/L on total VFA concentration over time

Day	Treatment	Total VFA mg/L	SD	Difference mg/L	p-value
0	Control	2,141	40		
	50 mg/L	2,141	40	0	1.0000
3	Control	1,731	30		
	50 mg/L	1,424	18	307	0.00001
5	Control	1,235	344		
	50 mg/L	1,161	481	74	0.81
10	Control	106	5		
	50 mg/L	121	3	-15	0.006
15	Control	102	15		
	50 mg/L	94	13	8	0.43

Difference was calculated as the value of the control minus the treatment; p-values were calculated with a t-test. Green color means significant difference between treatment and control with a p-value < 0.05; n=4.

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Appendix 7 Summary of the effect of 50 mg CEF/L on the VFA profile over time

Day	Treatment	Formate				Acetate				Propionate			
		mg/L	SD	Diff.	p-value	mg/L	SD	Diff.	p-value	mg/L	SD	Diff.	p-value
0	Control	2	2.9			1,665	36.9			326	17.2		
	50 mg/L	2	2.9			1,665	36.9			326	17.2		
3	Control	35	9.2	-3	0.70	1,413	31.8	172	0.0004	212	3.4	123	0.00001
	50 mg/L	38	10.0			1,241	16.5			89	8.2		
5	Control	41	4.2	-5	0.44	917	343.3	29	0.92	242	10.0	55	0.001
	50 mg/L	46	10.8			888	446.2			187	13.9		
10	Control	50	4.8	- 9	0.02	50	1.5	-7	0.002	1	0.6	0	0.64
	50 mg/L	60	1.4			57	2.0			1	0.9		
15	Control	24	16.4	3	0.72	76	7.9	8	0.23	0	0.5	0	0.24
	50 mg/L	21	7.3			68	9.4			0	0.0		

Day	Treatment	Butyrate				Isovalerate				Valerate			
		mg/L	SD	Diff.	p-value	mg/L	SD	Diff.	p-value	mg/L	SD	Diff.	p-value
0	Control	122	42.7			28	3.0			12	1.5		
	50 mg/L	122	42.7			28	3.0			12	1.0		
3	Control	28	5.8	18	0.006	39	4.6	15	0.005	5	0.2	-18	0.007
	50 mg/L	10	1.9			24	1.6			23	5.6		
5	Control	2	1.2	-3	0.35	32	3.8	3	0.32	2	2.9	-5	0.39
	50 mg/L	5	5.4			29	5.9			6	2.1		
10	Control	2	1.2	1	0.43	-	-	-1	0.39	3	3.0	1	0.39
	50 mg/L	1	1.3			1	1.9			2	1.1		
15	Control	1	0.9	-1	0.04	-	-	-1	0.52	0	0.2	-2	0.41
	50 mg/L	2	2.7			1	1.0			2	4.0		

Difference (Diff) is calculated as the value of the control minus the treatment; p-values were calculated with a t-test. Green color means significant difference between treatment and control with a p-value < 0.05; n=4.

Appendix 8 Summary of the effect of 50 mg CEF/L on organic matter removal over time

Day	Treatment	COD			dCOD			TS			VS		
		g/L	SD	p-value	g/L	SD	p-value	g/L	SD	p-value	g/L	SD	p-value
0	Control	36.6	1.4		6.5	0.2		15.0	0.3		22.6	0.3	
	50 mg/L	36.6	1.4		6.5	0.2		15.0	0.3		22.6	0.3	
3	Control	20.2	1.2	0.44	6.0	0.6	0.007	15.0	0.1	0.07	22.0	0.2	0.11
	50 mg/L	21.1	1.8		7.4	0.4		15.4	0.4		22.5	0.3	
5	Control	17.1	0.7	0.18	5.1	0.6	0.40	13.8	0.2	0.003	21.5	0.2	0.002
	50 mg/L	18.5	1.5		4.8	0.6		15.0	0.3		22.9	0.4	
10	Control	15.6	1.0	0.45	2.9	0.3	0.52	12.6	0.2	0.03	20.4	0.2	0.07
	50 mg/L	16.4	1.5		3.0	0.2		13.5	0.6		21.4	0.6	
15	Control	17.4	3.3	0.36	3.4	0.3	0.27	12.6	0.4	0.02	20.1	0.3	0.02
	50 mg/L	15.5	1.7		3.2	0.1		13.3	0.2		20.8	0.2	

Green color means significant difference between treatment and control with a p-value < 0.05; n=4.

Appendix 9 Summary of the effect of 250 mg CEF/L on CH_4 and CO_2 production

		CH_4								CO_2							
Day	Treatment	Cumulative production mL/gVS	SD	Reduction	p-value	Daily rate mL/d-gVS	SD	Reduction	p-value	Cumulative production mL/g VS	SD	Reduction	p-value	Daily rate mL/d-gVS	SD	Reduction	p-value
1	Control	21	2	26%	0.0001	21	2	26%	0.0001	16	1	36%	3E-07	16	1	36%	3E-07
	250 mg/L	16	1			16	1			10	1			10	1		
2	Control	48	7	40%	3E-05	26	5	51%	4E-05	32	3	50%	6E-07	16	2	64%	1E-06
	250 mg/L	29	3			13	2			16	1			6	0		
3	Control	69	17	47%	0.0007	28	6	64%	0.0007	43	8	56%	4E-05	14	2	73%	4E-05
	250 mg/L	36	4			10	1			19	2			4	0		
4	Control	100	20	54%	0.001	25	8	67%	0.003	58	8	61%	0.0001	12	3	76%	0.0008
	250 mg/L	46	4			8	2			22	1			3	0		
5	Control	120	33	58%	0.003	30	1	77%	4E-06	66	14	64%	0.0006	13	1	82%	1E-06
	250 mg/L	51	5			7	2			24	2			2	1		
6	Control	161	11	63%	0.0002	21	6	69%	0.01	83	5	68%	4E-05	9	2	72%	0.003
	250 mg/L	59	3			7	2			27	2			3	1		
7	Control	173	8	63%	2E-05	13	5	60%	0.04	89	4	67%	2E-06	5	2	64%	0.01
	250 mg/L	64	3			5	1			29	2			2	1		
8	Control	182	8	62%	8E-06	8	1	48%	0.005	92	4	67%	1E-06	4	0	55%	0.003
	250 mg/L	69	4			4	1			31	3			2	1		
9	Control	189	9	61%	6E-06	7	2	39%	0.08	96	5	66%	2E-06	3	1	49%	0.04
	250 mg/L	73	5			4	2			32	4			2	1		
10	Control	192	11	61%	3E-05	6	1	32%	0.16	98	6	66%	1E-05	3	0	44%	0.06
	250 mg/L	75	5			4	1			33	4			2	0		
15	Control	218	7	60%	0.02	4	0	47%	0.11	114	4	65%	0.003	2	0	63%	0.03
	250 mg/L	86	1			2	1			40	3			1	0		

p-values were calculated with a t-test. Green color indicates significant difference between treatment and control with a p-value <0.005.

Appendix 10 Summary of the effect of 250 mg CEF/L on H_2 levels

				H_2	
Day	**Treatment**	**Conc. (ppm)**	**SD**	**Difference**	**p-value**
1	Control	784	114	369	7E-06
	250 mg/L	416	94		
2	Control	339	97	106	0.02
	250 mg/L	233	22		
3	Control	183	48	16	0.38
	250 mg/L	167	9		
4	Control	143	24	7	0.57
	250 mg/L	136	14		
5	Control	121	16	-2	0.83
	250 mg/L	123	14		
6	Control	117	15	-31	0.48
	250 mg/L	148	76		
7	Control	114	5	-35	0.49
	250 mg/L	149	89		
8	Control	99	4	-30	0.28
	250 mg/L	129	46		
9	Control	97	4	-36	0.42
	250 mg/L	133	77		
10	Control	90	5	-13	0.06
	250 mg/L	103	9		
15	Control	79	6	0	0.94
	250 mg/L	78	1		

Difference (Diff) is calculated as the value of the control minus the treatment; p-values were calculated with a t-test. Green color means significant difference between treatment and control with a p-value < 0.05;

Appendix 11 Summary of the effect of 250 mg CEF/L on total VFA concentration

Day	Treatment	Total VFA			
		Conc. (mg/L)	SD	Diff	p-value
0	250 mg/L	1183	5	43	0.003
	Control	1140	12		
3	250 mg/L	902	33	-312	2E-05
	Control	1214	20		
5	250 mg/L	918	69	-40	0.42
	Control	958	61		
10	250 mg/L	121	9	68	4E-05
	Control	53	9		
15	250 mg/L	90	63	35	0.37
	Control	55	29		

Difference (Diff) is calculated as the value of the control minus the treatment; p-values were calculated with a t-test. Green color means significant difference between treatment and control with a p-value < 0.05; n=4

Appendix 12 Summary of the effect of 250 mg CEF/L on the VFA profile

		Formate				Acetate				Propionate			
Day	Treatment	Conc. (mg/L)	SD	Diff	p-value	Conc. (mg/L)	SD	Diff	p-value	Conc. (mg/L)	SD	Diff	p-value
0	Control	64	9	- 11	0.11	664	6	- 27	0.02	238	4	- 8	0.04
	250 mg/L	75	8			691	13			246	4		
3	Control	29	7	- 27	0.001	877	11	216	0.0001	277	5	263	2E-08
	250 mg/L	56	6			661	28			14	7		
5	Control	16	15	- 41	0.008	773	49	140	0.01	127	19	121	0.0009
	250 mg/L	57	5			634	59			6	2		
10	Control	20	8	3	0.50	32	4	- 20	0.03	1	0	- 0	0.59
	250 mg/L	16	6			52	11			1	0		
15	Control	14	12	- 4	0.54	23	4	- 22	0.28	0	0	- 0	0.81
	250 mg/L	19	2			45	33			0	0		

		Butyrate				Isovalerate				Valerate			
Day	Treatment	Conc. (mg/L)	SD	Diff	p-value	Conc. (mg/L)	SD	Diff	p-value	Conc. (mg/L)	SD	Diff	p-value
0	Control	117	12	- 3	0.66	25	2	- 2	0.32	32	9	8	0.22
	250 mg/L	120	3			27	3			25	6		
3	Control	5	7	- 60	0.0001	26	3	15	0.04	-	-	- 94	0.0005
	250 mg/L	65	10			11	9			94	11		
5	Control	2	1	- 91	0.0002	28	5	1	0.79	12	9	- 90	6.73E-06
	250 mg/L	93	9			27	1			102	7		
10	Control	-	-	- 28	0.006	-	-	- 13	0.0003	1	0	- 10	0.12
	250 mg/L	28	8			13	1			11	9		
15	Control	5	6	- 7	0.41	9	10	0	0.99	4	2	- 2	0.57
	250 mg/L	12	14			9	10			6	5		

Difference (Diff) is calculated as the value of the control minus the treatment; p-values were calculated with a t-test. Green color means significant difference between treatment and control with a p-value < 0.05; n=4.

Appendix 13 Summary of the effect of 250 mg CEF/L on COD, dCOD, TS, and VS removal

Day	Treatment	COD				dCOD				TS				VS			
		Conc. (g/L)	SD	Diff	p-value	Conc. (g/L)	SD	Diff	p-value	Conc. (g/L)	SD	Reduction	p-value	Conc. (g/L)	SD	Reduction	p-value
0	Control	22.1	1.2	- 1.6	0.03	6.8	0.4	- 0.1	0.35	23.9	0.2			18.2	0.3		
	250 mg/L	23.7	1.6			7.0	0.3			26.5	0.3			20.1	0.4		
3	Control	18.4	1.0	- 2.3	0.01	6.0	0.3	- 0.2	0.30	22.1	0.6	7%	0.04	15.3	0.6	16%	0.30
	250 mg/L	20.7	0.4			6.2	0.2			23.1	0.8	13%		16.4	0.7	19%	
5	Control	18.0	0.9	- 2.4	0.005	5.9	0.8	- 0.8	0.11	21.0	0.3	12%	0.46	14.8	0.2	19%	0.31
	250 mg/L	20.5	0.7			6.8	0.3			23.0	0.6	13%		16.6	0.5	17%	
10	Control	16.5	1.0	- 3.3	0.003	4.7	0.2	- 1.3	0.001	20.5	0.4	14%	0.44	13.5	0.4	26%	0.11
	250 mg/L	19.8	0.9			6.0	0.3			22.4	0.7	15%		15.6	0.5	22%	
15	Control	15.1	0.5	- 2.9	0.01	4.7	0.4	- 0.7	0.02	19.6	0.6	18%	0.22	14.4	0.4	21%	0.02
	250 mg/L	18.1	1.2			5.4	0.2			22.3	0.3	16%		17.0	0.1	16%	

Difference (Diff) is calculated as the value of the control minus the treatment; TS and VS reduction is calculated based on the initial conditions (day 0) of each treatment; p-values were calculated with a t-test. Green color means significant difference between treatment and control with a p-value < 0.05; n=4.

Appendix 14 Summary of the effect of 50 mg CEF/L on *cmy-2* levels

Day	**Treatment**	***Cmy-2***		
		No./ng DNA	SD	p-value
0	Control	2.24	0.55	
	50 mg/L	2.24	0.55	
3	Control	2.43	0.30	0.18
	50 mg/L	3.40	1.13	
5	Control	0.78	0.37	0.06
	50 mg/L	2.09	0.94	
10	Control	0.50	0.33	0.01
	50 mg/L	1.87	0.58	
15	Control	1.34	0.71	0.20
	50 mg/L	0.72	0.42	

Green color means significant difference between treatment and control with a p-value < 0.05; n=4.

Appendix 15 Summary of the effect of 250 mg CEF/L on *cmy-2* levels

Day	**Treatment**	**Copy No.**		
		No./ng DNA	SD	p-value
0	Control	4.96	1.49	
	250 mg/L	4.96	1.49	
3	Control	0.97	0.44	0.009
	250 mg/L	3.62	1.05	
5	Control	0.85	0.38	0.02
	250 mg/L	1.58	0.28	
10	Control	0.55	0.42	0.18
	250 mg/L	0.92	0.10	
15	Control	0.40	0.19	0.02
	250 mg/L	1.74	0.69	

Green color means significant difference between treatment and control with a p-value < 0.05; n=4.