Roles of extracellular polymeric substances on the fate of antibiotic resistance genes in anaerobic digestion of thermally hydrolyzed sewage sludge

by

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Abstract

Antibiotic-related contaminants have recently been detected globally in natural and engineered ecosystems. Due to high concentrations of residual antibiotics in wastewater and organic feedstocks used for anaerobic digestion (AD), wastewater treatment plants (WWTPs) and sludge are substantial antibiotic resistance genes (ARGs) reservoirs. The inappropriate disposal of antibiotics-contaminated sludge led to the continuous emergence of antibiotic resistant bacteria (ARB), ARGs, and residual antibiotics, which pose severe environmental and human public health threats. AD is a widely applied technology for sludge treatment in WWTPs; however, conventional mesophilic AD is often ineffective in efficiently removing ARGs. On the contrary, thermal hydrolysis pre-treatment (THP) of sludge was found to reduce ARGs abundance in AD efficiently. Extracellular polymeric substances (EPS) in sludge play a critical role in ARG propagation. Particularly, EPS-associated ARGs abundance and their propagation in AD are overlooked. However, the detailed characterization of EPS components and their impact on ARGs propagation is still unclear. This master's thesis focuses on understanding the roles of THP, and EPS components on ARGs abundance in AD by investigating the fate of EPS-associated ARGs and evaluating their effect on the total ARGs in AD of thermally pretreated sludge.

First, the positive impact of the THP of sewage sludge on ARGs removal during AD has been reported in the literature. However, little information is available on how changes in different EPS due to THP can influence ARG propagation during AD. This study focused on systematically correlating EPS components and ARG abundance in AD of sewage sludge pretreated with THP (80 °C, 110 °C, 140 °C, 170 °C). THP under different conditions improved sludge solubilization, followed by improved methane yields in the biochemical methane potential (BMP) test. The

highest methane yield of 275 ± 11.5 ml CH4/g COD was observed for THP-140 °C, which was 40.5 ± 2.5% higher than the control. Increasing THP operating temperatures showed a non-linear response of ARG propagation in AD due to the rebound effect. The highest ARGs removal in AD was achieved with THP at 140 °C. The multivariate analysis showed that EPS polysaccharides positively correlated with most ARGs and integrons, except for macrolides resistance genes. In contrast, EPS protein was only strongly correlated with β-lactam resistance genes. These results suggest that manipulating THP operating conditions targeting specific EPS components will be critical to effectively mitigating the dissemination of particular ARG types in AD.

Second, recent studies have found that EPS in sludge may be an important ARGs reservoir. Although intracellular ARGs have gained considerable attention since they could be transferred by transduction or conjugation in sludge, extracellular ARGs are overlooked. However, no information is available on the fate of EPS-associated ARGs and their effect on the total ARGs abundance during AD of TH pretreated sewage sludge. The abundances of intracellular ARGs and extracellular including EPS-associated and cell-free ARGs were investigated under low and high temperature THP (90° C and 140° C). Also, the EPS components (proteins, polysaccharides, and eDNA) were correlated with the intracellular and extracellular ARGs abundance before and after AD. THP at both temperatures could increase the sludge solubilization, hence enhancing methane production in AD. The maximum methane yield of 305.7±4.7 ml CH_4/g COD was observed for 140°C-AD. The lowest EPS-associated ARGs abundances were detected at 140°C-AD, where the highest abundances were observed at 90°C-AD and control-AD. The EPS-associated ARGs represented a higher ratio of the total extracellular ARGs (EPS-associated and cell-free ARGs) in all samples. The multivariate analysis showed that EPS-polysaccharides, proteins, and eDNA were strongly positively correlated with intracellular and EPS-associated ARGs. On the contrary, cell-

free ARGs exhibited a fairly weak positive correlation with EPS components. These results suggested that there is a functional link between intracellular and extracellular ARGs; however, they are correlated with EPS components. Also, EPS-associated ARGs reduction in AD of pretreated sludge was reflected in intracellular and cell-free ARGs reduction as well.

Preface

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Dedication

To my parents, sister, brothers and my husband.

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Abbreviations and Units

AD	Anaerobic digestion
Alk	Total alkalinity
ARB	Antibiotic resistance bacteria
ARGs	Antibiotic resistance genes
C/N	Carbon/ nitrogen
CH4	Methane
CO2	Carbon dioxide
CO2	Carbon dioxide
DIET	Direct interspecies electron transfer
EPS	Extracellular polymeric substances
FAN	Free ammonium ammonia nitrogen, NH3
H2	Hydrogen
H2S	Hydrogen sulfide
H2SO4	Sulfuric acid
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
HSAD	High-solids anaerobic digestion
MGEs	Mobile genetic elements
NaOH	Sodium hydroxide
OLR	Organic loading rate
SRT	Solid residence time

THP	Thermal hydrolysis process
VGT	Vertical gene transfer

Chapter 1

Introduction

1.1. Background

Antibiotic resistance is one of the most serious threats to public human health and the environment in every region of the world. Antibiotics are widely used medicines to effectively treat bacterial infections by either killing the pathogenic bacteria or inhibiting their reproduction (Baquero and Levin, 2021). The antibiotic resistance bacteria (ARB) can develop several antibiotic resistance mechanisms, such as antibiotic molecule inactivation, target site modification, drug efflux pump activation, and resistance of global cell adaptations (Carvalho and Santos, 2016; Shi et al., 2021). For instance, tetracycline resistance genes can encode specific proteins for bacterial cell wall permeability alteration, increasing the tetracycline efflux or inhibiting their intracellular penetration (Mahey et al., 2021; J. Zhang et al., 2020). The target site modification aims at antibiotic binding prevention, while antibiotic inactivation or destruction could be achieved by several antibiotic degrading enzymes (Aziz et al., 2022).

Due to the overuse and misuse of antibiotics by humans and animals, the antibiotic resistance situation is steadily worsening (Liu et al., 2021). Although effluents from antibiotic manufacturing plants have been found as the major source of residual antibiotics in the environment (Milaković et al., 2019), residential areas, livestock farms, research institutions, and hospitals are contributing to residual antibiotics release (Tiwari et al., 2017; Xiang et al., 2019). However, the poor degradation of antibiotics in human and animals' guts leads to a continuous emergence of residual antibiotics, ARB, and antibiotic resistance genes (ARGs) in the environment. High levels of antibiotics in the environment were found to elevate the resistance severity by enhancing horizontal gene transfer (HGT) of ARGs (Milaković et al., 2019). Through the HGT, ARGs can propagate among different microbial communities via mobile genetic elements (MGEs) (e.g., integrons, plasmids, and integrons) (Lu et al., 2019; Tian et al., 2016; H. Wang et al., 2021). Notably, the HGT involves three mechanisms: transduction occurs via bacteriophage that acts as an intermediate between two cells, transformation relies on the uptake of free resistant DNA from the surrounding environment, and conjugation involves genetic material direct transfer from one

cell to another via conjugative pilus (Aziz et al., 2022). The existence of residual antibiotics in the environment not only stimulates resistance dissemination by posing a selection pressure on the resistant strains but also has a toxic effect on the environmental living organisms (Hu et al., 2022; Shao et al., 2018). Recently, residual antibiotics, ARB, and ARGs have been found globally not only in natural environments but also in engineered ecosystems (Haffiez et al., 2022b).

Wastewater treatment plants (WWTP) are hotspots for ARGs that have been detected in all wastewater and sludge treatment processes (Jia et al., 2012; C. Sun et al., 2019). Uncontrolled land application of sludge poses serious threats to the public human health and the environment by enabling ARGs transmission to the natural ecosystems (Pazda et al., 2019; Zheng et al., 2019). Recently, it was found that extracellular polymeric substances (EPS), a critical component in sludge composed of various organic biopolymers, harbor a considerable abundance of ARGs (L. Wang et al., 2021). Therefore, ARGs are classified into intracellular and extracellular ARGs, the latter is produced as a result of cell lysis and/or live cells secretion in the form of cell-free ARGs, while the ARGs retained on EPS are EPS-associated ARGs (Sui et al., 2019; Wang et al., 2022). It was suggested that due to the structural and functional characteristics of EPS, it enables ample adsorption sites for various pollutants such as heavy metals and organic micropollutants (Yu, 2020). Recently, a positive correlation has been found between polysaccharides in EPS and various ARGs profiles (Haffiez et al., 2022a). Interestingly, EPS-associated ARGs were found to play a critical role in total ARGs propagation in sludge by stimulating the transformation mechanism (Wang et al., 2022). Therefore, studying the fate of EPS-associated ARGs and investigating their effect on the total ARGs in sludge is crucial to alleviate the environmental concerns posed by ARGs.

Anaerobic digestion (AD) is an energy-positive technology that is widely used for sludge processing in WWTPs (Jenicek et al., 2012). However, AD was found to be an efficient approach to sludge size reduction and pathogen removal prior to land application (Pei et al., 2016; Pilli et al., 2015). Several studies investigated the fate of ARGs during AD and found that conventional mesophilic AD is mostly inefficient in ARGs removal and enhances the HGT (Gurmessa et al., 2020; Jang et al., 2017; Yang et al., 2020). Therefore, exploring an effective approach for higher ARGs removal efficiency has gained considerable attention from many researchers. One of the extensively studied approaches is thermal hydrolysis pre-treatment (THP) of sludge prior to AD

(Ma et al., 2011; Pei et al., 2016; C. Sun et al., 2019; Tong et al., 2017). The THP is a widely used technology in several WWTPs as a pre-treatment for sludge solubilization before AD (C. Sun et al., 2019; Tong et al., 2017). High temperature and pressure during the THP were found to cause cell wall disintegration, ARGs hydrolytic destruction, and antibiotics degradation, hence reducing the selection pressure for ARGs propagation (Mao et al., 2015; C. Sun et al., 2019).

Although the fate of ARGs during AD has gained specific attention in recent years, several research gaps are associated with their different responses to operating conditions and sludge components. Most importantly, the detailed characterization and fate of EPS-associated, cell-free, and intracellular ARGs during THP and AD is still unclear. Therefore, the master's research attention is directed to the impact of several operating conditions on ARGs abundance, the correlation between sludge EPS components and ARGs, and the detailed characterization of intracellular and extracellular ARGs during AD of TH-pretreated sludge.

1.2. Scope and objectives

This master's thesis focuses on scrutinizing the influence of process parameters on ARGs abundance in AD and the collateral fundamentals behind their impact. Moreover, it added new insights into the correlation between EPS components and different ARGs profiles, the response of ARGs to low and high-temperature THP, and the abundance of EPS-associated ARGs and their effect on the total ARGs in AD. The specific objectives of this thesis were:

1. To study the propagation of antibiotic resistance genes during anaerobic digestion of thermally hydrolyzed sludge and their correlation with extracellular polymeric substances.
2. To explore the fate of intracellular, extracellular polymeric substances-associated, and cell-free antibiotic resistance genes during anaerobic digestion of thermally pretreated sewage sludge.

1.3. Thesis outline

This thesis consists of five chapters. Chapter 1 demonstrates the background of the master's thesis and discusses the scope and objectives of the master's research. Chapter 2 provides a critical review on exploring the influences of process parameters on antibiotic-resistant gene propagation in anaerobic digestion and the inherent fundamentals behind their effects. Also, it summarized critical research gaps and challenges to guide the prospects for future studies. Chapter 3

investigated the fate of ARGs in sewage sludge and their correlation with EPS in THP-AD. Moreover, it showed the effect of low and high-temperature THP (80° C to 170° C) on EPS characteristics and functional groups of the sludge. Chapter 4 focused on investigating the fate of EPS-associated ARGs and evaluating their effect on the total ARGs abundance during AD of thermally hydrolyzed pretreated sewage sludge. Chapter 5 summarizes the conclusion and the recommendation for future research.

Chapter 2

Literature Review

A version of this chapter was published in Bioresource technology, (2022), 354, 127189.

2.1. Introduction

Antibiotics are widely used to effectively treat infectious diseases caused by pathogenic bacteria (Aziz et al., 2022). The antibiotic inhibits bacterial infections by either killing the bacteria or stopping their reproduction (Baquero and Levin, 2021). The most frequently used antibiotics are quinolones, macrolides, beta-lactams, and aminoglycosides (Xiang et al., 2019). Tetracyclines and sulfonamides are commonly used antibiotics in livestock animals, while macrolides, penicillin, and fluoroquinolones are commonly prescribed for humans (Treiber and Beranek-Knauer, 2021). The incomplete metabolism of antibiotics in human and animal bodies results in the continuous release of antibiotics to the nature and subsequent proliferation of antibiotic-resistant bacteria (ARB) carrying antibiotic-resistant genes (ARGs) (Liu et al., 2021). Therefore, concerns regarding antibiotic resistance have attracted tremendous attention in recent years (Liu et al., 2021; Xiang et al., 2019). Especially, the therapeutic action of antibiotics against pathogens that causes infectious diseases has been reduced (Carvalho and Santos, 2016).

Antibiotics are released to the environment from different sources, including residential areas, hospitals, livestock farms, pharmaceutical industries, and research institutions (Fig. 2.1a) (Tiwari et al., 2017; Xiang et al., 2019). Antibiotics pose a selection pressure on resistant strains in different environmental media and stimulate resistance amplification (Shao et al., 2018). ARB can develop different antibiotic resistance mechanisms, such as drug efflux pumps activation, target sites modification, antibiotic molecules inactivation, and the resistance of global cell adaptations (Shi et al., 2021). Efflux pumps refer to bacterial cell wall permeability changes that can increase the efflux of antibiotics or stop their intracellular penetration (Zhang et al., 2020a). For instance, various tetracycline resistance genes encode proteins for tetracycline efflux (Mahey et al., 2021). Modifying target sites involve preventing antibiotic binding via modification or destruction by antibiotic degrading enzymes (Aziz et al., 2022). ARB can spread among the population by natural

selection, where antibiotics eliminate the sensitive bacteria (Read and Woods, 2014). ARGs can propagate via vertical gene transfer (VGT) attributed to the genetic information transmission from the parent cell to its offspring, and horizontal gene transfer (HGT) between different bacteria through mobile genetic elements (MGEs) (Tian et al., 2016; Wang et al., 2021a). MGEs, such as integrons, transposons, and plasmids mediate the DNA transfer by encoding specific proteins (Lu et al., 2019). HGT can occur via three mechanisms: transformation refers to the uptake of the free resistant DNA from the environment, transduction relies on bacteriophage as an intermediate between two cells, and conjugation is a direct transfer of the genetic material from one cell to another by conjugative pilus (Aziz et al., 2022).

Recently, antibiotics-related contaminants have been found globally in natural and engineered ecosystems. Notably, large quantities of antibiotics, ARGs, and MGEs have been found in different treatment trains in wastewater treatment plants (Nguyen et al., 2021; Pazda et al., 2019). For instance, residual antibiotics in sewage sludge could widely vary between ng to 100 mg/kg of dry solids (Ezzariai et al., 2018). Residual antibiotics in livestock manure were as high as 136 mg/kg of dry solids (Ezzariai et al., 2018). Land application of sludge and various manures having high residual antibiotics has a high potential to enter the food chain (Congilosi and Aga, 2021). It has already been confirmed that ARGs can be transmitted from soil to edible crops (Cerqueira et al., 2019) and detected in food waste (He et al., 2019a).

Anaerobic digestion (AD) is a widely used for stabilizing various organic wastes, including sludge and manures (Sui et al., 2018; Zhang et al., 2015). In addition to biogas production, digestate from AD presents a beneficial biofertilizer for land application (Rehman et al., 2018). However, organic wastes used for AD can be significant reservoirs of various ARGs and MGEs. Hence, understanding their fate during AD has received considerable attention. Like natural ecosystems, residual antibiotics in AD may increase ARG propagation by selection pressure (Yun et al., 2021). Several studies also confirmed the significance of HGT in ARG dissemination during AD (Fig. 2.1b), indicated by a positive correlation between ARGs and MGEs (Jang et al., 2020; Wu et al., 2016). Although AD can remove ARGs, HGT can be encouraged depending on specific operating conditions, leading to inadequate ARG removal (Yang et al., 2020). Previous studies reported the proliferation of various ARGs in AD of sewage sludge (Yun et al., 2021; Zang et al., 2020; Zhang et al., 2015). Thus, ARG abundances in digestate could also vary depending on the

AD operating conditions (Gurmessa et al., 2020; Pazda et al., 2019; Wallace et al., 2018). Various operating parameters, such as temperature, residence time, solid content, and the number of stages, could drastically influence the ARG abundances in AD (Diehl and Lapara, 2010; Shi et al., 2021; Zhang et al., 2017a, 2019b). Heavy metals and (nano)microplastics in AD feedstock could also stimulate ARG propagation (Zhang et al., 2021; Zhang et al., 2020b, 2017e). Moreover, using additive materials and pre-treatments of feedstock influenced ARG levels in digestate (Chen et al., 2021; Sun et al., 2018; Tong et al., 2017; Zhang et al., 2017b). Therefore, investigating AD operating parameters and process schemes on the ARG abundance changes is essential for minimizing their transmission risks via land application of digestate. A few review articles have recently been published on the fate of ARGs in various biological processes, such as composting and AD, for biowastes (Congilosi and Aga, 2021; Cui et al., 2020; Youngquist et al., 2016). However, an exclusive review of the significance of various AD operating parameters and process schemes on the fate of ARGs and MGEs is critically needed to identify research gaps and guide future research.

Hence, this review article provides a critical overview of various process parameters influencing the ARG propagation during AD and underlying fundamental mechanisms. Moreover, research gaps and current challenges associated with ARG control in AD are also thoroughly discussed. Overall, the review will guide researchers towards future research for optimization of AD process for remediation of ARG transmission via digestate.

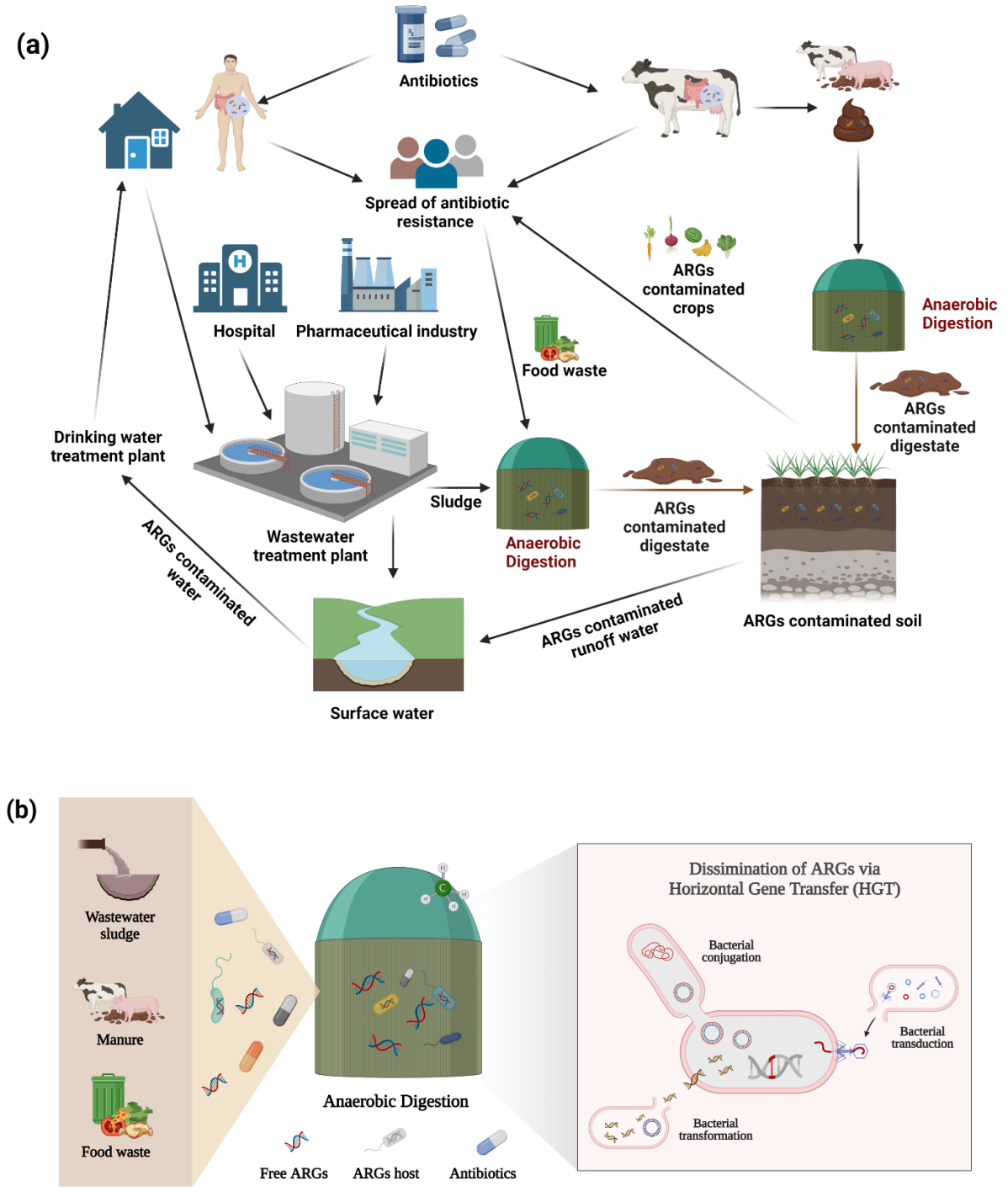

Figure 2.1. Illustrative representation of (a) the dissemination of ARGs in humans, animals, and the broader environment via different routes, including anaerobic digestion, and (b) horizontal gene transfer in anaerobic digestion.

2.2. Impact of process parameters on the fate of antibiotic resistance genes

2.2.1. Operating temperature

The digester microbial communities can influence ARG propagation (Sun et al., 2016), while the temperature is one of the critical operating parameters that shape the microbiome (Gurmessa et al., 2020; Sun et al., 2016a). Thus, temperature could influence ARB abundance and, consequently, the fate of ARGs and MGEs (Diehl and Lapara, 2010; Sun et al., 2016a). Previous studies reported that ARGs could respond differently to operating temperatures. In several cases, thermophilic AD outperformed mesophilic AD for ARG removal (Huang et al., 2019; Jang et al., 2017; Zou et al., 2020). Diehl and Lapara (2010) found that the most efficient reduction of *tet*X, *tet*A, *tet*L, *tet*O, *tet*W in the AD of municipal wastewater was achieved at 55°C, compared to other operating temperatures (22°C, 37°C, and 46°C). Similarly, for AD of dairy manure, Sun et al. (2016) reported superior ARG removal at 55°C than mesophilic (35°C) and moderate (20°C) temperatures. However, a few studies reported an opposite trend, i.e., inadequate ARG removal in thermophilic AD (Huang et al., 2019; Sun et al., 2019a; Zhang et al., 2015). For instance, Huang et al. (2019) observed the highest ARG abundance during AD of swine manure at 55°C than other temperatures (25°C and 37°C). Sun et al. (2019a) also reported a 23.7% higher ARG abundance in thermophilic solid-state AD of cattle manure than the mesophilic. These studies postulated that such observations could be attributed to the enhanced ARB activity under thermophilic conditions (Huang et al., 2019; Zhang et al., 2015). It was found that the abundance of Proteobacteria (potential ARG host) was higher in thermophilic AD (Li et al., 2015; Sun et al., 2019a). Also, Huang et al. (2019) observed a higher abundance of the total pathogenic bacteria (potential ARG hosts) in thermophilic digesters. Notably, thermophilic AD can boost the microbial biomass indicated by the higher 16S rRNA gene copies and enhance HGT under thermophilic conditions (Huang et al., 2019; Sun et al., 2016a). Moreover, studies revealed different responses of ARG subtypes under different temperatures. Zhang et al. (2015) compared the fate of ARGs in thermophilic and mesophilic AD of waste activated sludge. The mesophilic AD effectively removed various ARG subtypes (*tet*G, *tet*O, *tet*W, and *erm*B), while *sul*2 was effectively reduced in thermophilic AD. However, both temperatures were ineffective for *sul*1 removal. Recently, Jang et al. (2018) studied the removal of 21 targeted ARG subtypes in temperature-phased AD of

sewage sludge. Their results demonstrated that the mesophilic-thermophilic sequence could remove most targeted ARGs (*tet*M, *tet*B, *tet*Z, *tet*E, *tet*G, *tet*D, *tet*A, *tet*Q, *tet*X, *tet*H, *tetB*, *sul*1, *sul*2, *qnr*D, *aac(6')-Ib-cr*, bla_{SHV}, bla_{CTX}, bla_{TEM}, *flo*R, *oqx*A, *erm*B) better than the thermophilic-mesophilic sequence (discussed further in section 2.2). Despite the thermophilic-mesophilic sequence resulting in a better MGE (class 1 integrons) removal, some ARGs rebounded in the second stage (mesophilic). The authors concluded that the thermophilic-mesophilic sequence would still be effective for ARG and MGE removal and superior methane production. They recommended external/internal recirculation between two stages could possibly help in improving ARG and MGE removal, which warrants further investigation.

The effects of temperature on ARG removal have been widely investigated (Table 2.1) compared to other process parameters. Still, no consistent conclusions can be made on the optimum temperature for removing ARGs and MGEs during AD. Moreover, differences in other process parameters, such as feedstock type, solid content, residence times, could lead to different initial ARG/MGE levels and subsequent removal efficiencies. Notably, ARG/MGE types and abundances are not universal across different feedstocks (Huang et al., 2019; Sun et al., 2016a; Ma et al., 2011). Furthermore, a recent meta-analysis by Flores-Orozco et al. (2022) suggested that thermophilic AD would be more effective for reducing ARGs in pig manure but was not for cattle manure, indicating that feedstock type would influence the optimum operating temperature for ARG removal. Notably, high free ammonia levels in cattle manure might be attributed to the low ARGs removal compared to pig manure (Baek et al., 2020; Sui et al., 2018).

2.2.2. Two-stage (acidogenic-methanogenic) anaerobic digestion

The process of AD involves a syntrophic interaction between fermentative bacteria (acidogenic) and methanogens (Pasalari et al., 2021). However, they have differences in their growth kinetics, nutritional requirements, optimum environmental conditions. To provide optimal growth conditions for each microbial group, the acidogenic phase followed by the methanogenic phase (two-stage AD) has been proposed by researchers (Hou et al., 2021; Shi et al., 2021; Yuan et al., 2019). However, only a few studies looked into the effects of such two-stage configuration on ARG removal (Table 2.1). Wu et al. (2016) studied ARG and MGE removal in two-stage digesters operated under thermophilic and mesophilic conditions. In general, thermophilic AD provided superior ARG removal than mesophilic AD. However, 80% of the removed ARGs in the first stage

(thermophilic-acidogenic) rebounded in the second stage (thermophilic-methanogenic). Microbial diversity and abundances of potential ARB (Proteobacteria and Actinobacteria phyla) were correlated with ARG profiles in different stages. The study suggested that possibly a less-diverse microbiome established under a thermophilic-acidogenic environment would be unfavorable for ARG propagation. In contrast, neutral pH during the methanogenic phase might provide a favorable condition for ARB.

Recently, Shi et al. (2021) suggested that acidogenic and methanogenesis steps would influence ARG propagation differently. In their study, macrolide, lincosamide, and streptogramin (MLS) resistance genes were enriched in the first stage (alkaline fermentation under thermophilic and mesophilic conditions), while decreased in the second stage (methanogenesis under thermophilic and mesophilic conditions). Noteworthy, the potential hosts of MLS resistance genes showed higher abundance in the alkaline fermentation reactors than in the methanogenic reactors. Thus, the process conditions can shape the microbial community structure and affect the fate of ARGs (Shi et al., 2021). However, tetracycline resistance genes reduced during the first stage (thermophilic fermentation), while increased in the second stage (mesophilic methanogenesis). In contrast, tetracycline resistance genes increased in both stages of mesophilic fermentation followed by the mesophilic methanogenesis stage, which might be attributed to the mesophilic ARB hosting tetracycline resistance genes. Thus, different ARGs can respond differently to the process conditions. Nonetheless, overall ARG removal from two-stage digesters was higher than a single-stage mesophilic AD. Jang et al. (2020) investigated ARG removal in a full-scale two-stage (acidogenic-methanogenic) thermophilic AD treating food waste wastewater. The results were compared with a full-scale single-stage thermophilic AD operated with similar feedstock. Although the two-stage AD was operated at a lower residence time than the single-stage (15.5-17.5 vs. 39 days), it provided a superior ARG reduction. Thus, their results provided a full-scale demonstration of two-stage AD's effectiveness for ARG removal. Notably, *tet*G, *tet*H, *tet*M, *tet*Q, and *tet*X were completely removed in two-stage AD. However, as discussed earlier, various ARGs may respond differently depending on the operating conditions of the acidogenic stage. Hence, further research on various influential factors (e.g., pH, temperature) is required for two-stage AD.

2.2.3. Residence time

Several studies substantiated the critical importance of solids residence time (SRT) for digester microbiome and AD performance (Babaee and Shayegan, 2011; Bradley et al., 2019). However, only a few studies systematically investigated the impact of SRTs on the fate of ARGs (Ma et al., 2011; Sun et al., 2019b; Zhang et al., 2019b). Ma et al. (2011) investigated ARG removal in mesophilic co-digestion of primary and secondary sludge under 10-20 days of SRTs. Based on their results, a longer SRT of 20 days in mesophilic AD could provide higher removal of various ARG subtypes (e.g., *tet*C, *tet*G, *tet*X, *sul*I, and *sul*I) than shorter SRTs. Sun et al. (2019b) also reported similar trends. The authors suggested that higher ARG removal could be attributed to the low microbial diversity due to the oligotrophic condition developed under extended residence times. Thus, some ARB would be eliminated, and the reproduction probability of new cells might be restricted (Qian et al., 2021; Sun et al., 2019b). Moreover, higher antibiotic biodegradation under longer residence times could be another explanation (Nnadozie et al., 2017). However, Sun et al. (2019b) reported that residence times might have trivial effects on ARG removal depending on the operating temperatures. A marginal impact of retention times in thermophilic AD might be related to the limited ARB growth under high temperature; thus, the effect of retention time became intangible (Sun et al., 2019b).

A recent study by Zhang et al. (2019a) suggested that the responses of different ARGs to residence times would be different depending on their subtypes and process configuration. The authors studied the effects of different SRTs on the fate of ARGs in a single-stage AD, microwave pre-treatment followed by a single-stage AD, and microwave pre-treatment followed by two-stage (acidogenic-methanogenic) AD of sewage sludge. Their results showed that the shorter SRT of 15 days could provide effective ARG removal in single-stage AD and two-stage AD with microwave pre-treatment. In contrast, a single-stage AD with microwave pre-treatment required a longer SRT of 20 days for effective ARG removal. However, the responses of different ARG types and subtypes varied depending on the operating conditions. For instance, shorter SRT could reduce *erm*B and *tet*M, while increasing bla_{TEM}. Also, *ere*A and *tet*G were reduced in the single-stage AD but increased in the other configurations (i.e., microwave pre-treatment followed by single-stage and two-stage AD). Thus, ARG abundance variation can be attributed to several factors. Firstly, shorter SRT might limit the proliferation of ARB and thereby decline ARG abundance (Zhang et

al., 2019b). In contrast, often longer SRT may provide adequate residence times for microbes to facilitate HGT (Zhang et al., 2019b).

Furthermore, ARG hosts can be divergent depending on other factors. For instance, operating SRTs may influence the bioavailability of heavy metals (Thanh et al., 2016), thereby influencing selective pressure on ARG hosts and the subsequent fate of ARGs. As discussed later, heavy metals were identified as co-selection factors to ARG propagation (Guo et al., 2021; Zhang et al., 2019b). In summary, different factors could influence the optimum SRTs for ARGs removal. Therefore, a systematic optimization of SRTs considering other factors would be necessary to ensure adequate ARG removal during AD.

2.2.4. Solid content: high-solids vs. wet-type anaerobic digestion

In recent years, high-solids AD (also called solid-state or dry AD) became very popular for methane recovery from heterogenous high-solids organic feedstocks, such as the organic fraction of municipal solid waste, lignocellulosic biomass, etc. (Chowdhury et al., 2020; Dastyar et al., 2021b, 2021a; Di Capua et al., 2020; Wu et al., 2020). Notably, high-solids anaerobic digestion is known to be operated with ≥15% total solids (TS) (Sun et al., 2016b). In addition to higher volumetric methane productivity over wet-type AD, residuals from HSAD can often be used for land application without any dewatering process (Di Capua et al., 2020; Peng et al., 2020). A few recent studies suggested that HSAD digestate would have a relatively lower ARG level than wet-type AD (Sun et al., 2019a; Wang et al., 2021b).

Sun et al. (2019a) compared ARG removal in high-solids (22% TS) and wet-type (8% TS) mesophilic AD of cattle manure. They found that the HSAD was more effective in ARG removal than its wet-type counterpart. The abundances of at least 7 ARGs were reduced in the HSAD, along with the decreased abundance of ARB (Firmicutes and Proteobacteria). These results were consistent with another study that reported higher ARG and MGE removal in co-digestion of food waste and pig manure in HSAD (20% TS) than wet-type AD (5% TS) (Wang et al., 2021b). Interestingly, a recent study identified microbial mobility as a critical factor in HGT for ARG propagation (Zhang et al., 2018a). Typically, HSAD systems are mostly operated without active mixing, which could minimize ARG propagation potential.

However, Sui et al. (2018) reported contradictory results, where HSAD of swine manure (14% TS) showed a limited ARG removal compared to the wet-type AD (4% TS). In their study,

low ARG removal in HSAD was suggested to be attributed to high free ammonia nitrogen and volatile fatty acids (VFAs) accumulation due to high TS contents. This notion is also consistent with another study that demonstrated that high ammonia levels could enrich the MGEs (e.g., plasmid), enhancing HGT among ARGs of antibiotics target alteration (e.g., *erm*B) (Zhang et al., 2020a). Nonetheless, free ammonia has an inhibitory effect on the efflux system due to proton gradient alteration, thus reducing ARGs associated with antibiotics efflux (e.g., *tet*L) (Zhang et al., 2020a). Thus, more systematic research is needed to thoroughly understand the significance of process parameters on ARG propagation in HSAD systems.

2.2.5. Co-digestion

An efficient AD operation requires a balanced carbon-to-nitrogen ratio, dilution of toxic substances, pH and moisture content adjustment, and supplementation of trace elements (Tanimu et al., 2015; Wang et al., 2012; Xu et al., 2018). Co-digestion of two or more complementary feedstocks is often considered a feasible approach to achieve these objectives (Chowdhury et al., 2019; Xu et al., 2018). To date, a few studies investigated the fate of ARGs in co-digestion (Jiang et al., 2022; Song et al., 2017; Zhang et al., 2018a; Zhang et al., 2016; Zhang et al., 2018b). Different feedstock ratios in co-digestion could influence the evolution of bacterial communities and thereby impact ARG abundances (Jiang et al., 2022; Song et al., 2017). Furthermore, the dilution of antibiotics via feedstock mixing may reduce the selection pressure of ARG propagation (Jiang et al., 2022). In a recent study, Jiang et al. (2022) found that co-digestion of gentamicin mycelial residues with wheat straw could provide a dilution of antibiotics, ARGs, and MGEs in gentamicin mycelial residues along with balancing C/N ratios, which ultimately influenced microbial communities and subsequent ARG propagation. Song et al. (2017) suggested that co-digestion of swine manure and wheat straw at an optimum mixing ratio can benefit ARG removal. Furthermore, Zhang et al. (2018c) reported that adding Chinese medicinal herbal residues as a co-substrate with swine manure and wheat straw could inhibit HGT via MGEs. The authors postulated that antimicrobial compounds (e.g., flavonoids) in medicinal herbal residues could contribute to the inhibition of bacterial DNA synthesis. Nonetheless, it was evident that the selection of suitable co-substrate as well as their optimum mixing ratios would be critical to mitigating ARG dissemination (Jiang et al., 2022; Song et al., 2017). However, optimum mixing ratios for biogas production and ARG removal may vary considerably. Song et al. (2017) reported that an optimum

mixing ratio (7:3 mass ratio) for ARG removal was different from the optimum (5:5 mass ratio) for methane production. Such trade-off situations may often occur towards optimizing AD process parameters for ARG control.

2.2.6. Presence of heavy metals and (nano)microplastics in feedstock

Several studies examined the effect of heavy metals (e.g., copper, zinc, etc.) on ARG propagation in AD (Zhang et al., 2017e, 2020b; Zhou et al., 2021b). For example, high copper level (227 mg/L) in AD operated with swine manure increased the abundance of various ARGs (*tet*, *sul*, *erm*, *bla*) and MGEs (Zhang et al., 2020b). Also, copper affected the abundance of potential ARB (Firmicutes), which positively correlated with the propagation of *tet*W, *erm*F, and *erm*Q. A recent study also reported increasing of 8 ARGs subtypes (*tet*T, *tet*A, *tet*B, *tet*O, *erm*A, and *erm*B) after adding copper salt in the digester (Zhou et al., 2021b). Zhang et al. (2017e) reported that 125 mg/L of zinc could increase ARG abundance during AD of swine manure. Heavy metals in feedstocks can increase the tolerance levels of microbes towards antibiotics (Baker-Austin et al., 2006), while metal resistance is well linked to antibiotic resistance (Zhang et al., 2020b). In such cases, the heavy metals pose a selection pressure on ARGs by transferring the co-occurred ARGs and metal resistance genes between bacteria via MGEs (Baker-Austin et al., 2006). Nonetheless, monitoring heavy metals in feedstock for AD is not a common practice, while the presence of heavy metals can potentially influence ARG abundance.

Recent reports raised concerns that microplastics (MPs) and nano-plastics (NPs) can stimulate ARG propagation in natural and engineered systems (Dong et al., 2021; Azizi et al., 2021; Shi et al., 2020). MPs/NPs have been found in feedstocks (e.g., sewage sludge, food waste) frequently utilized for AD (Golwala et al., 2021; Luo et al., 2020; Zhang and Chen, 2020). In sewage sludge, MPs/NPs concentrations have been detected in the range of 1.5×10^3 to 2.4×10^4 particles/kg solids (dry) (Luo et al., 2020; Zhang and Chen, 2020). The accumulation and adsorption of various antibiotics (e.g., tetracyclines, sulfonamides, etc.) on MPs/NPs have been reported (Lu et al., 2021; Syranidou and Kalogerakis, 2022). Thus, the fate and biodegradability of antibiotics in such waste streams can be altered (Liu et al., 2019a), which will ultimately influence ARG propagation. First, antibiotics adsorption by MPs/NPs can enhance the selective pressure on ARGs (Yu et al., 2020). Second, biomass attachment or accumulation on the surface of MPs can promote HGT (Zhang et al., 2020c). Moreover, heavy metals that can drive ARG co-selection can also be adsorbed by

MPs/NPs (Mohsen et al., 2019; Wang et al., 2017). However, information on interactions between MPs/NPs and ARG propagation in AD is still limited (Azizi et al., 2022; Tang et al., 2022; Zhang et al., 2021). Zhang et al. (2021) studied ARG abundance in thermophilic (55 °C) and hyper-thermophilic (65 °C) AD of dairy wastes in the presence of 1 g/L of polyethylene MPs. Polyethylene MPs caused a significant increase in fold changes of ARGs (particularly *tet*C, *tet*G, and *tet*W) at 55 °C; however, there were no substantial changes in ARGs abundance (except for *tet*W) at 65 °C compared to the control. Thus, possibly higher operating temperature would be required to minimize the ARG propagation enhanced by MPs/NPs. A few recent studies further confirmed that depending on the initial concentrations, various (nano)microplastics such as polystyrene, dimethyl phthalate could increase ARG propagation during AD of sewage sludge (Azizi et al., 2022; Tang et al., 2022).

2.2.7. Impact of conductive additives

Recently, adding conductive materials (e.g., activated carbon, biochar, magnetite, etc.) in AD became a popular approach to improve methane production by enabling direct interspecies electron transfer (DIET), an efficient syntropy, between bacteria and methanogens (Barua and Dhar, 2017; Zhao et al., 2020). The detailed fundamental mechanisms of DIET by conductive additives can be found in the literature (Barua and Dhar, 2017; Zhao et al., 2020). Most studies reported positive impacts of conductive additives in terms of enhanced methane recovery and process kinetics (Barua and Dhar, 2017; Zhao et al., 2020). Interestingly, several studies also revealed how these conductive materials influence the fates of ARGs in AD (see Table 2.1).

Zhang et al. (2018a) reported that activated carbon in a mono-digestion of feedstock could provide a better ARG removal than the control (without activated carbon), which might be attributed to the retention of microbes on activated carbon, leading to less microbial mobility and reduced HGT potential. However, antibiotics in mixed feedstock for co-digestion may still pose selective pressure for ARGs (Jiang et al., 2022). This notion was also supported by enhanced HGT (indicated by high levels of *intl1*) and limited influence on ARG removal found in co-digestion in the presence of activated carbon (Zhang et al., 2018a). Regarding antibiotics in co-substrate, using a different additive may provide a feasible solution. Zhang et al. (2019a) suggested that powdered activated carbon could provide physical adsorption of various antibiotics (e.g., lincomycin, ciprofloxacin, erythromycin, etc.) during co-digestion of food waste and chicken manure, and

thereby promote ARG removal. However, some antibiotics, such as chlortetracycline and triclosan, were removed by biodegradation in their study. Regarding the impact of biochar, Sun et al. (2018) reported a non-linear relationship between different biochar dosages (0, 5, 20, and 50 g/L) and ARG removal during AD of cattle manure. The highest ARG and MGE removal was observed for 20 g biochar/L, followed by 5 g biochar/L. In contrast, 50 g biochar/L increased ARG abundance. It was suggested that heavy metals in biochar might increase selective pressure for ARGs, while antibiotics adsorption by biochar had an oppositive impact (Sun et al., 2018; Yang et al., 2021). Thus, understanding the relationship between chemical properties and dosages of biochar would be crucial for optimum ARG removal.

Similar to biochar, other additives, such as graphene oxide (GO), zerovalent iron (ZVI), magnetite, etc., also showed inconsistent impact on ARG removal during AD (Ma et al., 2019; Zhang et al., 2017b; Zhang et al., 2019e). Zhang et al. (2019b) reported the variability and insufficient ARG reduction with the amendment of magnetite in AD. Moreover, Zhang et al. (2017b) found that GO concentrations of 50 and 100 mg/L deteriorated ARG removal in AD of swine manure which may be due to the enhanced HGT by GO. In contrast, 500 mg/L showed a better ARG removal than the control (0 mg/L GO), while 5 mg/L had a trivial impact. It has been suggested that the absorption of ARGs by GO due to the noncovalent combination of GO-ARGs can inhibit the ARGs proliferation. However, all GO concentrations reduced methane production (2.68 – 17.07%) compared to the control, indicating that GO adversely affected microbial activity. While ARG removal is critical to minimize the environmental risks, ensuring stable or enhanced methane production is also vital to ensure the economic sustainability of using conductive additives. Therefore, additives should be carefully selected to achieve a ‘win-win’ situation for both. In summary, available studies suggested that some conductive additives can positively influence higher ARGs/MGEs removal during AD (see Table 2.1). However, a deeper understanding of the underlying mechanisms of their impacts correlating their physical/chemical properties and dosages would be necessary to reap the dual benefits of additives (i.e., methane enhancement and ARG/MGE removal).

2.2.8. Impact of feedstock pre-treatment

The enhancement of hydrolysis kinetics in AD with feedstock pre-treatment is a widely demonstrated approach that has also been applied in full-scale facilities (Tong et al., 2019).

Notably, thermal, chemical, and ultrasound pre-treatment methods have been extensively studied for AD (Cho et al., 2013; Dhar et al., 2012; Xu et al., 2014). The hydrolysis of particulate organics is usually considered the rate-limiting step in AD of complex feedstock (Jang et al., 2013; Ma et al., 2013). The pre-treatment of feedstock prior to AD can enhance the solubilization of complex organics and consequently accelerate the hydrolysis kinetics (Tyagi et al., 2014)**.** To date, several studies looked into how pre-treatment could influence ARG propagation during AD (Chen et al., 2021; Sun et al., 2019b; Tong et al., 2017).

In general, antibiotics removal from feedstock can reduce selection pressure for ARG propagation in AD (Mao et al., 2015; Zhang and Li, 2018). As antibiotics degradation may require exposure to a high temperature for a certain time, many studies have investigated the effect of thermal hydrolysis of feedstock on antibiotics degradation (Gurmessa et al., 2020; Li et al., 2017; Wallace et al., 2018; Zhang and Li, 2018). Zhang and Li (2018) reported effective tetracycline removal during thermal hydrolysis (120 °C for 60 minutes) of sewage sludge. Sun et al. (2019b) also reported a significant decline of several ARG types/subtypes and MGEs due to thermal hydrolysis (160 °C and 0.6 MPa for 30 NaOH), along with a reduction in some antibiotics (macrolide and tetracycline). Moreover, several studies reported the positive impact of thermal hydrolysis on ARG removal during AD (Ma et al., 2011; Sun et al., 2019b; Tong et al., 2017). ARGs removal by thermal hydrolysis may be attributed to the high temperature and pressure leading to the hydrolytic destruction of cell walls and DNA (Ma et al., 2011). Most recently, Azizi et al. (2022) found that thermal hydrolysis of sewage sludge could mitigate antibiotic resistance genes propagation introduced by polystyrene nanoplastics in AD of sewage sludge. In addition to traditional thermal hydrolysis, a few studies examined the impact of microwave-based thermal pre-treatment on ARG removal (Tong et al., 2016; Zhang et al., 2019b; Zhang et al., 2016; Zhang et al., 2019d). Zhang et al. (2016) also reported a positive impact of microwave (MW) on the ARG removal during co-digestion of food and sewage sludge. Moreover, Tong et al. (2016) compared MW and its combination with acid (HCl) and oxidant (H_2O_2 under alkaline pH) on AD of sewage sludge. The combined process of MW-HCl achieved the highest reduction of ARB. The reason for that might be because of the DNA helix stability, which can tolerate alkaline conditions more than acidic conditions (Williams et al., 2001).

Other pre-treatment methods, such as ozone and ultrasound, have also been examined for ARG

removal during AD (Chen et al., 2021; Oh et al., 2014; Tong et al., 2017). Oh et al. (2014) reported up to 90% ARG removal with 3 mg/L ozone. Tong et al. (2017) compared different pre-treatment methods (e.g., thermal hydrolysis, MW, and ozone) on AD of pharmaceutical waste sludge. Their study achieved the highest ARG and MGE removal with thermal hydrolysis (165℃ and 0.6 MPa for 30 min). The lowest reduction in absolute abundance of ARGs and MGEs was observed with ozone, which could be attributed to the utilization of ozone primarily by the soluble organic matters (Pei et al., 2016; Tong et al., 2017). Moreover, Wang et al. (2019a) compared alkaline, ultrasound, and thermal hydrolysis for AD of sewage sludge. Although thermal hydrolysis was the most effective in ARG removal during pre-treatment, ultrasound showed the highest ARG removal in subsequent AD (up to 75.07%). It has been suggested that cell disintegration resistance under low power density conditions during the ultrasound pre-treatment might have a limited impact on ARGs (Zhang et al., 2017d). On the other hand, despite significant ARG removal during pre-treatment, some ARGs might rebound during AD (Sun et al., 2019b). Such an observation could be attributed to the microbial community composition in the inoculum, which controlled the behavior of ARGs relative to the ARGs in influent. Moreover, the probability of residual DNA and HGT could be another reason behind rebounding (Ma et al., 2011). Thus, systematic optimization of different pre-treatment methods would be necessary for further development as a remediation method for ARGs in AD.

Table 2.1. Summary of investigated process parameters and their effects on ARG removal during anaerobic digestion.

Focus parameter	Feedstock	Temperature/Mode/Residence time[a]	Analytical method	Key features and major findings	Ref.
Temperature	Wastewater solids	22, 37, 46, and 55°C/Semi continuous/15 days	qPCR	- Tetracycline resistance genes (*tet*A, *tet*O, *tet*W, *tet*X) and class 1 integrons (*int*I1) were declined with an increase in operating temperatures. 55°C showed the highest removal of ARGs and *intI*1.	(Diehl and Lapara, 2010)
Temperature	Sewage sludge	35, and 55°C/ Semi continuous/15 days	Metagenomic analysis	- 35 ARG subtypes were detected in the feed; 8 and 13 ARGs were effectively (>90%) removed by thermophilic and mesophilic AD, respectively. - During thermophilic AD, *aad*A, *mac*B, and *sul*1 were enriched, while *erm*I, *sul*1, and *tet*M genes were increased during mesophilic AD.	(Zhang et al., 2015)
Temperature	Dairy manure	20, 35, and 55°C/Batch/60 days	qPCR	- Thermophilic AD (55°C) was more effective than other temperatures for ARG removal; 8/10 ARGs declined, and 5/10 reduced >1.0 log. - Thermophilic AD effectively reduced the abundance of potential ARG hosts (Bacteroidetes and Proteobacteria).	(Sun et al., 2016a)
Temperature	Sewage sludge	35 and 55°C/Batch/35 days	qPCR	- Compared sludges from conventional and $FeCl_3$-enhanced sedimentation; initial ARGs and MGEs levels were higher in sludge from $FeCl_3$-assisted sedimentation. - 21 ARG subtypes (7 types) and intI1 were monitored; *sul*1, *tet*X, *tet*M, *bla*TEM, and *tet*Z were the major (85.22-87.34% of total) ARGs in both samples.	(Jang et al., 2017)

				- Higher ARGs and MGEs removal were achieved in thermophilic AD than mesophilic AD; however, both sludges showed comparable ARG/MGE removal performance.	
Temperature	Swine manure	25, 37, and 55°C/Batch/30 days	qPCR	- Experiments were conducted with and without spiking a mixture of antibiotics (oxytetracycline + sulfamethazine + ciprofloxacin; 50 mg/kg wet weight for each); 9 ARGs and 2 MGEs were analyzed. - AD at 55°C provided the lowest ARG removal and considerable enrichment of *Streptococcus* (ARG host) than other temperatures.	(Huang et al., 2019)
Temperature	Pig manure	35 and 55°C/Batch/60 days	qPCR	- Thermophilic AD provided superior ARG removal than mesophilic AD (0.7-3 vs. 0.9-1.5 log removal). - Although thermophilic AD could reduce ARB quantities, ARB diversity was unaffected, indicating a lack of clear linkage between ARG types and potential ARB species. Notably, their results highlighted the limitations of the ARG host prediction method based on the correlation analysis between ARGs and the bacterial communities, which is currently widely used by the research community.	(Zou et al., 2020)
Temperature	Cattle manure	35, and 55°C/Batch/60 days	qPCR	- ARG removal in high-solids AD (22% TS) was assessed under mesophilic and thermophilic conditions. - ARG abundance was 23.7% higher in thermophilic AD than mesophilic AD.	(Sun et al., 2019a)

Temperature + Residence time	Sewage sludge	35, 37, 47, 52, 59°C/Semi continuous/10-20 days	qPCR	- 1 methicillin (*mec*), 1 vancomycin (*van*), 2 sulfonamide (*sul*), 3 erythromycin (*erm*), and 12 tetracycline (*tet*) genes were monitored. - Thermophilic AD at different temperatures (47, 52, 59°C) showed comparable performance; however, compared to mesophilic AD, thermophilic AD provided higher removal of *erm*B, *erm*F, *tet*O, and *tet*W genes. For other ARGs and *int*I1, thermophilic showed inferior or comparable performance. - Mesophilic AD could effectively reduce *sul*I, *sul*II, *tet*C, *tet*G, and *tet*X under both SRTs (10-20 days), while removal efficiencies increased at SRT of 20 days.	(Ma et al., 2011)
Residence time	Sewage sludge	37°C/Semi-continuous/15-20 days	qPCR	- Shortening SRT from 20 to 15 days could provide higher ARG removal. However, shorter SRT increased removal *erm*B and *tet*M while enriched $bla_{\text{OXA-1}}$. The removal of *sul*I, *sul*II, *erm*F, and *tet*X showed a similar pattern.	(Zhang et al., 2019b)
Two-stage AD + Temperature	Sewage sludge	35 and 55°C/Semi-continuous/13 days (3 and 10 days for first and second stage, respectively)	qPCR	- Two-stage AD (acidogenic-methanogenic) was investigated under mesophilic and thermophilic conditions. - 13 selected ARG subtypes and *int*I1 were analyzed. - Acidogenic phase in thermophilic AD provided the highest ARG removal, while some ARGs rebounded in the methanogenic phase. Overall, two-stage thermophilic AD provided 0.1–0.72 log removal for various ARGs.	(Wu et al., 2016)

				- ARG abundance was correlated with Proteobacteria and Actinobacteria. Moreover, ARGs were positively correlated with *int*I1, indicating the role of HGT on ARG propagation.	
Two-stage AD	Municipal waste activated sludge	35 and 55°C/Semi-continuous/13 days (Two-stage: 5 and 10 days for first and second stage, respectively; Single stage: 15 days)	Metagenomic analysis	- Thermophilic alkaline fermentation followed by mesophilic AD (two-stage), mesophilic alkaline fermentation followed by mesophilic AD (two-stage) and single-stage mesophilic AD were compared. - In general, both two-stage configurations provided better overall ARG removal than single-stage AD. However, thermophilic alkaline fermentation followed by mesophilic AD could reduce more differential ARG subtypes.	(Shi et al., 2021)
Two-stage AD	Food wastewater	50°C (Single-stage), 58.5°C (Two-stage)/Semi-continuous/50 days (Single-stage), 1.5–2.5, and 14–15 days for acidogenic, and methanogenic phases, respectively (Two-stage)	qPCR	- Full-scale single and two-stage (acidogenic-methanogenic) thermophilic digesters were compared for 10 selected ARG subtypes and *int*I1 removal. - Two-stage AD provided higher ARG removal. Notably, some ARGs (*tet*G, *tet*H, *tet*M, *tet*Q, and *tet*X) and *int*I1 were undetected after two-stage AD.	(Jang et al., 2020)
Solid content	Cattle manure	35°C/Batch/60 days	qPCR	- The impact of TS content (8% vs. 22%) on the removal of 20 ARG subtypes and 4 MGEs was compared in mesophilic AD.	(Sun et al., 2019a)

				- AD with high TS content was more effective for ARGs and MGEs removal; total abundances of ARG at 22% TS content was 59% of the total abundances of ARGs observed at 8% TS content. - The abundances of potential ARG hosts (Firmicutes and Proteobacteria) also decreased in high-solids AD.	
Solid content	Swine manure	37ºC/Batch/74-133 days	qPCR	- The impact of TS content (4% vs. 8%–14%) on the removal of 13 ARG subtypes and *int*Il was assessed. - High-solids AD showed lower ARG and MGE removal efficiencies than wet-type AD. ARG abundances were correlated with the abundances of Proteobacteria (ARG host). - Free ammonia and VFAs accumulation declined the removal of ARGs.	(Sui et al., 2018)
Solid content	Food waste + Pig manure	37ºC/Batch/64 days	qPCR	- High-solids AD (20% TS) provided 2.3 times higher ARG removal efficiencies than wet-type AD (5% TS). - A longer retention time was essential for ARG removal during the batch AD, as initial hydrolysis/fermentation could increase some ARGs and MGEs.	(Wang et al., 2021b)
Co-digestion	Swine manure + Wheat straw + Chinese medicinal herbal residues	37ºC/Batch/-	qPCR	- Mixing Chinese medicinal herbal residues with swine manure + wheat straw decreased ARGs and MGEs removal than the AD of swine manure + wheat straw. - Some antimicrobial compounds in medicinal herbal residue might inhibit HGT. - Firmicutes and Proteobacteria were identified as potential ARG hosts.	(Zhang et al., 2018b)

Co-digestion	Gentamicin mycelial residues + Wheat straw	37ºC/Batch/30 days	qPCR	- A mixing ratio of 1:1 (VS basis) of two feedstocks reduced ARG dissemination, while mono-digestion of gentamicin mycelial residues increased ARGs compared to the inoculum. - Firmicutes, Bacteroidetes, and Proteobacteria were identified as potential ARG hosts.	(Jiang et al., 2022)
Co-digestion	Swine manure + Wheat straw	37ºC/Batch/55 days	qPCR	- Different ratios of swine manure and wheat straw (3:7, 5:5, and 7:3, mass basis) were investigated. A mass ratio of 7:3 led to the highest ARG reduction, while the highest methane production was observed for 5:5. - Genera *Solibacillus*, *Enterococcus*, *Facklamia*, *Corynebacterium*_1, and *Acinetobacter* were identified as potential ARG hosts using Network analysis.	(Song et al., 2017)
Additive + Co-digestion	Food waste + Chicken manure, and Food waste + Waste activated sludge	35ºC/ Semi-continuous/30 days	qPCR	- With the addition of powdered activated carbon (15 g/L), ARGs removal was more effective in mono-digestion of food waste than its co-digestion with chicken manure and waste activated sludge (wet mass ratio 1:1).	(Zhang et al., 2018a)
Additive	Food waste	35ºC/ Semi-continuous/30 days	qPCR	- The addition of activated carbon (15 g/L) decreased *tet*A, *tet*M, *tet*W, *tet*O and *tet*X genes compared to the unamended control. However, compared to the control, activated carbon did not improve the removal of other ARGs (*tet*A, *sul*1, *sul*2, *cml*A, and *flo*R), and *int*I1.	(Zhang et al., 2017c)
Additive	Food waste	35ºC/ Semi-continuous/30	qPCR	- Powdered activated carbon (15 g/L) increased the removal of *tet*Q and *tet*W,	(Zhang et al., 2019a)

		days			while the abundance of other ARGs (*tet*A, *tet*M, *tet*O, *tet*X, *sul*1, *sul*2, *cml*A, and *flo*R) increased.	
Additive	Swine manure	37ºC/Batch/38 days	qPCR	-	Nano-magnetite (5-350 mmol) was ineffective for reducing many ARGs (*tet*M, *ere*A, *sul*I, *sul*II, and bla_{CTX-M}), which could be attributed to co-selection from heavy metals or enhanced HGT by nano-magnetite.	(Zhang et al., 2019c)
Additive	Cattle farm wastewater	35ºC/Batch/60 days	qPCR	-	Impact of 5-50 g/L of biochar was examined. 20 g/L was biochar was more effective for ARGs removal.	(Sun et al., 2018)
Additive	Cattle manure	35ºC/Batch/43 days	qPCR	-	Impact of nanoscale zero-valent iron (nZVI, 80 and 160 m/L) was assessed. 160 mg/L of nZVI provided higher ARGs removal.	(Ma et al., 2019)
Additive	Sewage sludge	35ºC/Continuous/ 10-30 days	qPCR	- Impact of ZVI (800, 1000, and 1200 mg/L) was assessed. - 1000 mg/L of ZVI and 10 days SRT was optimum for removal (56.58-97.39%) of 4 out of 5 antibiotics. - AAC (6′)-IB-CR and tetB could be effectively reduced.		(Zhou et al., 2021a)
Additive	Swine manure	35ºC/Batch/50 days	qPCR	-	The addition of 10 g/L of coal gasification slag (5-10 g/L) provided the highest removal of ARGs and 5 selected antibiotics.	(Liu et al., 2019C)
Additive	Swine manure	37ºC/Batch/22 days	qPCR	-	Compared to the control (33.7% removal), ARG removal deteriorated at the graphene oxide (GO) concentration of 50 mg/L (3.7% removal) and 100 mg/L (23.9% removal) and enhanced at 500 mg/L (40.2% removal), while no apparent changes at 5 mg/L (32.8% removal). However, all GO dosages decreased methane production compared to	(Zhang et al., 2017b)

					the control.	
Additive + Temperature	Food waste	35, and 55ºC/Batch/10-15 days	qPCR	-	Different dosages of nZVI (0.1-5 g/L) were investigated. The highest methane yield was observed for mesophilic AD amended with 5 g nZVI/L, while the highest ARG removal was observed for 2 g nZVI/L.	(Wang et al., 2019b)
Additive + Pre-treatment	Chicken manure	37ºC/Semi-continuous/-	qPCR	-	The combined impact of microwave and powdered activated carbon on AGR removal was assessed. The combined pre-treatment could provide higher ARG removal than the control (87-95% vs. 34-58%) along a 1.4-fold increase in methane yield.	(Zhang et al., 2019d)
Pre-treatment + Temperature	Sewage sludge	40, and 55ºC/Continuous/ 4-25 days	qPCR	-	Pilot-scale digesters were operated with thermal hydrolysis (160°C and 0.6 MPa for 30 min) of sewage sludge. Thermal hydrolysis could reduce most ARG and MGE subtypes (>94%); however, thermophilic digesters showed more effectiveness in alleviating rebounding of ARGs.	(Sun et al., 2019b)
Pre-treatment + Co-digestion	Sewage sludge + Food waste	37ºC/Batch/-	qPCR	-	Microwave-enhanced co-digestion could provide higher ARG removal than mono-digestion of sewage sludge. - Microwave-pre-treatment of sewage sludge provided better ARG removal than pre-treatment of food waste.	(Zhang et al., 2016)
Pre-treatment	Pharmaceutical sludge	38ºC/Batch/33 days	qPCR	-	The effects of microwave (pH=10, 100ºC, 5 min), thermal hydrolysis (160°C and 6 bar for 30 min), and ozone (0.11 g O_3/g TS) pre-treatment on ARGs removal were compared. Thermal hydrolysis was the most effective for ARGs and MGEs reduction and improving methane production.	(Tong et al., 2017)

Pre-treatment	Sewage sludge	39-40ºC/Continuous/ 18 days	qPCR	-	Impact of thermal hydrolysis (160 °C and 0.6 MPa for 30 min) on ARG removal in full-scale AD was assessed. - Thermal hydrolysis and AD could reduce *bla*$_{TEM}$, *tet*A, *tet*X, *sul*II, *int*I1, but induced the proliferation of *bla*$_{CTX-M}$, *erm*B, *erm*F, *mef*A/E, *qnr*S, *tet*M in the digested sludge.	(Tong et al., 2019)
Pre-treatment	Waste activated sludge	37ºC/Continuous/ -	qPCR	-	Impact of microwave (80ºC at pH 10) + H_2O_2 (0.2 g H_2O_2/g TS) pre-treatment on ARG removal during single-stage and two-stage (acidogenic-methanogenic) was assessed. - Microwave-H_2O_2 could reduce ARG gene copies but increase the abundance of most ARGs. - Two-stage AD was beneficial for ARGs abundance reduction.	(Zhang et al., 2017a)
Pre-treatment	Dairy wastewater	35ºC/Batch/32 days	qPCR	-	Ultrasound (200 W), ozone (4.2 mg O_3/L), and combined pre-treatment methods were assessed. These pre-treatments did not decrease the absolute concentration of ARGs but decreased their relative abundance.	(Chen et al., 2021)
Pre-treatment	Sewage sludge	38ºC/Batch/30 days	qPCR	-	Microwave (heated up to 100ºC at pH = 7.2, microwave/HCl (pH = 2.5 and heated up to 100°C), microwave/H_2O_2 (pH = 10 and heated up to 80ºC). - Microwave/HCl could efficiently reduce most ARGs during the pre-treatment. However, most ARGs rebounded by 0.10–2.04 log after AD.	(Tong et al., 2016)

[a]For batch, residence time indicates the duration of batch experiment

2.3. Research gaps and future direction

Based on this literature review, Fig. 2.2 summarizes the key take-home messages regarding the effects of different process parameters on ARG removal during AD. Previous studies established underlying fundamental mechanisms behind the impacts of various process parameters on ARG and MGE removal in AD. Nonetheless, establishing general trends on the effects of these parameters or recommending optimum process parameters is still challenging based on the existing literature. For instance, it is widely believed that high temperatures can reduce ARG propagation by HGT (Nguyen et al., 2021), while higher temperatures do not always result in higher ARG removal during AD (discussed earlier). The operating temperature may have different effects depending on other parameters, such as residence times, pre-treatment, and the number of stages (Ma et al., 2011; Sun et al., 2019b; Wu et al., 2016). Thus, interactions among multiple process parameters might contribute to some inconsistent trends reported in the literature. Therefore, more experimental investigations are needed to understand the interactions of various process parameters on the fate of ARGs and MGEs. Advanced tools like machine learning algorithms can help identify the most critical process parameters and provide advanced process optimization tools for ARG and MGE removal in AD.

Moreover, some of the inconsistencies could be attributed to the fact that different studies investigated different ARG and MGE types and subtypes. Different measurement methods (e.g., qPCR, metagenomic) may also result in variations (Liu et al., 2019b; Zhang et al., 2022). Most studies used the qPCR method (see Table 2.1), which may often limit the detection of unknown or untargeted ARGs (Zhang et al., 2022). Other methods also have pros and cons; more details could be found in a recently published review article (Zhang et al., 2022). Moreover, units used for reporting ARG abundance (e.g., copies/mL, ARGs copies/16S rRNA gene copies, ARG copies/g of solids) varied among studies (Zhang et al., 2022). For instance, Ma et al. (2011) found that SRT of 20 days was better than 10 days for ARG removal when ARG quantities were normalized to 16S rRNA gene copies; however, such difference was not evident when ARG quantities were normalized to grams of solids. Thus, adopting a standardized quantification method and unit by the AD research community is critically needed to allow systematic comparison among different studies.

The effect of different process schemes, such as two-stage AD, AD amended with additive materials, and AD integrated with feedstock pre-treatment, on ARG removal have been investigated. Notably, certain conductive additives and pre-treatment techniques showed promising results in simultaneously improving methane production and mitigating ARG propagation. As an emerging approach, some conductive additives could positively influence ARG propagation via: (i) biomass immobilization leading to reduced HGT, (ii) adsorption of antibiotics minimizing selection pressure (Zhang et al., 2019c, 2017c). However, different physicochemical characteristics (e.g., metal content, physical form, etc.) can have different impacts. Pre-treatment can substantially degrade antibiotics, ARGs, MGEs, and ARB before AD (Sun et al., 2019b; Tong et al., 2017). However, rebounding of ARB, ARGs can still be inevitable depending on digester operating conditions. Considering the potential rebounding of ARGs during AD, conductive additives may provide a better solution for enhancing methane production and ARG removal. However, research on conductive additives is still in earlier stages, while pre-treatment technologies like thermal hydrolysis have already been implemented in full-scale AD facilities. In fact, sustainable AD optimization for ARG removal requires integration with the economic element to enhance methane recovery and process kinetics while minimizing the environmental risks of ARG spread. Considering such multi-objective optimization requirements, pre-treatment and conductive additives require more in-depth investigation as they can potentially meet both objectives. However, it can often be quite challenging, as optimal conditions for ARG removal may not always be optimum for methane production and other economic and operational aspects (Song et al., 2017; Wang et al., 2019b). The development of decision support tools would be necessary for the sustainable resolution of such trade-off situations. For instance, post-treatment of digestate for ARG removal might be considered when methane production can not be compromised.

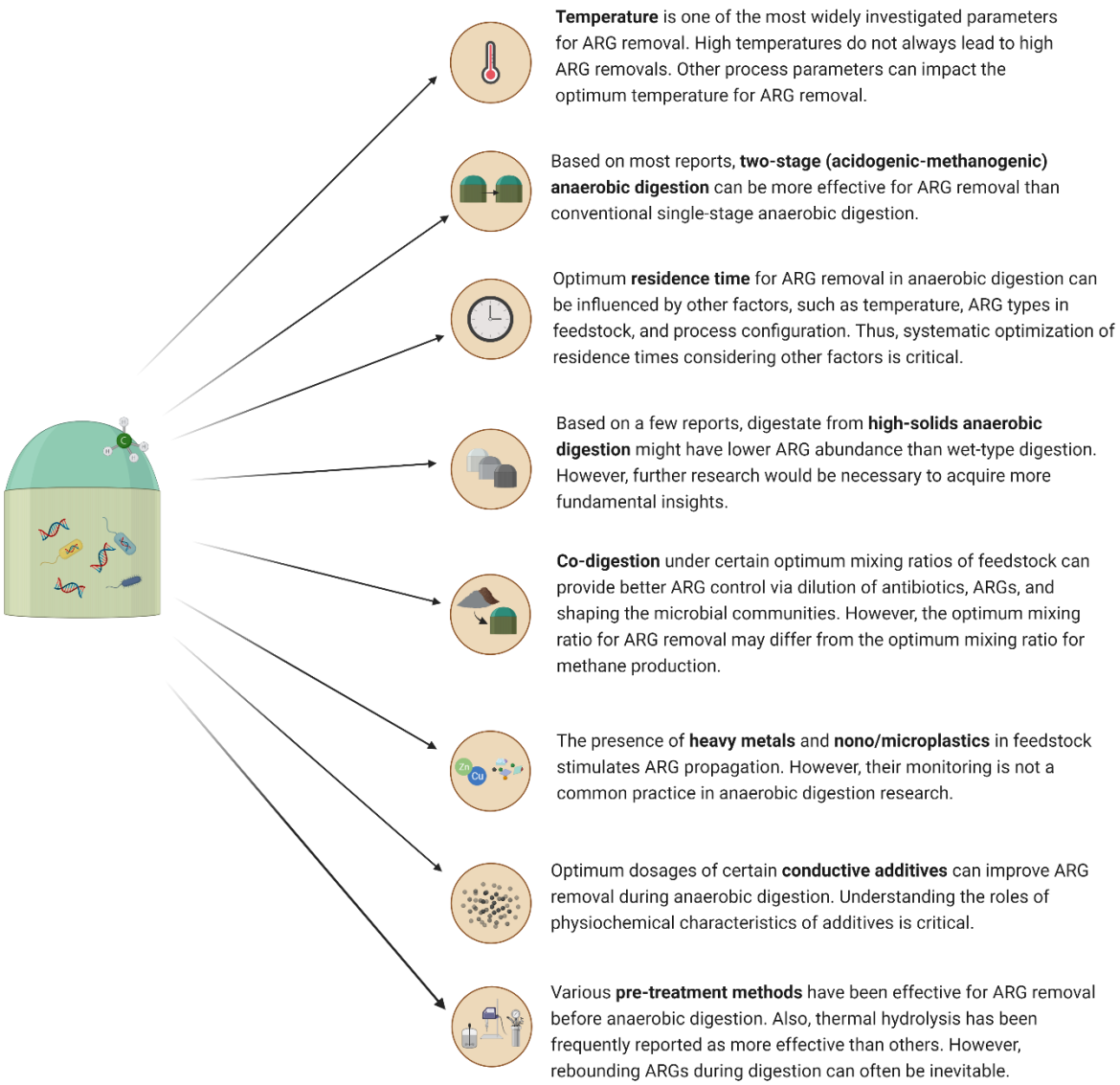

Figure 2.2. Key take-home messages regarding the effects of different process parameters on ARG removal during anaerobic digestion.

2.4. Conclusions

In summary, it was evident that optimizing various process parameters can effectively remove ARGs during AD. Previous studies revealed underlying fundamentals relating to the effects of different process parameters. However, interactions among various process parameters often resulted in inconsistencies in earlier reports. Thus, more research is still needed to understand how interactions among different process parameters influence ARG propagation in AD. Moreover, further development of conductive additives and pre-treatment methods to provide dual solutions for improved biogas production and ARG control is essential to ensure the economic sustainability of AD while minimizing the risk of the digestate for land application.

Chapter 3

Propagation of antibiotic resistance genes during anaerobic digestion of thermally hydrolyzed sludge and their correlation with extracellular polymeric substances

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3.1. Introduction

Concerns regarding the release of antibiotics and subsequent dissemination of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) in natural and engineered ecosystems have emerged in recent years (Pazda et al., 2019; C. Sun et al., 2019). Notably, engineered ecosystems like wastewater treatment plants (WWTPs) are considered as hotspots for ARGs propagation and transmission to the natural ecosystems (Pazda et al., 2019; Zhang et al., 2009). Residual antibiotics in wastewater and sludge can stimulate resistance expansion by selective pressure on the resistant strains (Zhang et al., 2009). Through horizontal gene transfer (HGT), ARGs are spread among microbial communities via mobile genetic elements such as integrons (Chen et al., 2016; Pazda et al., 2019). In WWTPs, ARGs have been detected in all stages of wastewater and sludge treatment processes (Jia et al., 2012; C. Sun et al., 2019). Remarkably, the land application of sludge from WWTPs has been identified as a significant route for ARGs transmission to the natural ecosystem, posing severe threats to the environment and human health (Pazda et al., 2019; Zheng et al., 2019).

Anaerobic digestion (AD) is a widely applied approach for sludge processing in WWTPs, which focuses on three critical aspects: energy recovery, sludge reduction, and pathogen removal prior to land application or disposal of sludge (Pei et al., 2016; Pilli et al., 2015). Based on several reports, the conventional mesophilic AD is often ineffective in adequately removing ARGs and encourages HGT (Gurmessa et al., 2020; Jang et al., 2017; Yang et al., 2020). For instance, Yang et al. (2020) reported an increase in the abundance of various ARGs (e.g., *sul*1, *sul*2, *drf*A7, and *qnr*S*)* after mesophilic AD of swine manure. Also, several mesophilic AD studies reported increased tetracycline resistance genes in digestate (Jang et al., 2017; Ma et al., 2011; Yang et al., 2014). Thus, considerable momentum has been gained towards exploring effective remediation

methods for sufficient ARG removal. Notably, the effects of the thermal hydrolysis process (THP) on ARG removal have received specific attention, which has been implemented in many WWTPs as a pretreatment method for the sludge solubilization before the AD (Ma et al., 2011; Pei et al., 2016; C. Sun et al., 2019; Tong et al., 2017). The application of THP can overcome the rate-limiting hydrolysis during AD, leading to efficient biogas production. Moreover, THP improves sludge dewaterability and provides pathogens sterilization (Bougrier et al., 2008; Carrère et al., 2008; Pilli et al., 2015; C. Sun et al., 2019). THP is often considered more economically attractive than other pretreatment methods (e.g., mechanical, chemical methods) due to energy recovery as heat from pretreated sludge (Pei et al., 2016). To date, a few studies demonstrated that THP could provide removal of ARGs before AD (Azizi et al., 2022; Ma et al., 2011; C. Sun et al., 2019; Tong et al., 2017). It has been suggested that applying high temperature and pressure during THP can result in disintegration of cell walls and hydrolytic destruction of ARGs (Mao et al., 2015; C. Sun et al., 2019). Furthermore, THP was found to be effective in degrading several antibiotics in AD feedstock, such as tetracycline, lincosamides, and macrolides, thereby reducing the selection pressure for ARG propagation during AD (Mao et al., 2015; Zhang and Li, 2018). However, it has also been reported that some ARGs removed during THP could still rebound in the subsequent AD (Ma et al., 2011; C. Sun et al., 2019; Tong et al., 2017). Despite such rebounding, THP-AD could provide better ARG removal than conventional AD without THP (Ma et al., 2011; C. Sun et al., 2019; Tong et al., 2017).

A critical feature of sewage sludge is the presence of extracellular polymeric substances (EPS), consisting of various organic biopolymers such as carbohydrates, proteins, and humic substances produced by microorganisms (Eskicioglu et al., 2006; Liu and Fang, 2002; Peng et al., 2021). High ARG abundance was found in the EPS matrix in aerobic activated sludge flocs in WWTPs (L. Wang et al., 2021), which could be attributed to the high DNA adsorption ability of EPS (Saeki et al., 2011). Although extracellular ARGs can be primarily restricted in the EPS layer, they play a critical role in the ARG dissemination via HGT (L. Wang et al., 2021). Notably, EPS-associated ARGs have exhibited higher transformation abilities than cell-free ARGs in the activated sludge process (L. Wang et al., 2021). Another report suggested a positive correlation between ARGs abundances in membrane foulant and EPS content (protein and polysaccharide) in an anoxic/aerobic membrane bioreactor (Zhu et al., 2018). To the best of the authors' knowledge,

related information for AD or THP-AD has not been examined or reported in the literature. Particularly, THP of sludge prior to AD can significantly influence the solubilization of sludge EPS (Azizi et al., 2021a; Pei et al., 2016; Xue et al., 2015). Under different THP operating temperatures, EPS solubilization may respond differently (Bougrier et al., 2007; Wilson and Novak, 2009). However, the correlation between THP operating temperatures, EPS composition, and ARGs has not been investigated for AD.

Based on the aforementioned research gaps, this study investigated the fate of ARGs in sewage sludge and their correlation with EPS in THP-AD. First, the effects of low and high-temperature THP (80° C to 170° C) on EPS characteristics and functional groups of the sludge were studied. Second, the quantitative and qualitative characteristics of microbial communities were analyzed. Third, EPS composition and ARGs abundances before and after AD were explored. To the best of the authors' knowledge, this study is the first to provide new insight towards a better understanding of EPS composition and the ARG abundances in AD integrated with THP.

3.2. Material and methods

3.2.1. Sludge and inoculum

Primary sludge, waste activated sludge, and anaerobically digested sludge were collected from the Gold Bar Wastewater Treatment Plant (Edmonton, Alberta, Canada) and stored at 4°C before use. The primary sludge (i.e., settled solids from primary clarifier) was mixed with waste activated sludge at a volume ratio of 1:1 and used for the experiment. The detailed characteristics of sludge and inoculum are provided in Table 3.1.

Table 3.1. Characteristics of substrates and inoculum

Parameters	**Inoculum**	**Substrate**		
	Digested sludge	**PS[a]**	**TWAS[b]**	**PS+TWAS[c]**
TSS (mg/L)	16,625± 125	33,875 ± 125	26,500 ± 250	30,188 ± 63
VSS (mg/L)	16,625 ± 1,250	30,500 ± 1,500	24,875 ± 375	30,188 ± 937
TCOD (mg/L)	24,188 ± 18	50,126 ± 750	39,385 ± 345	44,756± 210
SCOD (mg/L)	3,280 ± 97	5,152 ± 75	2,047 ± 51	3,600 ± 13

TVFA (mg COD/L)	125 ± 2	1,125 ± 13	103 ± 3	614 ± 4
TAN (mg/L)	1,244 ±57	79.45 ± 1.9	199 ± 1.5	139.2 ± 1.4
pH	7.50 ± 0.01	6.4 ± 0.01	6.6± 0.01	7.4 ± 0.04

[a] Primary sludge (PS).
[b] Thickened waste activated sludge (TWAS).
[c] Mixture of PS and TWAS (volume ratio of 1:1).

3.2.2. Thermal hydrolysis and biochemical methane potential (BMP) test

2 L bench-scale hydrothermal reactor (Parr 4848, Parr Instrument Company, Moline, IL, USA) was used for thermal hydrolysis of sludge at four different temperatures (80°C, 110°C, 140°C, 170°C) for 60 min exposure time. This exposure time is within the range reported in the literature (Abu-Orf and Goss, 2014; Pilli et al., 2015). For each experimental condition, 500 mL of feedstock (mixture of primary sludge and waste activated sludge) was fed to the hydrothermal reactor. The detailed operating protocol has been described elsewhere (Azizi et al., 2021a).

The biomethane potential of raw and pretreated sludge was appraised with the BMP test. The BMP tests were performed with glass anaerobic bioreactors (working volume of 300 mL) equipped with mechanical agitators and electric motors (ISES-Canada, Vaughan, ON, Canada). The feedstock and inoculum volumes were used based on the food to microorganism ratio (F/M) of 2 g of total chemical oxygen demand (TCOD) of sludge/g of volatile suspended solids (VSS) of inoculum. Furthermore, a blank test (inoculum + deionized water) was performed to evaluate methane production from the inoculum. Before start-up, the reactors were purged with nitrogen gas for 3 minutes and then placed in water baths at 37±2°C. The liquid was continuously mixed at 300 rpm. All tests were conducted in triplicate. The BMP tests were operated in a batch mode for 38 days, and the samples were taken before (day 0) and after the BMP tests (day 38) for analyses. Methane production was monitored daily using gas bags connected to sequestration bottles for capturing acidic gases (e.g., CO_2, H_2S). These bottles contained 3 M NaOH solution and a thymolphthalein indicator (Ryue et al., 2019). The methane gas volume was measured by a frictionless glass syringe.

3.2.3. Analytical methods

TCOD, soluble chemical oxygen demand (SCOD), and total ammonia nitrogen (TAN) concentrations were measured with Hach reagent kits (Hach Co., Loveland, Colorado, USA) using UV-spectrophotometer (Model DR 3900, HACH, Germany). For SCOD and TAN, the samples were filtered using 0.45 µm membrane syringe filters. Total suspended solids (TSS) and VSS were measured according to the standard method (APHA, 2005). The free ammonia nitrogen (FAN) concentrations were calculated according to the literature (Koster, 1986). Ion chromatography (DionexTM ICS-2100, Thermos Scientific, USA) equipped with an electrochemical detector and microbore AS19, 2 mm column was used for volatile fatty acids (VFAs) concentrations measurement; the samples were filtered using 0.2 µm membrane syringe filters. A bench-top pH meter (AR15 pH meter, Fisher Scientific, Pittsburgh, PA) was used for pH measurement. The student's paired t-test was performed to manifest the statistical difference between the results obtained from different conditions using Microsoft Excel. Fourier-transform infrared spectroscopy (FTIR) analysis was performed as previously described in the literature (Azizi et al., 2021a).

3.2.4. Microbial community and DNA extraction

For microbial community analysis, digested sludge samples were collected after the BMP test on day 38, followed by centrifugation at 5000 rpm for 15 min, then 0.5 g of the pellet was taken for the DNA extraction. The genomic DNA extraction was accomplished by PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer's instructions. For sequencing, the extracted DNA samples were stored at -70 °C. The purity and concentration of DNA were detected by using the Nanodrop spectrophotometer (Model 2000C, Thermo Scientific, USA). The universal primer set 515F/806R has been used to target 16S rRNA using Illumina Miseq sequencing (Table A1). For microbial diversity evaluation, the nucleotides sequence reads were stored out by using a data analysis pipeline. A denoising and chimera detection steps were carried out to remove short sequences, chimeric sequences, and noisy reads. After that, each sample was run using the analysis pipeline to determine the taxonomic information for each component in the sample. Quantitative Insights Into Microbial Ecology (QIIME) pipeline (QIIME2, Version 2021.2) was used to analyze microbial communities' taxonomy according to Zakaria and Dhar (2021).

3.2.5. Quantification of ARGs

Quantitative polymerase chain reaction (qPCR) was used for quantifying thirteen frequently detected ARGs including 7 tetracycline resistance genes (*tet*A, *tet*B, *tet*C, *tet*W, *tet*M, *tet*Q, *tet*X), 2 sulfonamide resistance genes (*sul*1, *sul*2), 2 macrolide resistance genes (*erm*B, *erm*C) and 2 ß-lactam resistance genes (bla_{AOX}, bla_{TEM}). In addition, integrons (*intl1*, *intl2*) and 16S rRNA were also quantified. The primers of the selected genes are provided in Table A1. QuantiFast SYBR Green PCR Kit (Qiagen, CA) was used for the preparation of qPCR mixtures in 25 µL reactions as following: 2 µL of the DNA template, 12.5 µL 2x master mix, 2.5 µL forward and reverse specific primer, and 5.5 µL nuclease-free water. Then, the CFX 96 real-time PCR system with a C1000 Thermal Cycler (Bio-Rad, USA) was used for the quantification process according to the QuantiFast SYBR Green PCR Kit's protocol. The PCR initial heat activation cycle at 95˚C for 5 min, 35 cycles at 95˚C for 10 sec and 60˚C for 30 sec, and one cycle at 40˚C for 30 seconds. All samples were run in triplicate.

3.2.6 EPS characterization

The EPS extraction was carried out by heating method due to its high performance reported in the literature (Xu et al., 2013). The biomass samples were centrifuged at 3000×g for 15 min at 21°C. Then, the supernatant was removed, and the pellet was washed with 0.1 M PBS (pH 7.4) three times. After washing, pellets were collected for cell lysis rate examination by Glucose-6-Phosphate Dehydrogenase kit (Sigma-Aldrich, USA). The details of EPS extraction and analytical methods were performed as previously described in the literature (Zakaria and Dhar, 2021). Carbohydrates were measured using the phenol-sulfuric acid method using glucose as a standard; details could be found in the literature (Zakaria and Dhar, 2021). After mixing 2 mL and 5 mL of EPS and concentrated H_2SO_4, respectively, 0.05 mL of 80 wt.% phenol was added. Then, the sample was left at room temperature for 10 min before shaking and incubating at 30°C for 20 min. After cooling down to room temperature and using the UV-spectrophotometer (Model DR 3900, HACH, Germany), the absorbance measured 490 nm. The EPS protein content was determined by using the Pierce Modified Lowry Protein Assay Kit (Thermo Fisher, USA) according to the manufacturer's instructions.

3.2.7. Multivariate analysis

Using Spearman's rank correlation, ARG abundance, EPS components, and microbial communities were correlated with different operating conditions (Khafipour et al., 2020). The correlation coefficient values ranged from -1 to +1, the higher positive values indicating stronger correlation and lower negative values indicating weak correlation. The alpha value for the correlation confidence intervals was set up as 0.05. Correlation analyses were visualized in principal component analysis (PCA) generated by JMP software (SAS Institute Inc., Cary, NC, US) and R software (RStudio v.1.4.1103).

3.2.8. Kinetic analysis

The methanogenesis kinetics for different conditions were assessed based on the BMP test experimental data using the modified Gompertz model. The detailed methodology for estimation of kinetic parameters can be found elsewhere (Azizi et al., 2021a; P. Zhou et al., 2021).

3.3. Results and discussion

3.3.1. Effect of THP on sludge solubilization

As shown in Fig. 3.1a, an increase in SCOD levels points to the disintegration of particulate biopolymers to soluble monomers and the release of the water-soluble components due to THP (Pilli et al., 2015). Notably, SCOD concentrations increased (p = 0.00004) from 3,599±12 mg/L for the raw sludge to 20,998±132 mg/L after THP at 170°C (Fig. 3.1a). However, TCOD concentrations remained unchanged, indicating no volatilization of organics occurred during THP. Thus, SCOD/TCOD ratios in pretreated sludge dramatically increased compared to the raw sludge (Fig. A1). After THP, TSS and VSS concentrations also considerably decreased in the pretreated sludge (Fig. 3.1b). All pretreatment conditions significantly decreased VSS/TSS ratios (Fig. A1). The VSS reductions were 24.4%, 32.5%, 39.3%, and 49.3% for THP at 80°C, 110°C, 140°C, and 170°C, respectively. Thus, noticeably, increasing THP operating temperature increased the solubilization of particulate organics linearly.

As shown in Fig. 3.1c, TVFA concentration increased from 604±2.8 mg COD/L (raw sludge) to 1,091±11.3 (p = 0.003), 1,269±5 (p = 0.001), 1,752±7.3 (p = 0.005), and 2,275±14.9 (p = 0.003) mg COD/L for THP at 80°C, 110°C, 140°C, and 170°C, respectively. Similar to SCOD, VFA concentrations also increased with increasing THP operating temperature. These results agree with

previous reports on increasing organics and suspended solids solubilization with increasing THP temperatures in different ranges (50-170ºC) (Azizi et al., 2021a; Bougrier et al., 2007; Xue et al., 2015). As suggested in the literature, enhanced hydrolysis could ultimately increase the downstream acidogenesis process for the production of VFAs (Fu et al., 2021; Yang et al., 2019).

Compared to the raw sludge, TAN concentrations increased in all pretreated samples (Fig. 3.1d). Notably, TAN concentration reached up to 436.3±8.9 mg/L (p = 0.008) at 170 ºC, while TAN concentration in raw sludge was 139.15±1.2 mg/L. A significant increment of TAN concentrations is typically attributed to the hydrolysis of nitrogenous compounds, such as proteins (Azizi et al., 2021a). Despite remarkable increments in TAN after THP, TAN levels in all the samples were lower than 440 mg/L, which was much lower than inhibitory TAN concentrations (4.2 g/L) previously reported for AD (Jarrell et al., 1987). Due to the further hydrolysis during AD, TAN concentrations increased >1,000 mg/L in digestate samples after the BMP test (Fig. A2b), with the highest concentration of 1446.05±0.95 mg/L (p = 0.001) was observed for the digested THP sample at 170°C. However, the digester operating conditions, methanogenic communities can be inhibited if FAN concentration is around 215-1,450 mg/L (Yenigün and Demirel, 2013). In both raw and pretreated samples, FAN concentrations were <1 mg/L (Fig. A2a). Although FAN concentrations increased in all digestate samples, the pretreated digestate samples showed considerably lower FAN levels than the control. Notably, the highest FAN of 209±0.77 mg/L was observed for the digested control sample, while the lowest concentration of 169±0.13 mg/L (p = 0.004) was observed for the digested THP 140°C. As the operating temperature was the same for all conditions, estimated FAN concentrations (Fig. S2c) were correlated with TAN and pH values (Fig. A2b).

3.3.2. Changes in EPS and macromolecules

The changes in EPS were characterized in terms of polysaccharides and proteins as they are considered the most dominant EPS components in sludge (Dai et al., 2013). As shown in Fig. 3.1e, polysaccharides and proteins contents in sludge decreased after the THP. Polysaccharide and protein contents in the THP-80ºC sample were 173.5±3.5 and 306±6 mg/g sludge, which is 7% and 37%, respectively, lower than the control (i.e., raw sludge). The highest reduction of polysaccharide and protein (45% and 64%, respectively) was observed for THP-170°C. Noticeably, EPS contents decreased gradually with increasing THP temperature. Previous studies

also reported that THP could disrupt the EPS network, releasing intra- and extracellular organics in the aqueous phase (Eskicioglu et al., 2006; J. Sun et al., 2016). Furthermore, FTIR analysis of solids was carried out to identify the effects of THP on functional groups associated with macromolecular compounds (Fig. A3). FTIR results also confirmed solubilization of macromolecular organics after the THP. Moreover, the gradual decrease of the absorption peaks with increasing the THP operating temperature accentuated the relationship between THP operating temperatures and solubilization efficiencies.

Both protein and polysaccharide contents in the extracted EPS from the digestate (after BMP) from THP-110ºC and THP-140ºC were less than those in control, THP-80ºC, and THP-170ºC samples (Fig. A4). Interestingly, THP-110ºC and THP-140ºC also showed higher methane production than other conditions (discussed later). Notably, a dramatic shift in the EPS composition was observed for the digestate THP-170ºC sample. That might be attributed to the microorganisms' protection mechanism that involves polysaccharides secretion to form a protective layer against the recalcitrant or inhibitory compounds commonly formed at high temperatures (S. Li et al., 2019; Liu and Fang, 2002). Moreover, for the THP-170ºC sample, the increased EPS contents during AD and decreased methane generation (discussed later) suggest that THP at 170ºC might form some recalcitrant/inhibitory compounds. Increasing EPS in the form of proteins can have a positive impact, as proteins can act as electron shuttles due to the exoenzyme's existence, enhancing the extracellular electron transfer and improving AD performance (S. Li et al., 2019). However, there is no evidence of such a positive impact of EPS polysaccharides (S. Li et al., 2019).

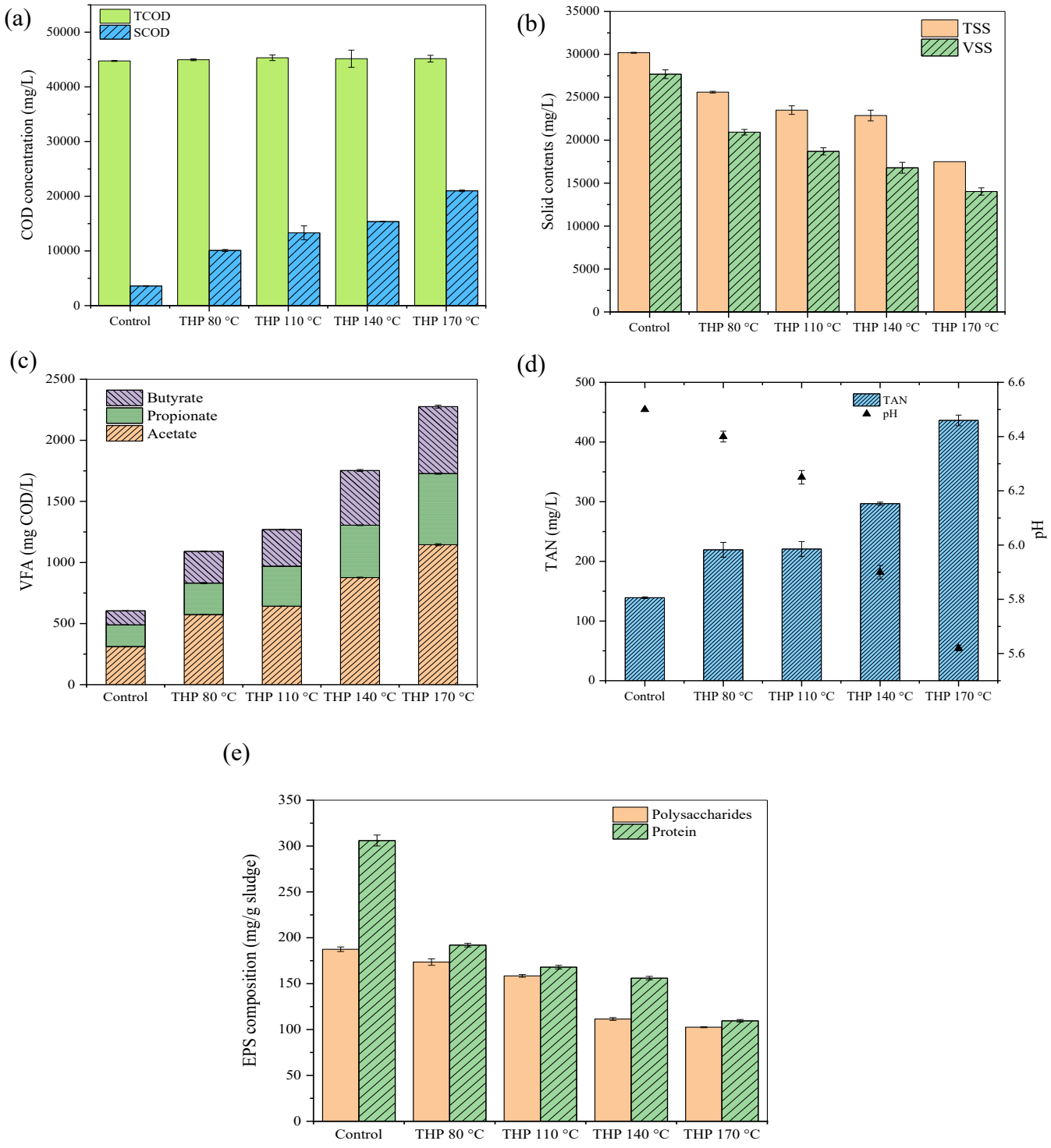


Figure 3.1. Effects of THP on (a) TCOD and SCOD, (b) TSS and VSS, (c) VFAs, (d) TAN, (e) EPS concentrations.

3.3.3. Methane production

As shown in Fig. 3.2, THP under different temperatures significantly improved the total cumulative methane yields than the control. For control, THP-80°C, and THP-110°C, methane production started immediately without any noticeable lag phases. In contrast, minor lag phases appeared for THP-140°C and THP-170°C. The estimated lag phases with the modified Gompertz model were also consistent with these experimental observations (Table A2). These results may attribute to the period that microorganisms need to adapt to the thermally pretreated sludge (P. Zhou et al., 2021). Particularly, high-temperature THP may release some refractory and inhibitory compounds that can extend the lag phases during AD (Zhu et al., 2021). Nonetheless, ultimately, all THP-treated samples led to higher total cumulative methane yields than the control. Despite higher lag phases than the control, THP-140ºC and THP-170°C ultimately led to higher methane yields than the control, attributed to the higher maximum methane production rate than the control (see Table A2). The accumulated methane production increased by 20.6±1.9%, 32.3±1.7%, 40.5±2.5% and 19.3±0.2% for THP-80°C, THP-110°C, THP-140°C, and THP-170°C, respectively, compared to the control. Among the THP samples, the maximum methane yield (p = 0.03) of 275±11.5 ml CH_4/g COD was obtained for THP-140°C, while the least methane yield (p = 0.07) of 203±6.9 ml CH_4/g COD was observed for THP-170°C. Thus, the cumulative methane yields increased linearly with temperature increment except for THP-170°C. The negative effect of THP at 170°C on methanogenesis kinetics might attribute to recalcitrant compounds or toxic intermediates (e.g., melanoidins) formation (Carrere et al., 2012).

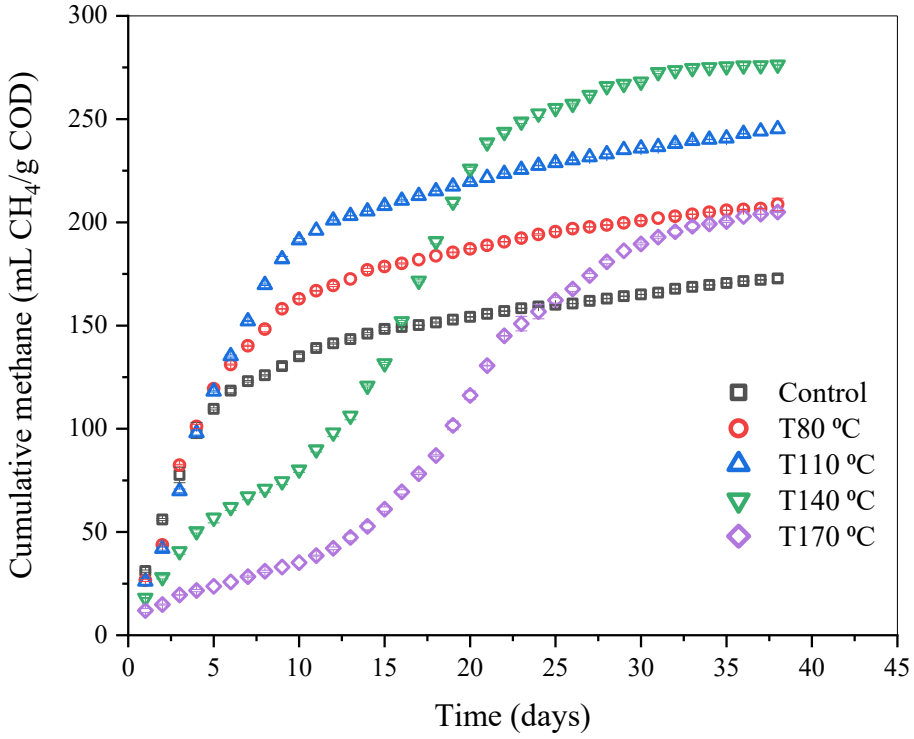


Figure 3.2. Cumulative methane production for raw and pretreated samples. Note: yields were calculated based on the initial COD of substrate.

3.3.4. Fate of ARGs

All targeted ARG subtypes were found in raw and pretreated sludge, as well as in the digestate after AD (Fig. 3.3). The absolute copy number of ARGs in the initial sludge sample was 3.31×10^5 copies/g sludge (Fig. 3.3a). Compared to the raw sludge, a significant reduction (p = 0.000002 - 0.000009) of total ARGs was observed after THP. The ARGs copy number after THP at 80°C, 110°C, 140°C, 170°C were 1.54×10^5, 9.96×10^4, 1.09×10^5, 1.06×10^5, respectively (Fig. 3.3a). Thus, THP could remarkably reduce ARGs abundance prior to AD, while THP-110ºC provided the highest total ARGs removal (70%). The total ARGs also decreased in the subsequent AD except for the THP-110°C sample (Fig. 3.3a). After AD, digestate from THP-140ºC and THP-

170ºC showed lower ARG abundances than the digestate from control, while THP-140ºC was the most effective for overall ARG removal (79%) in the final digestate. Despite THP-110ºC being the most effective for ARG removal from before AD, the corresponding digestate sample showed an increase in ARG abundance (Fig. 3.3a). The increase in total ARGs for THP-110°C sample indicates that rebounding of ARGs occurred during AD. Previous studies also reported similar ARG rebounding for thermally hydrolyzed sludge (Pei et al., 2016).

As shown in Fig. 3.3b-c, various ARG subtypes (e.g., *tet*W*, tet*M*, tet*B*, tet*A, and *sul*1) might rebound during AD. Some potential ARG host microbes (e.g., *Treponema* (W. Sun et al., 2019), *Pseudomonas* (W. Sun et al., 2019), *Desulfotomaculum* (Shi et al., 2020) can resist extreme environmental conditions (e.g., high temperature and pressure up) up to a certain limit during THP by forming endospores to cope with stressful conditions (O'Sullivan et al., 2015). Thus, some host microbes might also exist in the pretreated sludge. Such a survival mechanism may happen under moderate temperature (110°C) than high temperature (140°C and 170°C). When favorable conditions return, these endospores sprouts and active bacterium release to proliferate (Rampelotto, 2010). Thus, host microbes may proliferate in subsequent AD. Moreover, the possibility of residual DNA and horizontal gene transfer (HGT) may be a reason behind such rebounding (Ma et al., 2011). Noteworthy, the HGT is mediated by the MGEs, such as integrons (e.g., *intl*1, *intl*2) that control the DNA movement by encoding specific proteins (Lu et al., 2019).

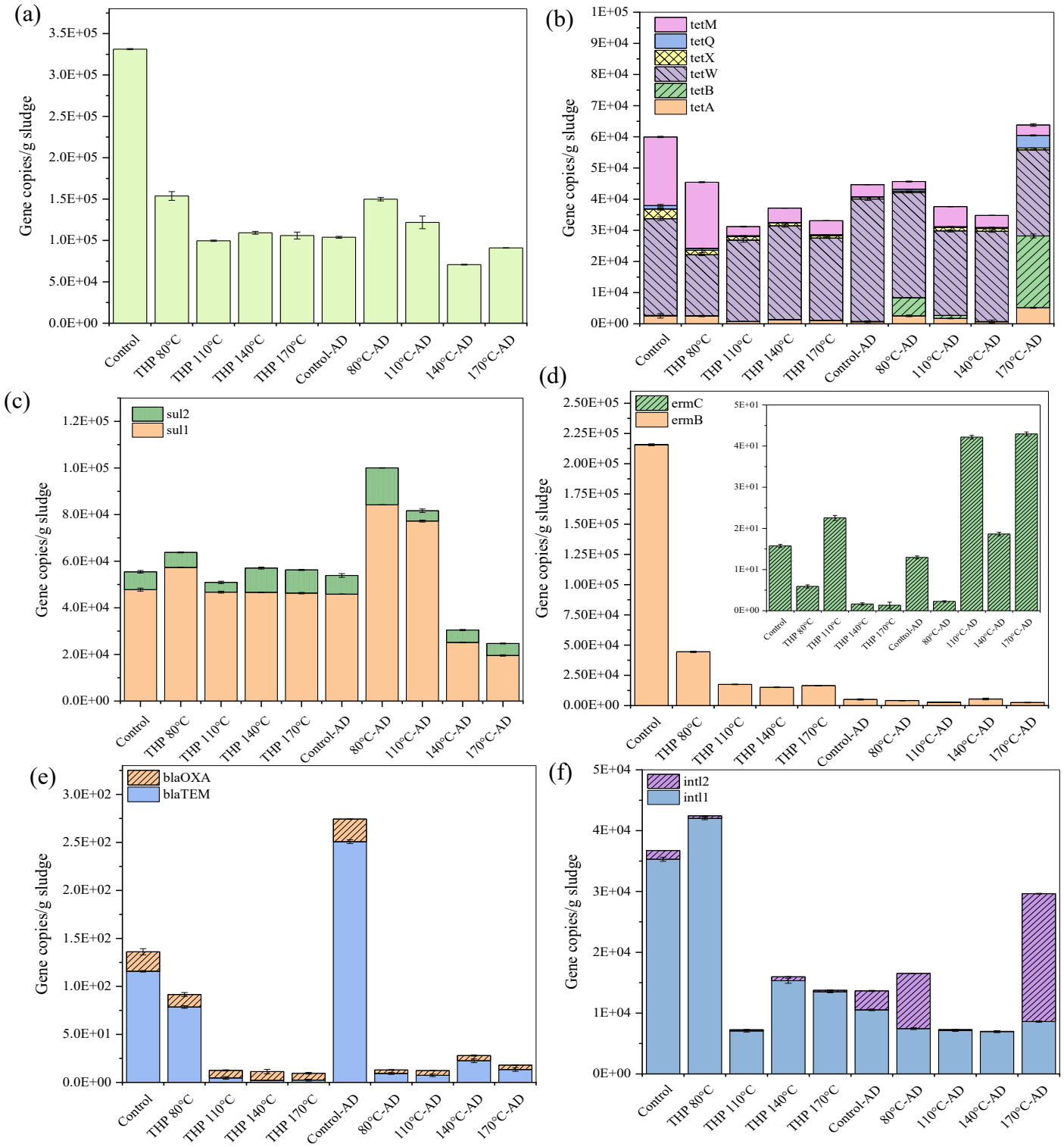


Figure 3.3. (a) Total ARGs, (b) tetracycline resistance genes, (c) sulfonamide resistance genes (d), macrolide resistance genes (e) β-lactam resistance genes, and (f) integrons in pretreated and digested sludge.

3.3.5. Microbial quantity, diversity, and richness

The quantitative qPCR analysis was performed for the initial (inoculum + sludge) and final digestate. Due to the pretreatment, 16S rRNA gene copies remarkably decreased from 8.71×10^9 gene copies/g sludge in control to as low as 3.84×10^6 gene copies/g sludge for THP-170°C (Fig. 3.4). However, 16S rRNA gene copies increased after AD. Notably, 16S rRNA gene copies gradually increased in digestate samples from THP-80°C to THP-140°C. The solubilization of organics via THP led to the proliferation of microbial communities. In contrast, the formation of recalcitrant or toxic compounds (Carrere et al., 2012) at 170°C might decrease microbial propagation.

The estimated alpha diversity indices were provided in Table A3. Compared to the control, all the indices were decreased after the THP except for the Chao1 and OTUs for THP-80°C. The highest reduction in the microbial alpha diversity was observed for THP-170°C. For instance, Chao1, Shannon, Pielou, and observed OTUSs were reduced from 171, 6.4, 0.86, and 170 to 95, 5.1, 0.79, and 95, respectively. Thus, THP could mostly reduce microbial community diversity and richness. This result agrees with previous studies that reported that temperature is the major factor affecting microbial alpha diversity (Wu et al., 2020). Compared to the control, digestate for THP samples exhibited higher microbial diversity and richness. This might attribute to the enhanced sludge solubilization due to THP, which subsequently enhanced microbial diversity and richness during AD (Niu et al., 2020).

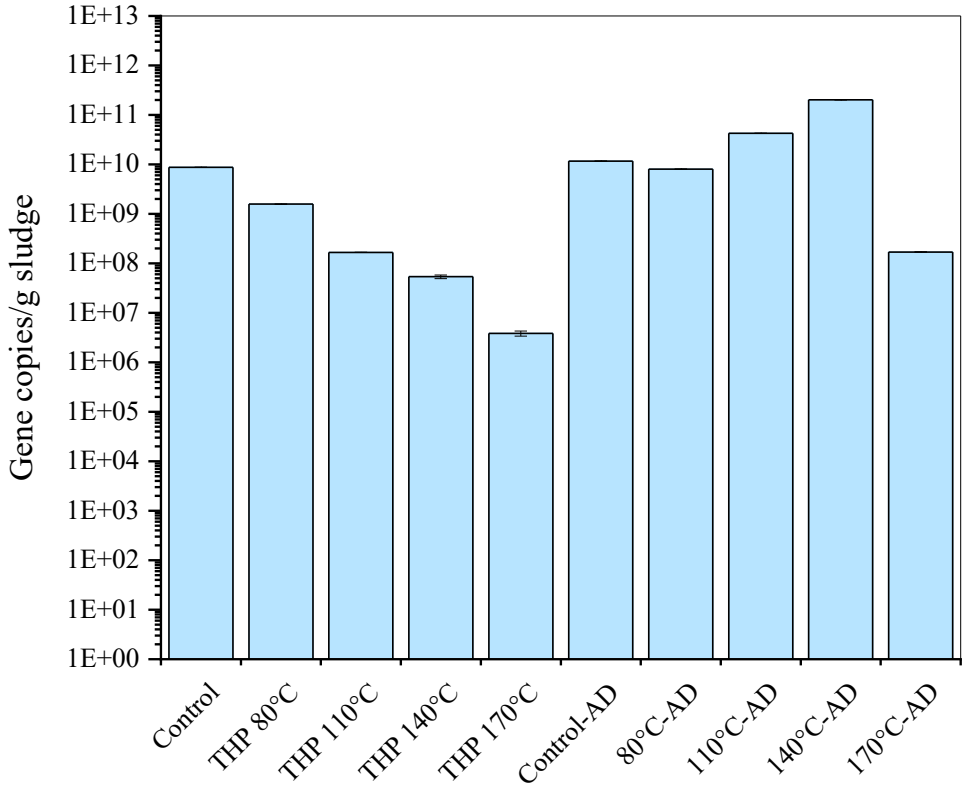


Figure 3.4. 16S rRNA gene copies in raw, pretreated, and digestate sludge samples

3.3.6. Bacterial community

Among the pretreated digested samples, dominant bacterial phyla were *WWE1, Firmicutes, Chloroflexi, Bacteroidetes*, and *Proteobacteria* (Fig. A5). Notably, members of *WWE1* were the most dominant in all samples. They are known for the fermentation of sugars in AD (Limam et al., 2014). *Firmicutes* are syntrophic bacteria involved in VFAs degradation (Feng et al., 2019). Compared to the control, their relative abundance increased in digested THP samples. *Chloroflexi* species are known to hydrolyze carbohydrates (Feng et al., 2019). Their relative abundance in control (21%) was higher than all digested THP samples. *Bacteroidetes* and *Proteobacteria* can degrade various organics, including cellulose and proteins (Zhou et al., 2020). Their relative abundances were higher digested THP samples than the control. Notably, their highest abundance was observed for the digested THP-170°C sample (12% and 13%, respectively). *Bacteroidetes* and *Proteobacteria* are known as potential carriers of tetracycline resistance genes and other ARGs in general (Chen et al., 2018; Li et al., 2020; W. Sun et al., 2016). Thus, the highest abundance of tetracycline resistance genes observed in digestate of THP-170°C sample was consistent with their high abundance.

At the genus level (Fig. 3.5a), the most dominant genera were *W22* (family *Cloacamonaceae*) and *T78* (family *Anaerolinaceae*) in all samples. Their highest abundances were observed in the control (47% and 20%, respectively), while both showed a remarkable decrease in the digested THP samples. The members belonging to *W22* (family *Cloacamonaceae*) were reported as syntrophic VFAs oxidizers (Dennehy et al., 2017). Moreover, their potential roles in hydrolysis and acidogenesis have also been suggested (Jiang et al., 2019). The genus *T78* can contribute to hydrolysis/acidification, including carbohydrates and oil organics degradation (Feng et al., 2019; Wang et al., 2016).

Like control, *W22* was still the most dominant genus (20-35%) in digested THP samples. However, their relative abundances noticeably decreased for higher THP operating temperatures (140-170°C). Other dominant bacterial genera in digested THP samples include *Bacteroides* (6-8%), *T78* (9-12%), *Clostridium* (5-9%), and *Treponema* (4-6%). Members belonging to the genus *Clostridium* and *Bacteroides* are obligate anaerobes and can contribute to the fermentation of organics in AD (Chen et al., 2005; Ziganshina et al., 2021). Among all digested THP samples, the lowest relative abundances of these bacterial genera were observed for THP-170°C. However, the

abundance of *Syntrophomonas* and *Acidovorax* increased for this condition. Thus, these results indicate that increasing temperature led to distinct differences in bacterial communities.

3.3.7. Archaeal community

Fig. 3.5b shows the relative abundances of archaeal communities. The digested control sample was dominated by the genus *Methanosaeta* (41%), the family *Methanospirillaceae* (29%), followed by genera *Methanoculleus* (16%), and *Methanobacterium* (14%). The relative abundance of acetoclastic *Methanosaeta* (Z. Guo et al., 2021) was reduced in all digested THP samples. In contrast, various known hydrogenotrophic methanogens (*Methanoculleus*, *Methanospirillaceae,* and *Methanobacterium*) were dominant in these samples. *Methanoculleus* was the most prevalent in the digested THP-140°C sample (41%). Also, hydrogenotrophic *Methanospirillaceae* (19%) and metabolically versatile *Methanosarcina* (20%) were dominant in this sample. The digested THP-170°C sample was dominated by *Methanosarcina* (28%), and *Methanobacterium* (38%). Among the digested THP samples, the relative abundances of acetoclastic *Methanosaeta* species were higher in the digested THP-80ºC and THP-110ºC samples. Hydrogenotrophic methanogens usually have a higher ability to resist environmental changes than acetoclastic methanogens (Merlin Christy et al., 2014). Thus, it appeared that high-temperature THP (140 and 170ºC) might have more pronounced effects on the archaeal community distribution and methanogenesis pathways.

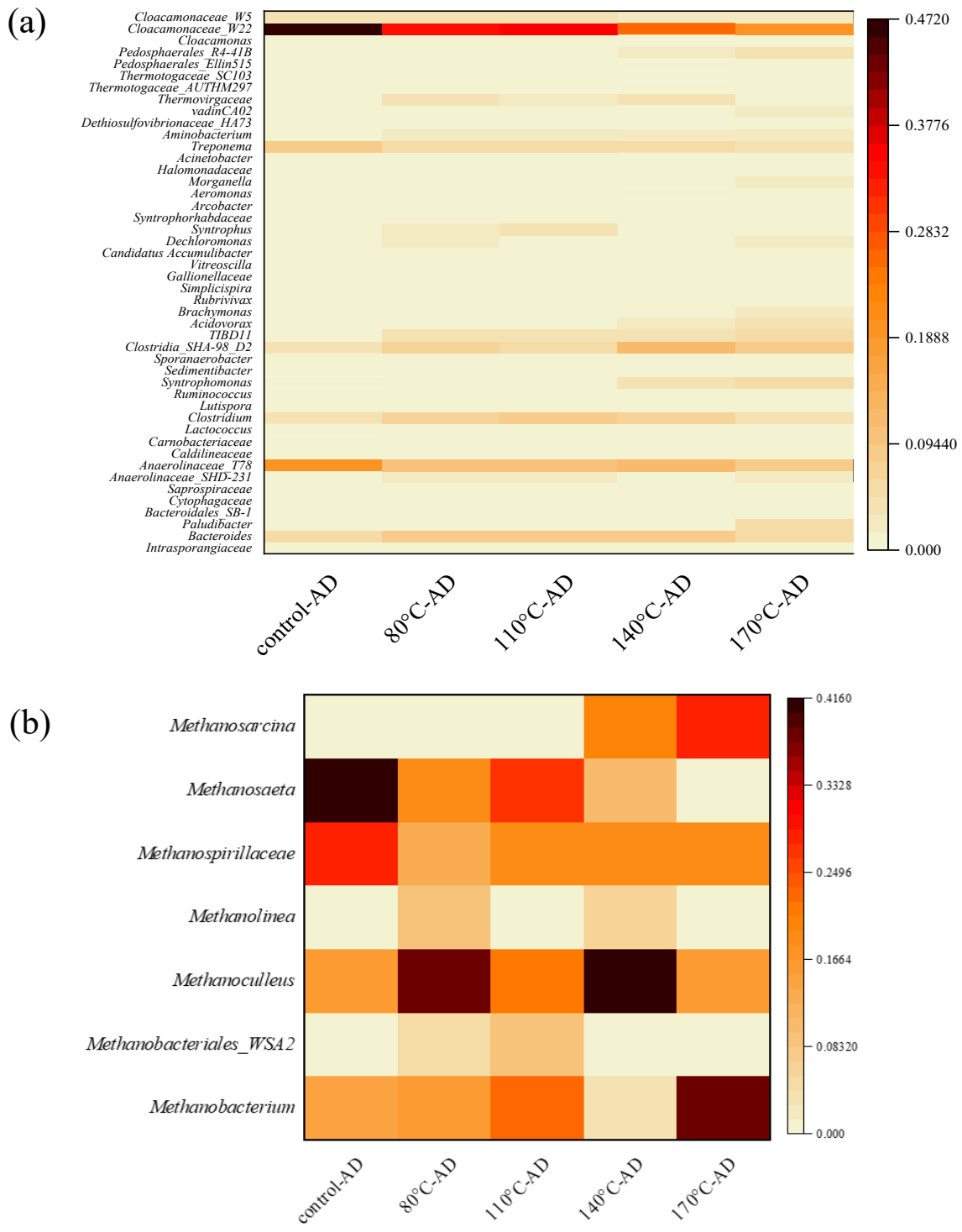


Figure 3.5. Relative abundance of (a) bacterial, (b) archaeal communities at the genus level.

3.3.8. Multivariate analysis

The multivariate PCA was performed to evaluate the correlation between ARG abundance and bacterial communities in digested samples (Fig. 3.6a). For THP-110°C and THP-80°C, *Clostridium*, *Bacteroides*, *Thermovirgaceae*, and *Syntrophus* were closely associated with sulfonamide resistance genes. *Cloacamonaceae_W22*, *Cloacamonaceae_W5*, *Treponema*, and *Anaerolinaceae* were clustered in a different quadrant close to the control and associated with β-lactam and macrolide resistance genes. The tetracycline resistance genes, strongly correlated with integrons, were close to THP-170°C, where *Acidovorax*, *Paludibacter*, and *Syntrophomonas* genera were dominant. Based on previous reports, *Clostridium*, *Bacteroides*, *Treponema*, *Paludibacter*, *Syntrophomonas, Acidovorax* species are potential ARG hosts (Han et al., 2020; Kenyon, 2018; Sun et al., 2021; Wen et al., 2021). For instance, the prevalence of macrolide resistance genes in *Treponema* species has already been widely reported (Kenyon, 2018). Thus, THP played a critical role in shaping the bacterial communities and consequently changed the ARG profiles.

Interestingly, digested THP-80ºC, THP-110ºC, and THP-140ºC samples showed a similar decreasing trend for EPS (discussed earlier) and total ARGs, indicating a possible positive correlation between them. Therefore, the relationships between ARG abundances and EPS composition were further analyzed (Fig. 3.6b). EPS proteins, *int*l1, *sul2*, bla_{TEM}, $bla_{OXA,}$ and *erm*B were located close to the control and THP-80°C samples. On the other hand, *intl2*, *tet*B, *tet*Q, and *tet*A were associated with EPS polysaccharides and were in a different quadrant close to the THP-170°C sample. However, *sul*1 was close to the THP-140°C sample, while *tet*M and *tet*X were located in a different quadrant close to the THP-110°C sample. However, different functional components of the EPS layers could affect the ARGs abundance. For instance, humic acids have strong adsorption to the DNA molecules by ligand binding, hydrophobic interactions, precipitation, and aggregation (Saeki et al., 2011). These results indicate that ARG profiles responded differently to the THP operating temperatures. Moreover, different EPS components (polysaccharides and proteins) were correlated with different ARG types/subtypes.

To further understand the relationship between ARG and EPS, correlation analysis was performed. Fig. 3.6c shows the correlation coefficients of THP-AD, EPS components, and ARG abundances in digested sludge. Obviously, there was a positive correlation between the EPS

protein component and all variables except for the THP-AD (r = -0.76). In contrast, all the process variables showed a high negative correlation with THP-AD except for the tetracycline resistance genes and integrons (r = 0.29 and 0.30), respectively. For instance, a strong positive correlation between the EPS protein component exhibited a strong positive correlation with β-lactam resistance genes (r = 0.75), while the correlation with other ARGs, such as sulfonamides and macrolides (r = 0.17 and 0.33, respectively) was fairly weak. On the other hand, EPS polysaccharides showed a positive correlation with all ARGs, especially with tetracycline resistance genes (r = 0.87), while macrolides resistance genes were the only exception (r = -0.31). Also, the integrons, which are considered biomarkers for ARG spread (Chen et al., 2010), exhibited a very strong correlation with tetracycline resistance genes (r = 0.99). In contrast, the integrons showed a relatively weak correlation with other ARGs.

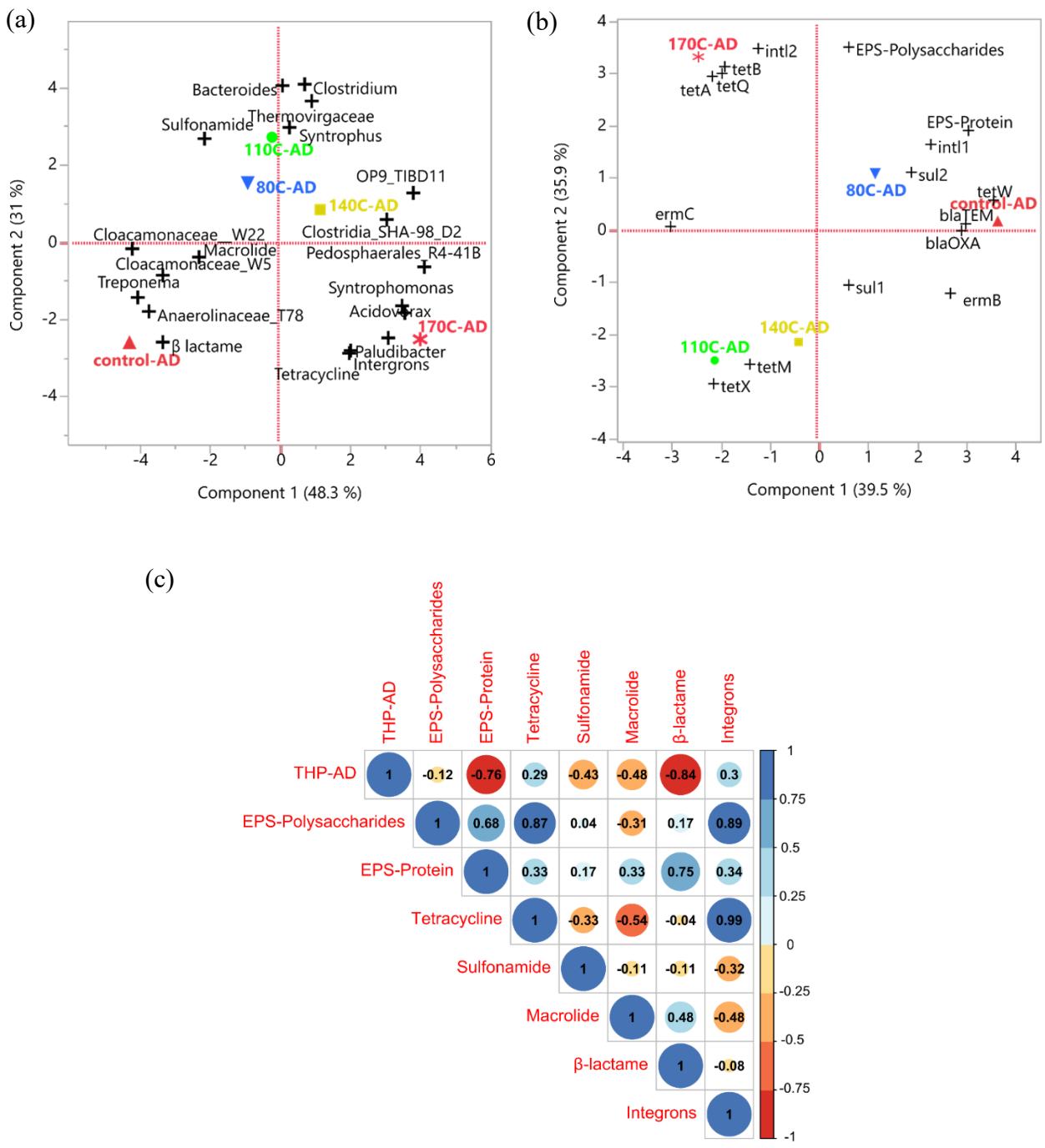


Figure 3.6. (a) PCA of ARGs and microbial communities, (b) PCA of ARGs and EPS components, and (c) Correlation analysis between EPS components and ARG abundance under different conditions.

3.4. Implications

Our results suggest that different EPS components (proteins and polysaccharides) correlate with different ARGs and MGEs. As an important component of sludge, EPS may provide ample adsorption sites for ARGs and play a critical role in their propagation (He et al., 2019b; L. Wang et al., 2021; Zhu et al., 2021). Different sludge EPS components have different functional groups, such as carboxyl, phenolic, hydroxyl, etc. (Wang et al., 2018). Thus, the adsorption of different ARGs and MGEs onto various EPS components can be different. Interestingly, the digestate THP-140^{o}C sample had the lowest level of proteins and polysaccharides among all digestate samples, exhibiting the lowest ARG abundance. Overall, these results infer a functional link between EPS (proteins and polysaccharides) composition and ARGs in AD of thermally hydrolyzed sludge under different temperatures. Although a recent report suggested that EPS-associated ARGs would present a most significant portion of ARGs in sludge (L. Wang et al., 2021), the relationships observed in the multivariate analysis in this study remain correlational. Thus, further research should focus on the detailed characterization and changes of EPS-associated, intracellular, and cell-free ARGs under THP. Also, different layers of EPS might affect the fate and abundance of ARGs (He et al., 2019b), which require further investigations.

Chapter 4

Fate of intracellular, extracellular polymeric substances-associated, and cell-free antibiotic resistance genes in anaerobic digestion of thermally hydrolyzed sludge

A version of this chapter was submitted to a journal for peer-review and publication

4.1. Introduction

The spreading of antibiotic resistance genes (ARGs) in our ecosystem is considered as one of the most threatening global public health concerns these days. Particularly, high concentrations of residual antibiotics and ARGs have been found in different liquid and solid treatment trains in wastewater treatment plants (Haffiez et al., 2022a; Wang et al., 2020; R. Wang et al., 2021). It was reported that ARGs could be classified into intracellular and extracellular in the sludge flocs (Sui et al., 2019; Wang et al., 2022). Although intracellular ARGs have gained considerable attention since they could be transferred by transduction or conjugation in sludge, extracellular ARGs are often overlooked. Cell lysis and/or live cell secretion lead to the extracellular ARGs proliferation in the form of cell-free ARGs; however, when extracellular ARGs adsorbed on extracellular polymeric substances (EPS) in sludge, EPS-associated ARGs are formed (Wang et al., 2022). A few recent studies highlighted the significance of EPS on ARGs propagation (Haffiez et al., 2022a; He et al., 2019b; L. Wang et al., 2021).

The functional and structural characteristics of EPS enable abundant adsorption sites of several pollutants, such as organic micropollutants and heavy metals (Yu, 2020). For instance, humic and proteinaceous substances effectively bind to heavy metals and antibiotics via several functional groups, such as carboxyl and amine groups (Haffiez et al., 2022a; Yu, 2020). Interestingly, the adsorbed pollutants could be degraded in the EPS matrix, which continuously avails new adsorption sites (Yu, 2020). In addition, some proteinaceous bacterial flagella and type IV pili in EPS bind to extracellular DNA (eDNA) by cross-β structured proteinaceous amyloid (Aqeel et al., 2019; Yu, 2020). ARGs adsorption capacity on EPS could be influenced by the adsorbed heavy metals that decrease the EPS pore size, increasing the ARGs settling on EPS, consequently enhancing the resistance dissemination between microbial communities (Yu, 2020). A recent study

showed a positive correlation between polysaccharides in EPS and several ARGs profiles (Haffiez et al., 2022a). Also, a recent study revealed that EPS-associated ARGs are the major source of extracellular ARGs in sludge since they induce the resistance spreading by transformation mechanism (L. Wang et al., 2021). However, Wang et al. (2022) found that the transformation ability of EPS-associated ARGs is much higher than cell-free ARGs, which elucidates the high environmental concerns posed by EPS-associated ARGs.

Anaerobic digestion (AD) is widely used in centralized wastewater treatment plants for sludge handling and management due to its efficiency in pathogen removal, bioenergy recovery, and sludge reduction (Azizi et al., 2022, 2021a; Haffiez et al., 2022a; P. Zhou et al., 2021). Although the fate of ARGs during AD has been extensively studied (Haffiez et al., 2022c; Jang et al., 2017; Ma et al., 2011; Tong et al., 2017), the distribution of EPS-associated ARGs and their effect on total ARGs in digestate has not been studied yet. Moreover, preteratment of sludge using the thermal hydrolysis process (THP) under certain operating temperatures has shown promising results in terms of ARGs reduction during AD (Haffiez et al., 2022a; Ma et al., 2011; Tong et al., 2017). Recently, it has been found that THP as a pretreatment prior to AD could decrease EPS polysaccharides and proteins along with the reduction in ARGs abundance (Haffiez et al., 2022a). Therefore, a fundamental understanding of the fate of EPS-associated ARGs during AD of thermally hydrolyzed sludge is crucial to developing THP for ARGs dissemination control in AD.

To address these fundamental knowledge gaps, the specific objectives of this study were to (1) investigate intracellular and extracellular ARGs, including EPS-associated ARGs, and evaluate their contributions to the total ARGs in sewage sludge and their fate during thermal hydrolysis (90° C and 140° C) and subsequent AD, (2) characterize of various EPS components (proteins, polysaccharides, and eDNA) and microbial communities, and (3) finally, explore the possible correlations between extracellular ARGs, intracellular ARGs, and EPS components. The results will provide critical insights into the extracellular ARGs and their role on overall ARG propagation in AD of thermally hydrolyzed sludge. and correlated with the intracellular and extracellular ARGs.

4.2. Material and methods

4.2.1 Experimental operation and sampling

Thickened waste activated sludge (TWAS) and anaerobically digested sludge were collected from the Gold Bar Wastewater Treatment Plant (Edmonton, Alberta, Canada). After collection, the samples were stored at 4°C in a cold room. The digested sludge was pre-incubated at 37°C for 5 days for microbial communities' acclimation and residual organics reduction. The detailed sludge and inoculum characteristics are provided in Table B1.

For thermal hydrolysis pretreatment, 500 mL of TWAS was placed in a 2 L bench-scale hydrothermal reactor (Parr 4848, Parr Instrument Company, Moline, IL, USA) for pretreatment under temperatures of 90°C and 140°C for 60 min exposure time. The experimental exposure time and temperature ranges are within the ranges reported in the literature (Abu-Orf and Goss, 2014; Pilli et al., 2015). The detailed operating protocol for THP has been described elsewhere (Azizi et al., 2021a; Haffiez et al., 2022a).

The biomethane potential test (BMP) tests for the raw and pretreated sludge samples were conducted with anaerobic glass bioreactors with a working volume of 300 mL. The bioreactors were equipped with electric motors and mechanical agitators (ISES-Canada, Vaughan, ON, Canada) to ensure the continuous mixing at 300 rpm during operation of 30 days. The BMP tests were performed based on food to microorganism ratio (F/M) of 2 g of total chemical oxygen demand (TCOD) of TWAS/g of volatile suspended solids (VSS) of inoculum (Haffiez et al., 2022a). Prior to the start-up, N_2 was purged for 3 min in the bioreactors and then placed in water baths at 37±2°C. The BMP tests were operated in triplicate. The methane produced from each bioreactor was monitored and collected daily by 1 L gas bags connected to the CO_2 sequestration unit. In each sequestration bottle, 3 M NaOH solution and a thymolphthalein indicator for CO_2 capturing (Haffiez et al., 2022a; Ryue et al., 2019). The volume of methane was measured using a frictionless syringe. Moreover, a blank test of inoculum and deionized water was conducted to determine the methane production from the inoculum.

4.2.2 Extracellular polymeric substances (EPS) extraction and characterization

The EPS extraction was performed using the heating method. According to the literature, the heating method is highly effective for EPS extraction, especially for proteins, carbohydrates, and extracellular DNA (Haffiez et al., 2022a; Xu et al., 2013). After the centrifugation of the biomass sample at 3000 × g for 15 min at 21°C, the supernatant was discarded, and the pellet was washed three times with 0.1 M PBS (pH 7.4). Then, the pellets were washed twice with 0.9% NaCl (w/v) and resuspended in 0.9% NaCl (w/v) prior to be heated at 60°C water bath for 30 min. After the heating time, the sample was centrifuged at 20,000 × g at 4°C for 20 min. Then, the supernatant was filtered by a 0.2 µm filter and then the EPS samples were stored at -20°C for further analysis. The cell lysis was measured using Glucose-6-Phosphate Dehydrogenase kit (Sigma-Aldrich, USA) prior to the EPS components analysis. The protein content was measured by the Pierce Modified Lowry Protein Assay Kit (Thermo Fisher, USA) based on the manufacturer's instructions. Also, carbohydrates were measured according to the phenol-sulfuric acid method and using glucose as a standard (the detailed method can be found elsewhere (Haffiez et al., 2022a; Zakaria and Dhar, 2021)). The extracellular DNA (eDNA) was isolated from EPS samples using PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer's instructions. Then, the quality and quantity of eDNA concentrations were measured using the Nanodrop spectrophotometer (Model 2000C, Thermo Scientific, USA).

4.2.3. DNA extraction and microbial community analysis

After the 30 days of BMP test, the digested samples were collected and centrifuged for 15 min at 2000 × g. Then, the supernatant was discarded and the pellet was taken (~0.5 g) for the genomic DNA extraction using PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer's instructions. Nanodrop spectrophotometer (Model 2000C, Thermo Scientific, USA) was used to determine the DNA quality and quantity. Prior to the sequencing, the extracted DNA samples were stored at -70 °C. 16S rRNA gene was sequenced using Illumina Miseq with universal primer set 515F/806R at the Research and Testing Laboratory (Lubbock, TX, USA) (Table B2). For the microbial community taxonomy and diversity, the raw sequencing data have been processed using Quantitative Insights Into Microbial Ecology (QIIME)

pipeline (QIIME2, Version 2021.2) after the filtration and denoising process. The detailed method can be accessed elsewhere (Zakaria and Dhar, 2021).

4.2.4 ARGs quantification

Seven tetracycline resistance genes (*tet*W, *tet*A, *tet*B, *tet*Q, *tet*M, *tet*X), two ß-lactam resistance genes (bla_{AOX}, bla_{TEM}), two sulfonamide resistance genes (*sul*1, *sul*2), two macrolide resistance genes (*erm*B, *erm*C), two MGEs (*intl1*, *intl2*), and 16S rRNA were targeted to be analyzed by quantitative polymerase chain reaction (qPCR). Details of the primers for the selected genes are given in the Supplementary Information. The preparation of qPCR mixtures was carried out by QuantiFast SYBR Green PCR Kit (Qiagen, CA) (Haffiez et al., 2022a). The quantification process was performed using CFX 96 real-time PCR system with a C1000 Thermal Cycler (Bio-Rad, USA) according to the QuantiFast SYBR Green PCR Kit's protocol. The qPCR protocol can be found elsewhere (Haffiez et al., 2022a). All samples were run in triplicate.

4.2.5. Multivariate analysis

Intracellular ARGs, extracellular ARGs, and EPS components were correlated with different THP conditions using Spearman's rank correlation (Haffiez et al., 2022a; Khafipour et al., 2020). The correlation coefficient values ranged from -1 to +1. The alpha value for the correlation confidence intervals was set up as 0.05. Correlation analyses were visualized using R software (RStudio v.1.4.1103). Also, EPS-associated ARGs and EPS components with THP conditions were visualized in principal component analysis (PCA) generated by JMP software (SAS Institute Inc., Cary, NC, US).

4.2.6. Analytical methods

The raw and pretreated samples were analyzed for total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), and total ammonia nitrogen (TAN) concentrations using Hach reagent kits (HACH Co., Loveland, Colorado, USA) and measured using UV-spectrophotometer (Model DR 3900, HACH, Germany). The preparation of SCOD and TAN samples was performed using 0.45 µm membrane syringe filters. Both total suspended solids (TSS) and VSS concentrations were determined according to the standard method (APHA, 2005). Also, the samples were filtered by 0.2 µm membrane syringe filters prior to volatile fatty acids

(VFAs) measurement. Then, the VFAs concentrations were measured using Ion chromatography (DionexTM ICS-2100, Thermos Scientific, USA) equipped with an electrochemical detector and microbore AS19, 2 mm column. The concentrations of free ammonia nitrogen (FAN) were calculated according to the literature (Koster, 1986). pH was measured by a bench-top pH meter (AR15 pH meter, Fisher Scientific, Pittsburgh, PA). Fourier-transform infrared spectroscopy (FTIR) analysis was performed on the raw and pretreated samples according to the literature (Azizi et al., 2021a). Student's paired t-test was used to compute the significance and statistical analysis using Microsoft Excel.

4.3. Results and discussion

4.3.1. Sludge solubilization

The impact of THP on sludge solubilization was assessed in terms of changes in COD, suspended solids, VFAs, ammonia-nitrogen, EPS, and functional groups associated with various macromolecules. The SCOD concentrations increased from 3,705±218 mg/L (raw sludge) to 7365±36 mg/L (p = 0.001) and 11926±734 mg/L (p = 0.0008) after THP at 90°C and 140°C, respectively (Fig. 4.1a). However, TCOD concentrations remained almost unchanged in all samples, indicating negligible volatilization of organics during THP (Azizi et al., 2021a). Thus, SCOD/TCOD ratios increased from 0.1 (raw sludge) to 0.2 (p = 0.04) (THP-90ºC) and 0.3 (p = 0.03) (THP-140ºC) (Fig. 4.1b). Both TSS and VSS concentrations significantly declined after THP refers to significant hydrolysis of the suspended solids content in the sludge (Fig. 4.1c). Correspondingly, VSS/TSS ratios were decreased by 12.6% and 25.9% for THP- 90°C and THP-140°C, respectively. TVFA concentration considerably increased from 134.7±0.27 mg COD/L (raw sludge) to 824.5±4.5 mg COD/L (p = 0.0004) and 1354±27 mg COD/L (p = 0.0007) for THP-90°C and THP- 140°C, respectively (Fig. 4.1d). Mostly, higher VFA concentration after THP could be attributed to the breakdown of the unsaturated lipids (Azizi et al., 2021a).

Compared to the raw sludge, TAN concentrations slightly increased from 210.7±0.9 to 222.5±7.5 mg/L (p = 0.07) for THP-90°C, while remarkably increased to 299±6 mg/L (p = 0.03) for THP-140°C (Fig. 4.1e) due to nitrogenous compounds breakdown (Azizi et al., 2021a). However, among all samples, TAN concentrations were lower than the inhibitory TAN concentration (4.2 g/L) for AD reported in the literature (Jarrell et al., 1987; Yenigün and Demirel,

2013). Moreover, FAN is a critical factor in terms of methane production inhibition and AD failure (Yenigün and Demirel, 2013). The estimated FAN concentrations decreased after THP from 1.28 mg/L for the raw sludge to 0.63 mg/L (p = 0.02) and 0.29 mg/L (p = 0.01) for THP- 90°C and THP- 140°C, respectively. Thus, FAN concentrations were also less than the inhibitory FAN concentrations (215-1,450 mg/L) reported in the literature (Yenigün and Demirel, 2013). After AD (i.e., BMP test), TAN concentrations significantly increased for all conditions (see Supplementary Information). Notably, the highest TAN concentration was observed for the THP-140°C (1349±20 mg/L, p = 0.03), indicating superior hydrolysis for this condition. FAN concentrations also increased after AD (Fig. B1). However, the highest FAN concentration of 230±18 mg/L (p = 0.04) was observed for the control.

The major EPS components (i.e., polysaccharides, proteins, and eDNA) decreased after the THP (Fig. 4.1f). THP-140ºC led to higher EPS reduction than THP-90ºC. Compared to the raw sludge, polysaccharides, proteins, and eDNA were reduced by 42%, 45%, and 62%, respectively, in the THP- 140°C sample. EPS disruption and hydrolysis into low molecular substances due to THP have been reported by several studies (Choi et al., 2018; Eskicioglu et al., 2006; Haffiez et al., 2022a; J. Sun et al., 2016). Moreover, EPS provides protection for the adsorbed ARGs against degradation (Saeki et al., 2011; M. Wang et al., 2019); thus, the breakdown of the EPS network due to THP might avail the removal of EPS-associated ARGs. For the digested samples, the EPS extracted from the THP samples showed lower EPS levels than the control (Fig. B2). Moreover, the THP-140°C sample had the lowest EPS levels and showed the highest methane generation (discussed later). Compared to the control-AD, polysaccharides, proteins, and eDNA in the 140°C-AD sample were reduced by 20%, 28%, and 58%, respectively. Additionally, FTIR analysis of digested sludge samples showed a gradual decline in the absorption peaks of various functional groups associated with organic macromolecules (see Supporting Information), which provided a further indication of hydrolysis due to THP.

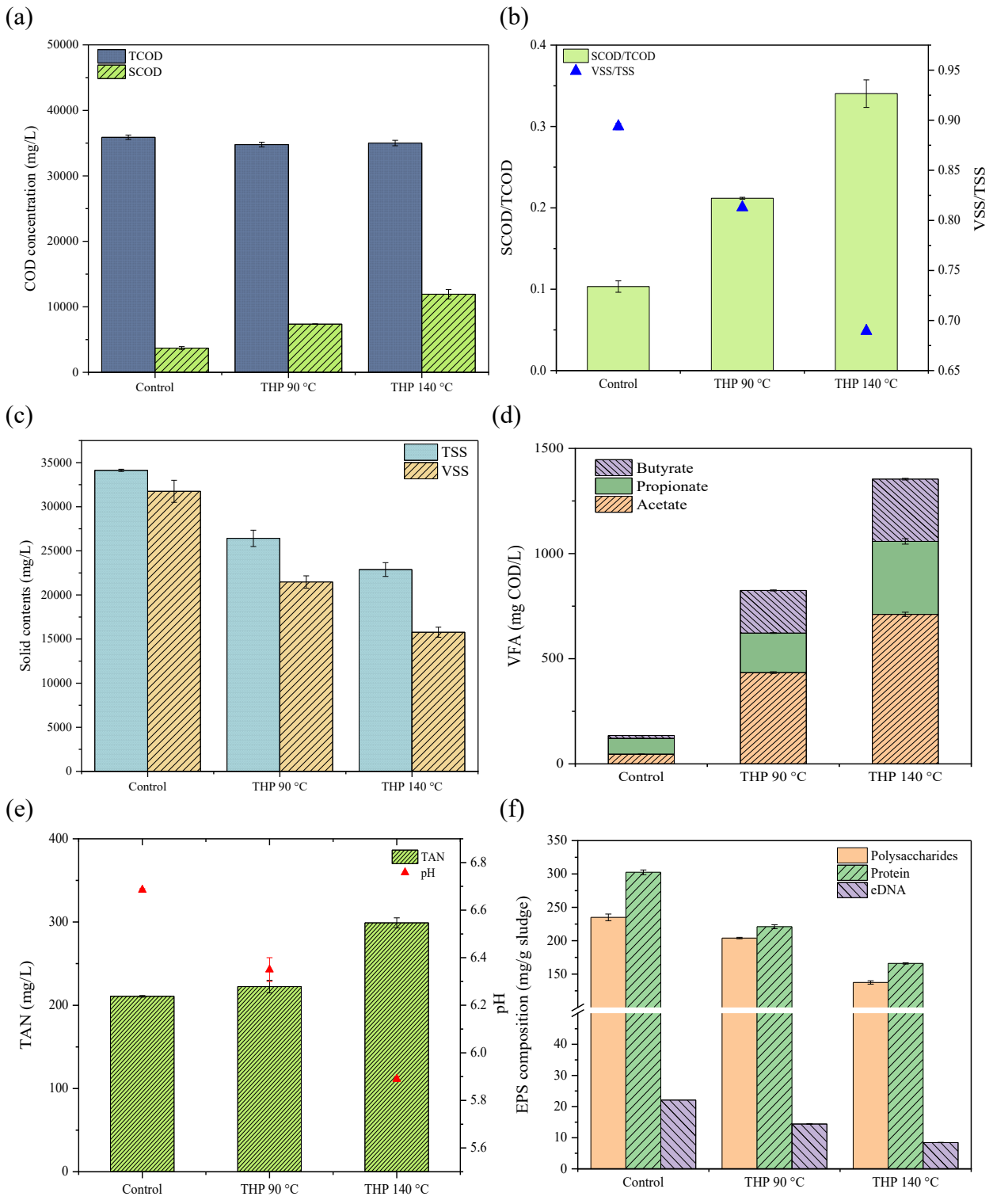


Figure 4.1. Effects of THP on (a) TCOD and SCOD, (b) TSS and VSS, (c) SCOD/TCOD, (d) VFAs, (e) TAN, (f) EPS composition

4.3.2. Methane production

Figure 4.2 shows the cumulative methane production profiles. Notably, THP at both temperatures remarkably enhanced the cumulative methane production than the control. For instance, the cumulative methane production increased by 14.49±1.2% (p = 0.005) and 52.79±3.4% (p = 0.001) for 90°C-AD and 140°C-AD, respectively. The maximum methane yield of 305.7±4.7 ml CH_4/g COD (p = 0.002) was observed for THP-140°C, while THP-90°C achieved 229±4.2 ml CH_4/g COD (p = 0.006). Previous studies also suggested 140°C as an optimal THP operating temperature for methane generation in AD (Haffiez et al., 2022a; Park et al., 2020). Notably, 140°C-AD achieved the highest methane generation and the lowest EPS levels (discussed earlier). Previous studies also suggested a positive impact of EPS solubilization on methanogenesis in AD (Azizi et al., 2021a; Choi et al., 2018; Haffiez et al., 2022a; Park et al., 2020). Therefore, the hydrolysis of high molecular compounds such as EPS into low molecular ones due to THP availed more biodegradable organics for fermentation and consequently enhanced methanogenesis (Choi et al., 2018; Y. Li et al., 2019). For instance, a previous study reported a higher relative abundance of acetoclastic and hydrogenotrophic methanogens in THP-AD than in conventional AD (Choi et al., 2018).

4.3.3. Intracellular, EPS-associated, and cell-free ARGs

The twelve targeted ARGs, including tetracycline resistance genes (*tet*W, *tet*A, *tet*B, *tet*Q, *tet*M, *tet*X), sulfonamides resistance genes (*sul*1, *sul*2), β-lactam resistance genes (bla_{AOX}, bla_{TEM}) and macrolide resistance genes (*erm*B, and *erm*C), and two MGEs (*intl1*, *intl2*) were detected in intracellular, cell-free, and EPS-associated DNA of all digested samples (Fig 4.3a-e). Among all ARGs, *sul*1 (51-79%) and *tet*W (15-32%) were the most abundant in all digested samples, followed by *sul*2 (6-14%), where the other ARGs represented with low abundance < 2% (Fig. 4.3g). For EPS-associated ARGs, *sul1* (68%, 79%, 67%) was the most dominant in the digested samples from the control, THP-90°C, and THP-140°C, respectively. A similar observation was reported by Wang et al. (2021) that EPS-associated ARGs (i.e., *sul*1 and *sul*2) showed high absolute abundance in activated sludge.

The absolute abundances of intracellular ARGs were 1.01×10^5, 1.14×10^5, and 5.14×10^4 copies/g sludge (p = 0.00002) in the digestate from control, THP-90°C, and THP-140°C samples,

respectively (Fig. 4.3f). Thus, THP at 90°C might encourage the rebounding effect during the subsequent AD. The absolute abundance of EPS-associated and cell-free ARGs compared to intracellular ARGs was much lower. Specifically, the abundances in control-AD, 90°C-AD, and 140°C- AD samples were 1.63×10^4, 1.90×10^4, and 6.67×10^3 copies/g sludge of EPS-associated ARGs ($p = 0.002$), while 6.43×10^3, 9.92×10^3, and 4.10×10^3 copies/g sludge of cell-free ARGs ($p = 0.001$), respectively. The lowest EPS-associated ARGs abundances were detected for THP-140°C-AD, with higher abundances observed for the digestate from the control and THP-90ºC. This might be attributed to the protection of the adsorbed ARGs against degradation, provided by the high EPS levels for these conditions (Saeki et al., 2011; L. Wang et al., 2021) (discussed earlier). Moreover, the abundance of cell-free ARGs was the least, which were easily degraded by DNA degrading enzymes such as DNase that secreted by many pathogenic bacteria (Sumby et al., 2005; L. Wang et al., 2021). Additionally, the fractured DNA molecules of the cell-free ARGs are hardly detected by gene targeted PCR (Y. Zhang et al., 2018). Similar trends were observed for MGEs (*intl1* and *intl2*) since the absolute abundance of intracellular MGEs was much higher than EPS-associated and cell-free MGEs.

For the total ARGs, THP-140°C could avail the highest ARGs reduction by 66% ($p = 0.001$) compared to the control (25%) and THP-90°C (33%). Also, the most effective reduction of EPS-associated and cell-free ARGs were observed for THP-140°C (59% and 36%, respectively) compared to the control. Interestingly, EPS-associated ARGs represented a higher ratio of the total extracellular ARGs (EPS-associated and cell-free ARGs) by 71.77%, 65.74%, and 61.90% in the digestate samples of the control, THP-90°C, and THP-140°C, respectively, which indicate that EPS-associated ARGs are the main source of extracellular ARGs that might contribute in horizontal gene transfer (HGT) in sludge (L. Wang et al., 2021). As a result, EPS-associated ARGs reduction in THP-140°C-AD also decreased the abundance of intracellular and cell-free ARGs. To further understand the ability of the microbial communities to carry ARGs in the digested samples, the relative abundances of ARGs (ARGs copies/16S rRNA) were computed (Fig. 4.3h). A lower relative abundance was observed for THP-140°C-AD (7.53×10^{-3} copies/16S rRNA gene copies) ($p = 0.00002$), compared to THP-90°C-AD (1.67×10^{-2} copies/ 16S rRNA gene copies) and control-AD (1.48×10^{-2} copies/ 16S rRNA gene copies) samples. This may indicate a higher proliferation of ARB in the digester operated with THP-90°C sludge than in other conditions.

Overall, the lowest EPS-associated ARGs abundance was observed in the digested sludge from THP-140°C-AD, which could be attributed to the higher EPS solubilization as well as enhanced AD performance leading to increased ARGs reduction. A higher reduction of eDNA in the EPS components in THP-140°C-AD than in other conditions might be a probable reason behind the lower abundance of EPS-associated ARGs. A recent study also demonstrated that the high abundance of EPS-associated ARGs could ultimately increase the total ARGs abundances (intracellular and extracellular) by enhancing HGT (L. Wang et al., 2021). Thus, the lowest intracellular and cell-free ARGs abundances observed in the digestate from THP-140°C-AD could be attributed to the reduced HGT due to EPS solubilization.

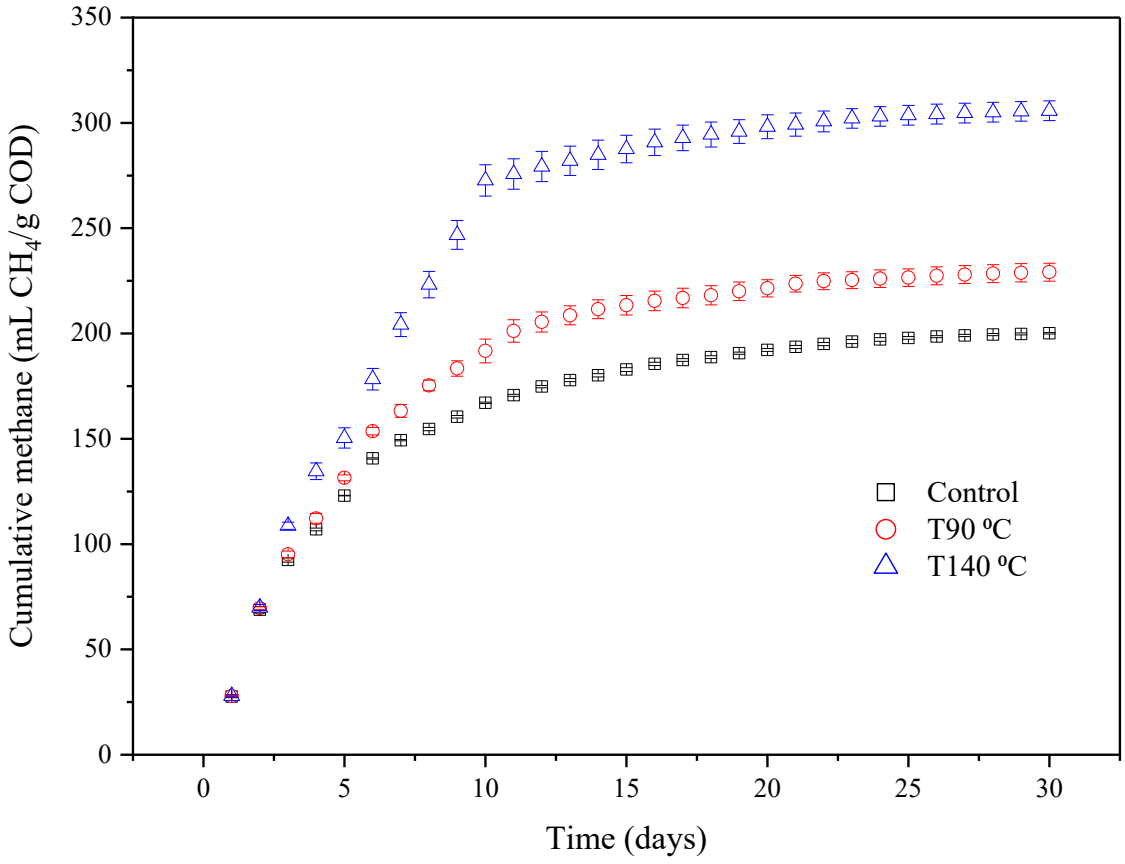
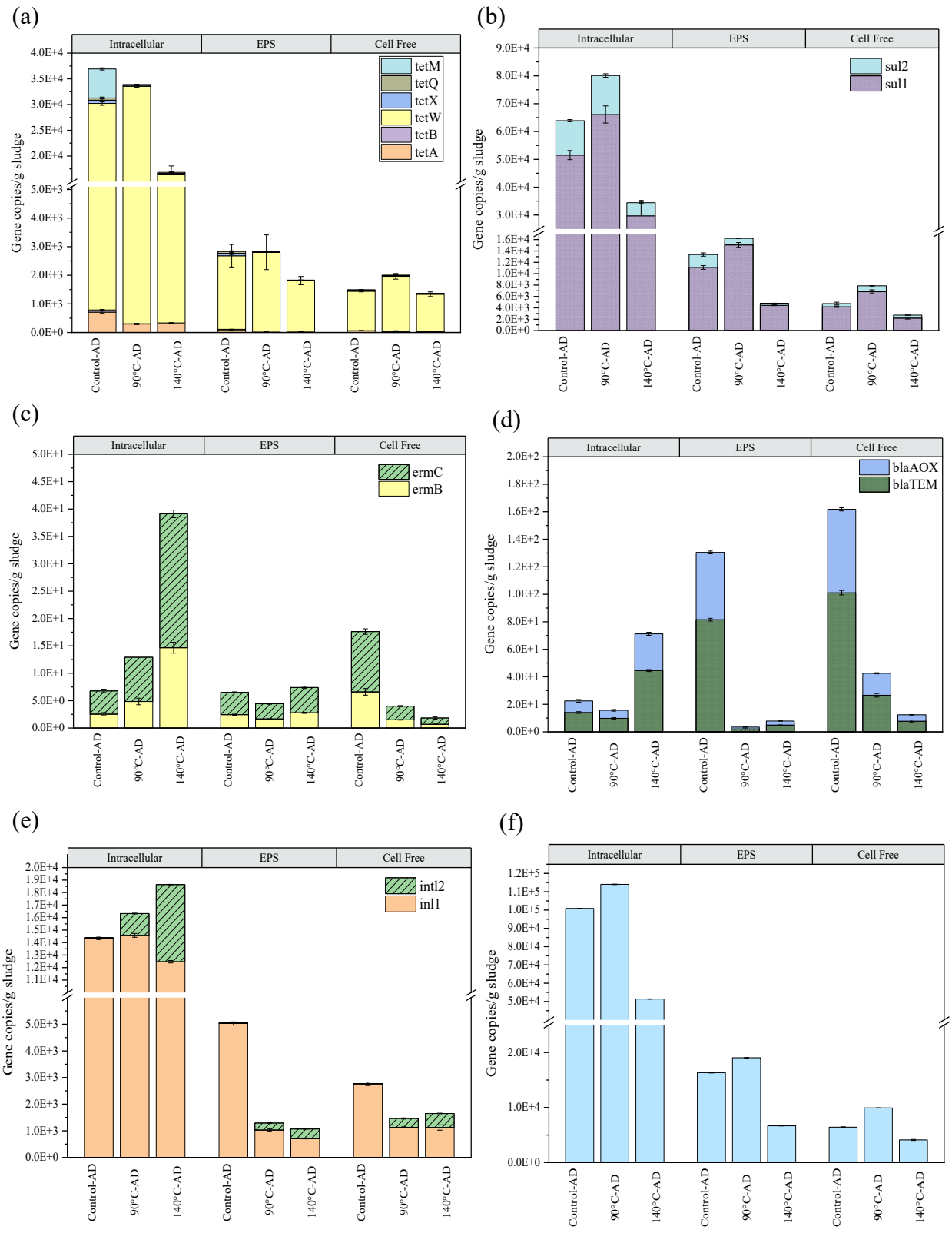


Figure 4.2. Cumulative methane production for raw and pretreated samples. Note: yields were calculated based on the initial COD of substrate.


(a)
Intracellular
EPS
Cell Free
tetM
tetQ
tetX
tetW
tetB
tetA
Gene copies/g sludge
Control-AD
90°C-AD
140°C-AD
(b)
sul2
sul1
(c)
ermC
ermB
(d)
blaAOX
blaTEM
(e)
intl2
inl1
(f)

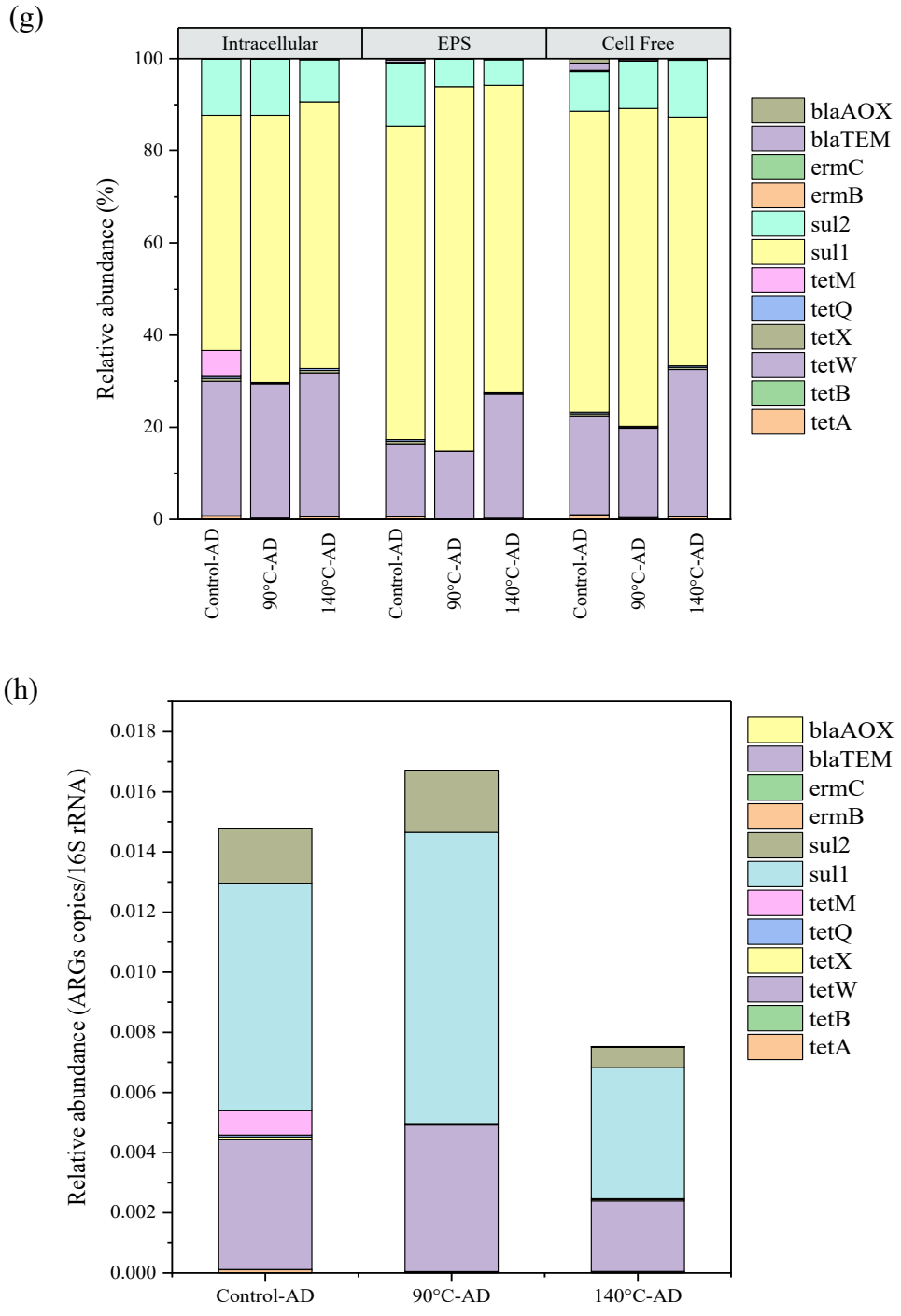


Figure 4.3. (a) tetracycline resistance genes, (b) sulfonamide resistance genes, (c) macrolide resistance genes, (d) β-lactam resistance genes, (e) integrons, (f) Total ARGs, (g) relative abundance (%) and (h) relative abundance (ARG copies/16S rRNA), in digested sludge.

4.3.4. Microbial quantity, diversity, and richness

4.3.4.1. Microbial diversity

Table 4.1 shows the estimated alpha diversity indices of the microbial community. Notably, Shannon and Pielou's evenness indicates microbial diversity, while OTUSs and Chao1indicate microbial richness. Compared to the control-AD sample, all pretreated digested samples showed lower indices except for the Pielou's evenness of THP-90°C-AD, which indicates higher microbial diversity for this condition. Notably, THP-140°C-AD showed the least diversity and richness indices. For instance, Shannon, Pielou, and Chao1 declined from 7.18, 0.90, and 252 for the control-AD to 6.51, 0.87, and 177 for THP-140°C-AD, respectively. Thus, THP decreased the microbial diversity and richness in subsequent AD of pretreated sludge. Some previous studies also reported similar results (Hosseini Koupaie et al., 2021; L. Zhang et al., 2021).

Table 4.1. The diversity and richness of the microbial communities.

	Chao1	Pielou_evenness	OTUS	Shannon
Control-AD	252	0.90	252	7.18
THP-90°C-AD	205	0.91	205	7.00
THP-140°C-AD	177	0.87	177	6.51

4.3.4.2. Bacterial community

Firmicutes (25%) and *Proteobacteria* (15%) were dominant phyla in the control-AD reactor, followed by *Chloroflexi* and *Euryarchaeota* (10%) (Fig. 4.4a). Compared to the control, *Firmicutes* remarkably enriched to 32% and 40% in THP-90°C-AD and THP-140°C-AD, respectively. Notably, *Firmicutes* are syntrophic fermenting bacteria able to degrade VFAs (Walter et al., 2019), and showed the highest abundance in all digesters. Increasing *Firmicutes* abundance with higher THP temperatures was previously observed by Niu et al. (2020). *Euryarchaeota* showed the same increasing trend from 10% in control to 14% and 16% in THP-90°C-AD and THP-140°C-AD, respectively. However, *Chloroflexi* was enriched in the digesters with pretreated sludge, while *Proteobacteria* exhibited the opposite trend. Notably, the bacterial phylum *Proteobacteria* was reported to be a tetracycline resistance gene host (Chen et al., 2018; Li et al., 2020; W. Sun et al., 2016), which had the highest abundance in the control-AD. The control-AD also showed a higher abundance of *tet* genes than other conditions.

At the genus level, *W22*, a member of the family *Cloacamonaceae,* was the most dominant genera in the control-AD (9%), while it showed a relatively lower abundance in THP- 90°C-AD (7%) and THP-140°C-AD (5%) (Fig. 4.4b). In contrast, acidogenic *Bacteroides* belonging to *Bacteroidetes* were the most dominant genera in THP-90°C-AD (14%) and THP-140°C-AD (17%), while their abundance in the control was relatively lower (8%). Noteworthy, a recent study identified *Bacteroidetes* as a potential secretor of EPS-associated DNA (Wang et al., 2022). Therefore, the relative abundance of *Bacteroides* in the pretreated digested samples indicated that more attention should be paid to their proliferation in sludge since they are potential contributors to EPS-associated ARGs secretion (Wang et al., 2022).

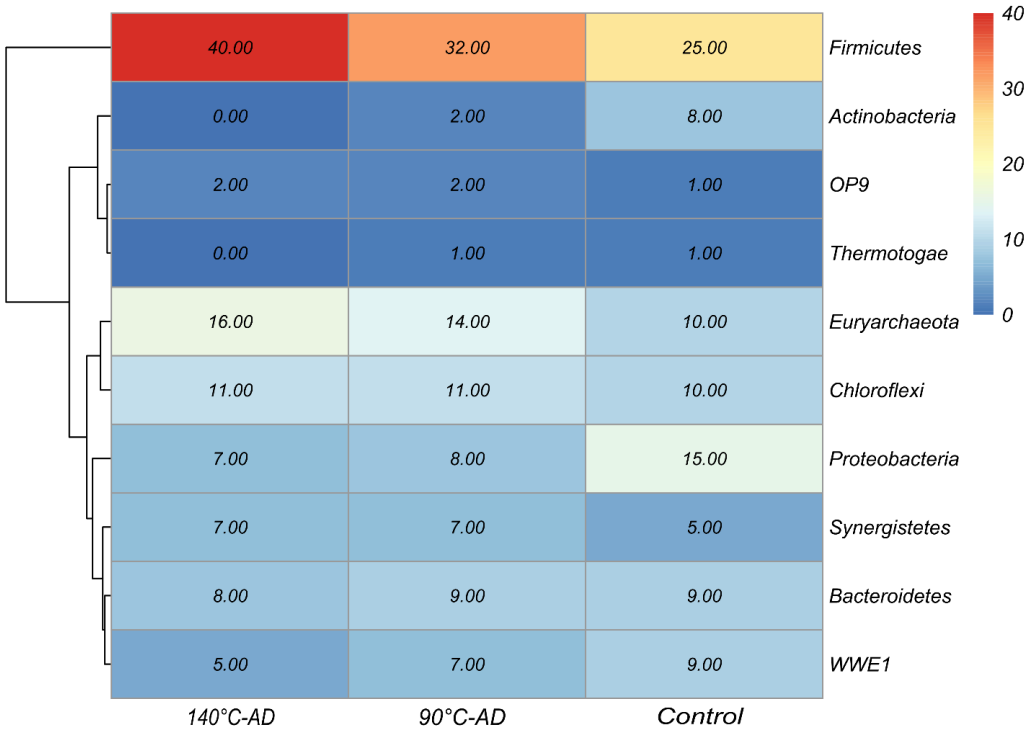
Dechloromonas (7%), T78 (7%), and *Sedimentibacter* (6%) were abundant in the control samples. Also, other genera were enriched in control, such as *Clostridium*, *Caldicoprobacter*, and *vadinCA02*, with less than 5%. In THP-90°C-AD and THP-140°C-AD, *T78* (10% and 11%), *Clostridium* (8% and 11%), *Sedimentibacter* (9% and 12%), and *vadinCA02* (6% and 7%) were the other dominant genera, respectively. Also, *Caldicoprobacter,* which are members of the order *Clostridiales*, were enriched in the THP samples with 7% and 5%, respectively. Therefore, THP is a critical factor in terms of shaping the microbial communities in AD reactors. However, implementing THP with AD encouraged the proliferation of organics degrading bacteria such as

Firmicutes and *Bacteroides* belongs to *Bacteroidetes.* A recent study observed an increased abundance of *Firmicutes* and *Bacteroidetes* due to THP of sludge (Niu et al., 2020).

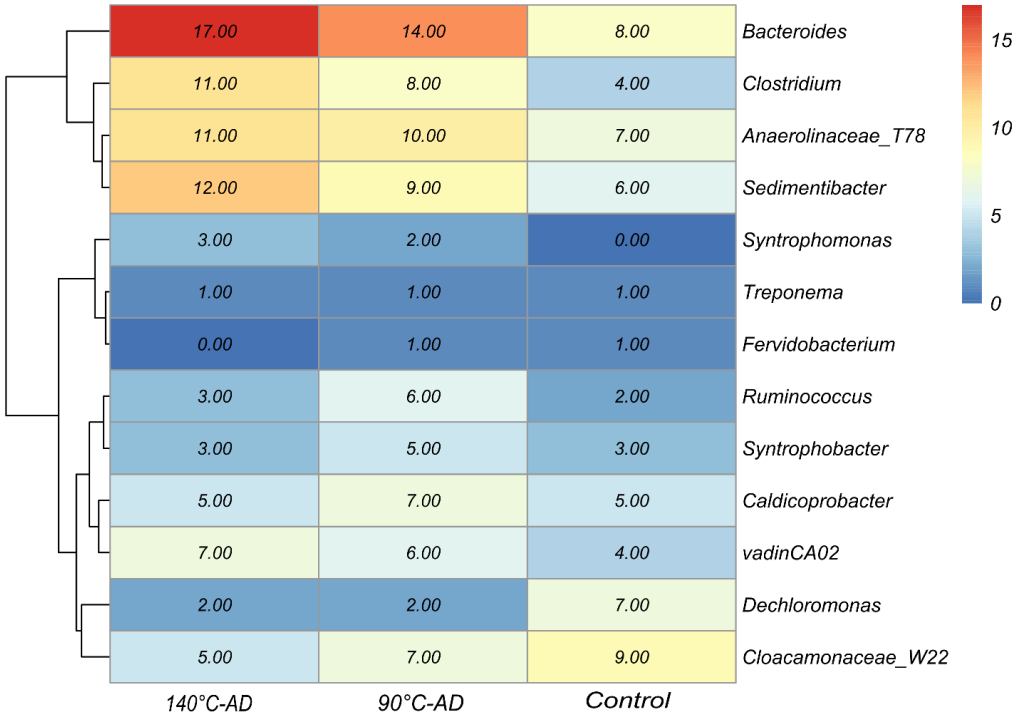
4.3.4.3. Archaeal community

The microbial sequencing data showed that the phylum *Euryarchaeota* was enriched in all digesters with the highest abundance in THP-140°C-AD (16%), followed by THP-90°C-AD (14%) and control-AD (10%) (Fig. 4.4a). Among the archaeal genera, *Methanosaeta* was the most dominant genera in THP-140°C-AD (47%) and THP-90°C-AD (47%), while the control had relatively lower abundance (31%) (Fig. 4.4c). In digesters with THP sludge, the high methane generation might be attributed to the high abundance of the acetoclastic *Methanosaeta*. *Methanosaeta* high abundance in AD with thermally hydrolyzed pretreated sludge was also observed by previous study (Choi et al., 2018). However, *Methanoculleus,* a known hydrogenotrophic methanogen, was the most enriched genera in the control samples with 42%, compared to the THP samples of 90°C (24%) and 140°C (29%) AD reactors. Also, another known hydrogenotrophic methanogen, *Methanobacterium,* existed in all the samples with high abundances; control-AD (23%), THP-90°C-AD (26%), and THP-140°C-AD (17%). In addition, *Methanosarcina* was enriched only in the THP-140°C-AD reactor (4%) with no existence in other reactors. These results suggested that the methane generation in pretreated samples was mainly via acetoclastic methanogenesis. However, microbial groups contributed in methane production were greatly affected by THP. For instance, altering sludge characteristics such as increasing COD solubilization due to THP could enhance the substrate supply (H_2 and acetate) for hydrogenotrophic and acetoclastic methanogens, hence enhance their proliferation (Niu et al., 2020).

(a)


40.00 32.00 25.00 Firmicutes
0.00 2.00 8.00 Actinobacteria
2.00 2.00 1.00 OP9
0.00 1.00 1.00 Thermotogae
16.00 14.00 10.00 Euryarchaeota
11.00 11.00 10.00 Chloroflexi
7.00 8.00 15.00 Proteobacteria
7.00 7.00 5.00 Synergistetes
8.00 9.00 9.00 Bacteroidetes
5.00 7.00 9.00 WWE1
140°C-AD
90°C-AD
Control
40
30
20
10
0

(b)


17.00 14.00 8.00 Bacteroides
11.00 8.00 4.00 Clostridium
11.00 10.00 7.00 Anaerolinaceae_T78
12.00 9.00 6.00 Sedimentibacter
3.00 2.00 0.00 Syntrophomonas
1.00 1.00 1.00 Treponema
0.00 1.00 1.00 Fervidobacterium
3.00 6.00 2.00 Ruminococcus
3.00 5.00 3.00 Syntrophobacter
5.00 7.00 5.00 Caldicoprobacter
7.00 6.00 4.00 vadinCA02
2.00 2.00 7.00 Dechloromonas
5.00 7.00 9.00 Cloacamonaceae_W22
140°C-AD
90°C-AD
Control
15
10
5
0

(c)

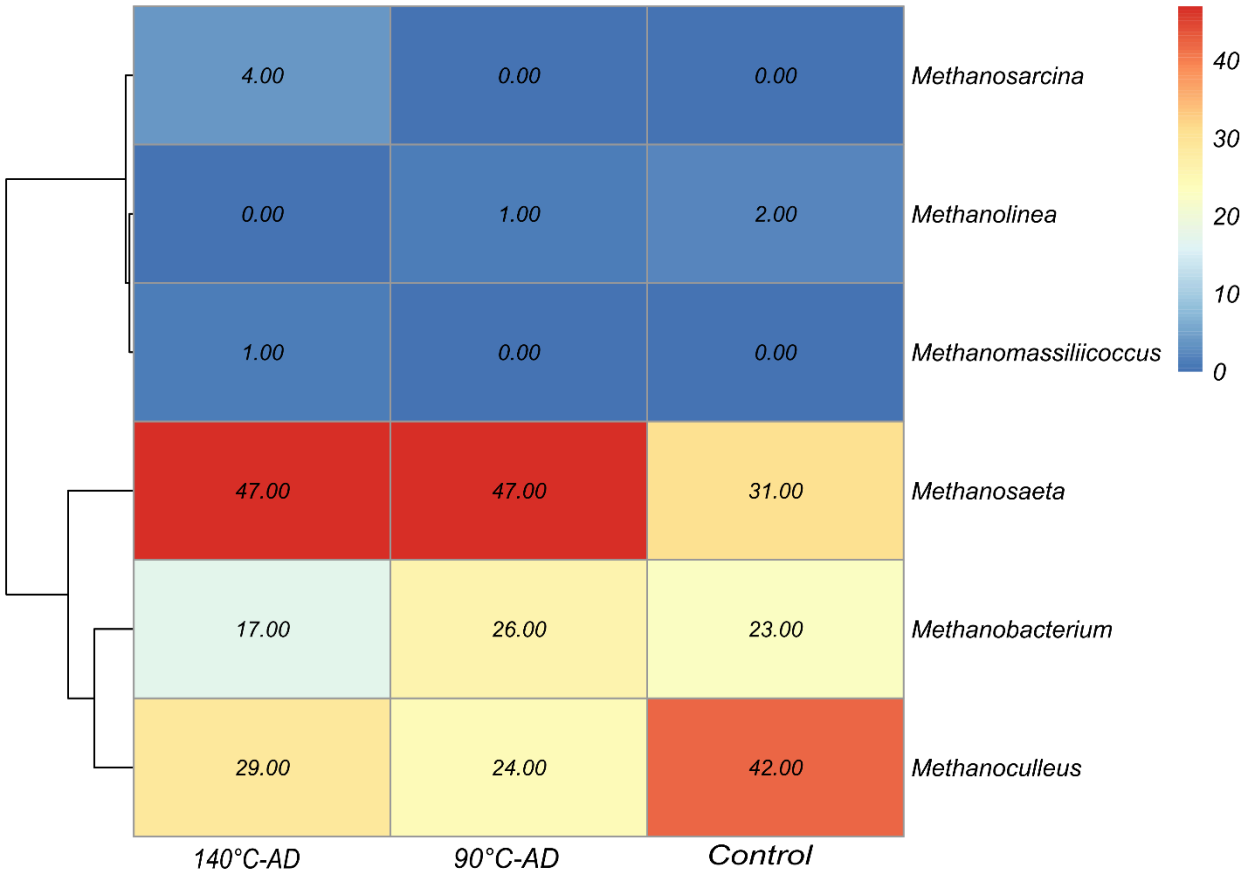


Figure 4.4. Microbial relative abundance. (a) phylum level, (b) bacterial genus level, (c) archaeal genus level under different operating conditions.

4.3.5. Multivariate analysis and significance of results

Figure 4.5 shows correlation coefficients of EPS components, cell-free, intracellular, EPS-associated ARGs, and THP-AD. The analysis showed that all the variables were negatively correlated with THP-AD. For instance, EPS-polysaccharides, proteins, and eDNA exhibited a very strong negative correlation with THP-AD (r = -0.97, -0.99, and -1), respectively. However, the effect of THP-AD on intracellular (r = -0.63) and EPS- associated ARGs (r = -0.62) was remarkably higher than that on cell-free ARGs (r = -0.24). In contrast, EPS components showed a positive correlation with intracellular and extracellular (EPS-associated and cell-free) ARGs. Specifically, PCA of EPS-associated ARGs and EPS components (Fig. 4.6) showed that EPS-protein and EPS-eDNA were closely clustered with β-lactam resistance genes in the control sample. A positive correlation between EPS-proteins and β-lactam resistance genes was previously reported in the literature (Haffiez et al., 2022a). In contrast, macrolide resistance genes were not located in any quadrant with EPS components. Moreover, the PCA showed that the majority of EPS-associated ARGs were clustered close to control and THP-90°C-AD samples (Fig. 4.6). Notably, EPS-polysaccharides, proteins, and eDNA were strongly positively correlated with intracellular ARGs (r = 0.8, 0.54, and 0.62) and EPS-associated ARGs (r = 0.8, 0.53, 0.61), respectively. In contrast, the correlation with cell-free ARGs (r = 0.47, 0.13, 0.22) was fairly weak. That may be attributed to the cell-free ARGs tendency to be degraded by specific degrading enzymes or adsorbed onto microbial cells or/and sludge particles, which result in gene pattern changes (L. Wang et al., 2021). Interestingly, there was a significant positive correlation between EPS-associated and cell-free ARGs (r = 0.91), suggesting the importance of EPS-associated ARGs as a potential source of extracellular ARGs. Also, intracellular ARGs were strongly positively correlated with EPS-associated ARGs (r = 1) and cell-free ARGs (0.91). Thus, these results suggest a functional link between extracellular and intracellular ARGs which may be secreted extracellularly as in genetic transformation or cell lysis to form cell-free and EPS-associated ARGs.

To the best of the authors' knowledge, this study is the first to investigate the fate of extracellular ARGs (EPS-associated and cell-free ARGs) in AD of thermally hydrolyzed sludge. Notably, the results revealed that EPS-associated ARGs could significantly affect the overall abundance of intracellular and cell-free ARGs in AD. Moreover, the correlational results between

EPS components with intracellular and extracellular ARGs demonstrate that understanding the detailed characterization and changes of EPS due to different operating temperatures of THP is crucial. Nonetheless, limited information is available in the literature on the transformation ability of EPS-associated ARGs during AD. Therefore, further research is needed to clearly understand the proliferation of EPS-associated ARGs in conventional AD as well as AD with pretreatment of feedstock to alleviate the potential risks of ARGs spreading.

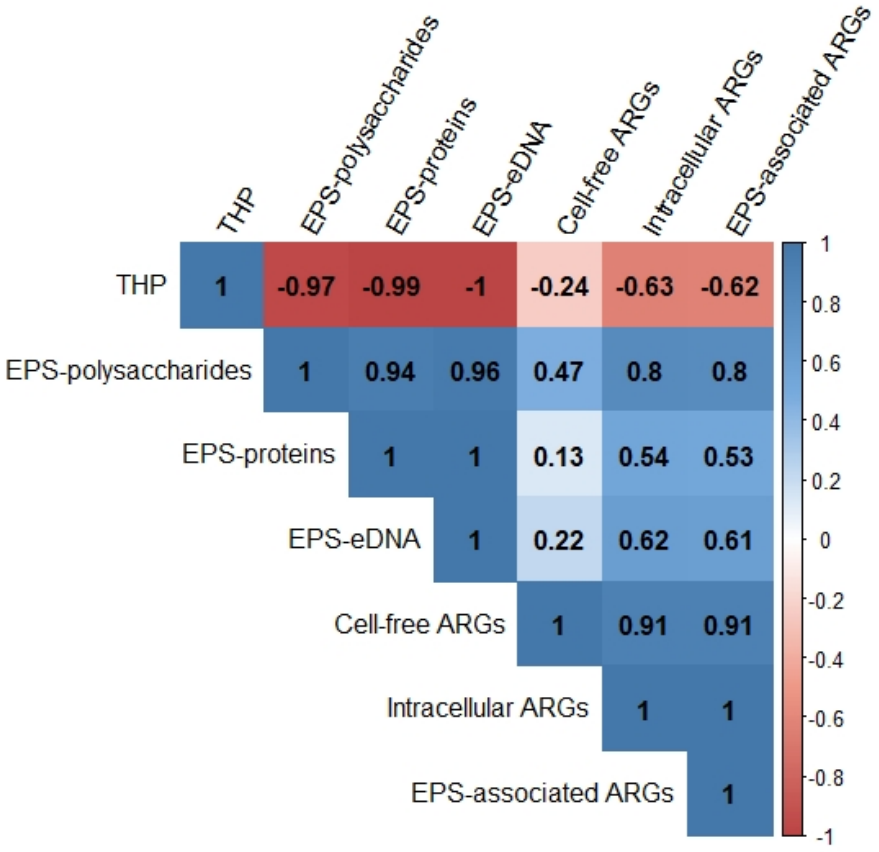


Figure 4.5. Correlation of EPS components, intracellular ARGs, EPS-associated ARGs, and Cell-free ARGs under different THP conditions.

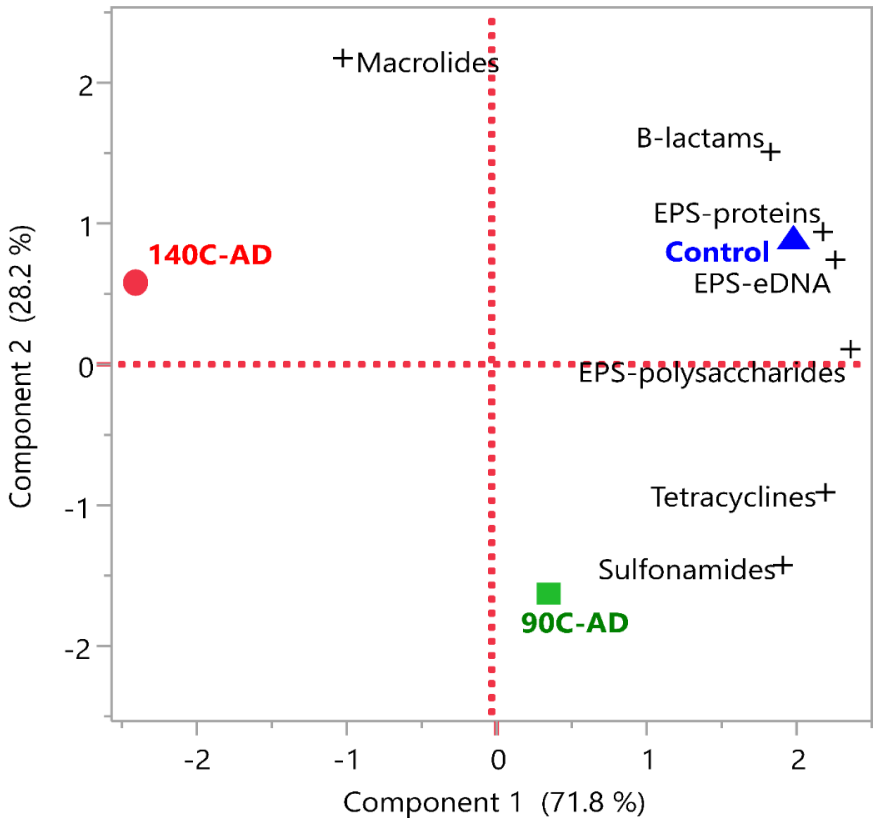


Figure 4.6. PCA of EPS-associated ARGs, EPS components, and THP conditions.

4.4. Conclusions

Overall, this study elaborated the detailed structure and fates of the extracellular ARGs in AD, where EPS-associated ARGs represented the largest portion compared to cell-free ARGs. EPS-associated *sul*1 genes showed the highest abundance in all digested samples. However, THP-140°C led to the lowest abundance of EPS-associated ARGs, while cell-free ARGs were substantially lower in all samples. EPS was significantly correlated with intracellular and extracellular ARGs. Furthermore, EPS-associated ARGs propagation greatly affected the abundance of intracellular and cell-free ARGs. These findings highlight the significance of EPS-associated ARGs in AD; therefore, further research is needed to control their propagation.

Chapter 5

Conclusions and Recommendations

5.1. Conclusions

This master's thesis focused on evaluating the impact of different operating conditions on the fate of ARGs in AD and summarizing the research gaps to guide the prospects for future studies. Also, it showed the impact of different THP reaction temperatures on ARGs abundance and EPS characteristics. Moreover, it gave new insights into correlating the EPS composition with intracellular, cell-free, and EPS-associated ARGs to further understand the relationship between EPS and ARGs.

This thesis presents a study on ARGs propagation during AD of thermally hydrolyzed sludge and their correlation with EPS components. The digestate of the pretreated sample at 140ºC showed the lowest concentrations of proteins and polysaccharides, and also had the lowest ARGs abundance. Therefore, a functional link between EPS composition and ARGs abundance was predictable. However, the relationships between ARGs abundance, EPS components, and different operating conditions observed in the multivariate analysis were correlational. Particularly, EPS polysaccharides were positively correlated with most targeted ARGs and integrons, while EPS proteins were strongly correlated with β-lactam resistance genes. THP improved the sludge solubilization, enhancing methane generation in the biochemical methane potential test. The results of this study suggest that manipulating THP operating conditions targeting specific EPS components will be critical to effectively mitigating the dissemination of particular ARG types in AD.

The detailed characterization of intracellular and extracellular ARGs in AD of thermally hydrolyzed sludge at 90°C and 140°C was investigated. THP-140°C led to the lowest abundance of EPS-associated ARGs. However, EPS-associated *sul*1 genes were the most dominant in all digested samples. On the contrary, cell-free ARGs showed relatively low abundance in all samples. Compared to cell-free ARGs, EPS-associated ARGs had the greater portion of total extracellular ARGs. The results of this study showed that EPS components were significantly correlated with

intracellular and extracellular ARGs. Also, a strong correlation was observed between EPS-associated ARGs, and intracellular and cell-free ARGs. Thus, EPS-associated ARGs have a significant role in AD in terms of ARGs propagation, and their control is crucial to avoiding the potential environmental concerns posed by ARGs.

5.2. Recommendations

Given that the structural and functional characteristics of EPS may provide ample adsorption sites for ARGs and play a critical role in their propagation in sludge, the results of this study infer a functional link between ARGs and EPS composition (proteins and polysaccharides) in AD of thermally pretreated sludge. However, EPS-associated ARGs were found to be the major source of extracellular ARGs in sludge and play an important role in HGT. Therefore, further research is needed to clearly understand the detailed characterization of EPS and the behavior of intracellular, EPS-associated, and cell-free ARGs in AD.

The multivariate analysis results of this study indicated the critical role of EPS-associated ARGs in affecting the propagation of cell-free and intracellular ARGs in AD of thermally pretreated sludge. Also, it showed that EPS-associated ARGs represented the higher ratio of total extracellular ARGs (cell-free and EPS-associated ARGs), which elaborate that EPS-associated ARGs are the major source of extracellular ARGs in sludge. However, further research should focus on comprehensively understanding the dissemination and proliferation of EPS-associated ARGs and evaluating control pathways to alleviate the potential environmental risks caused by extracellular ARGs.

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Appendix A

Supplementary Information for Chapter 3

Table A 1. Primers used for studying 16S rRNA and ARGs

	Forward (5'-3')	**Reverse (5'-3')**
*tet*A	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG
*tet*B	GGCAGGAAGAATAGCCACTAA	AGCGATCCCACCACCAG
*tet*C	GCGGGATATCGTCCATTCCG	GCGTAGAGGATCCACAGGACG
*tet*W	GAGAGCCTGCTATATGCCAGC	GGGCGTATCCACAATGTTAAC
*tet*M	ACAGAAAGCTTATTATATAAC	TGGCGTGTCTATGATGTTCAC
*tet*Q	AGAATCTGCTGTTTGCCAGTG	CGGAGTGTCAATGATATTGCA
*tet*X	CAATAATTGGTGGTGGACCC	TTCTTACCTTGGACATCCCG
*sul*1	CGCACCGGAAACATCGCTGCAC	TGAAGTTCCGCCGCAAGGCTCG
*sul*2	TCCGGTGGAGGCCGGTATCTGG	CGGGAATGCCATCTGCCTTGAG
*erm*B	GATACCGTTTACGAAATTGG	GAATCGAGACTTGAGTGTGC
*erm*C	TTTGAAATCGGCTCAGGAAAA	ATGGTCTATTTCAATGGCAGTTACG
bla_{OXA}	ATATCTCTACTGTTGCATCTCC	AAACCCTTCAAACCATCC
bla_{TEM}	ATCAGCAATAAACCAGC	CCCCGAAGAACGTTTTC
*intl*1	CCTCCCGCACGATGATC	TCCACGCATCGTCAGGC
*intl*2	GTTATTTTATTGCTGGGATTAGGC	TTTTACGCTGCTGTATGGTGC
16S rRNA	CCTACGGGNGGCWGCAG	GACTACHVGGGTATCTAATCC

Table A 2. Kinetic parameters estimated with the modified Gompertz model.

Experimental condition	Maximum methane yield, V_m^* (mL/g COD)	Maximum methane production rate, R (mL/g COD/d)	Standard error for R	Lag phase, λ (d)	Standard error for λ
THP-80 ºC-AD	208.7	13.15	0.82	-3.03	0.61
THP-110 ºC-AD	245.24	16.58	0.91	-1.27	0.47
THP-140 ºC-AD	276.3	14.24	0.83	3.53	0.62
THP-170 ºC-AD	205	10.62	0.59	8.18	0.59
Control-AD	172.76	9.39	0.66	-5.35	0.85

*V_m were fixed at the experimental total cumulative methane yields

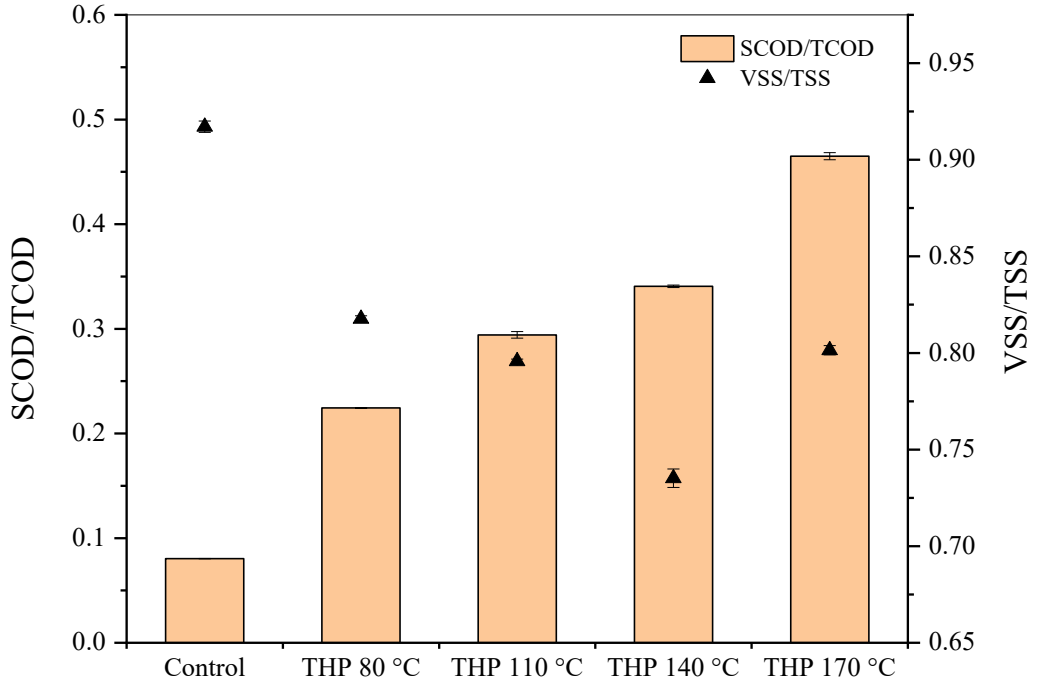

Figure A 1. Changes in SCOD/TCOD and VSS/TSS ratios after THP.

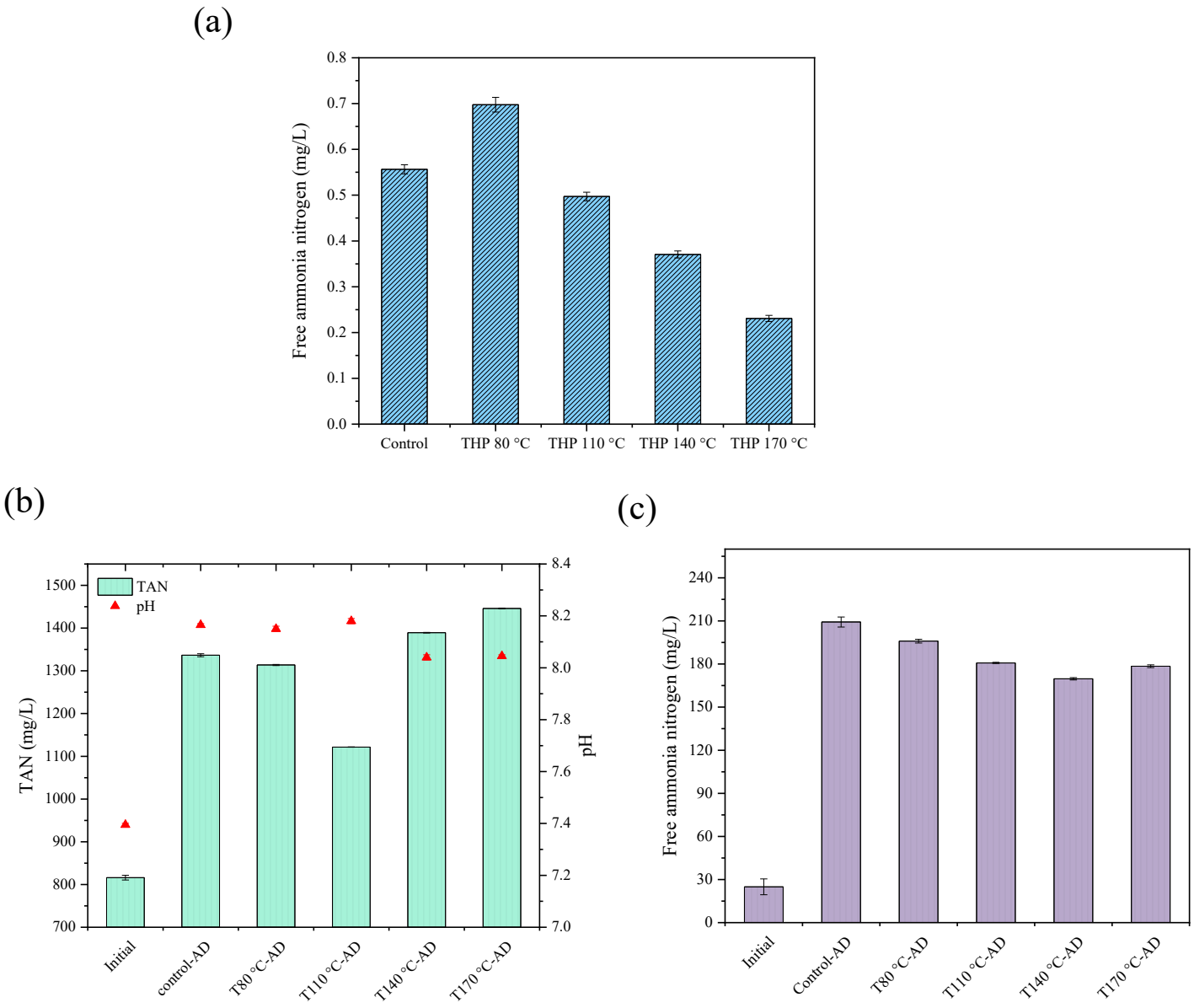

Figure A 2. (a) Estimated free ammonia nitrogen (FAN) after THP, (b) measured TAN concentrations and pH after BMP test, and (c) estimated FAN after BMP test.

Changes in functional groups of macromolecules

FTIR analysis of solids was carried out to identify the effects of THP on functional groups associated with macromolecular compounds. The raw sludge sample showed the highest absorbance peaks. In contrast, the peaks intensity decreased for THP samples, indicating that THP could induce changes in functional groups and chemical structures of macromolecular compounds. The peak in the range of 3200-3600 cm^{-1} indicates the presence of O-H vibration of alcoholic and carboxylic groups in addition to amide hydrogen vibrations (Azizi et al., 2021b). However, the absorption peaks between 2800-2980 cm^{-1} refer to the stretching of aliphatic C-H bonds, indicating lipid content (Castaldi et al., 2005; Chowdhury et al., 2019). For the region of 1300-1700 cm^{-1}, the peak at 1600-1700 cm^{-1} is for conjugated C=O and C=C in aromatic compounds, indicating the presence of aldehydes, ketones, proteins, and carboxylic acids, while the peak at 1540 cm^{-1} is for the amide and carboxylate C=O (Ramesh et al., 2006). Additionally, peaks that appeared in the ranges of 1030-1200 cm^{-1} present the stretching of a single C-O bond in esters, ethers, polysaccharides, and carboxylic acids (Castaldi et al., 2005; Chowdhury et al., 2019). Noticeably, all the absorption peaks intensities decreased after the THP. For instance, the remarkable reduction in the peak intensity in the range 2800-2980 cm^{-1} indicates the degradation of lipids and fats after THP. Also, peak intensities at 1600-1700 cm^{-1} are considerably reduced with temperature raising attributed to proteins and other aromatic compounds solubilization. The same decreasing trend in the peak's intensities at 1000-1050 cm^{-1}, indicates carbohydrates degradation. Thus, FTIR results further confirmed solubilization of macromolecular organics after the THP. Moreover, the gradual decrease of the absorption peaks with increasing the THP operating temperature indicates the relationship between temperature and solubilization efficiencies.

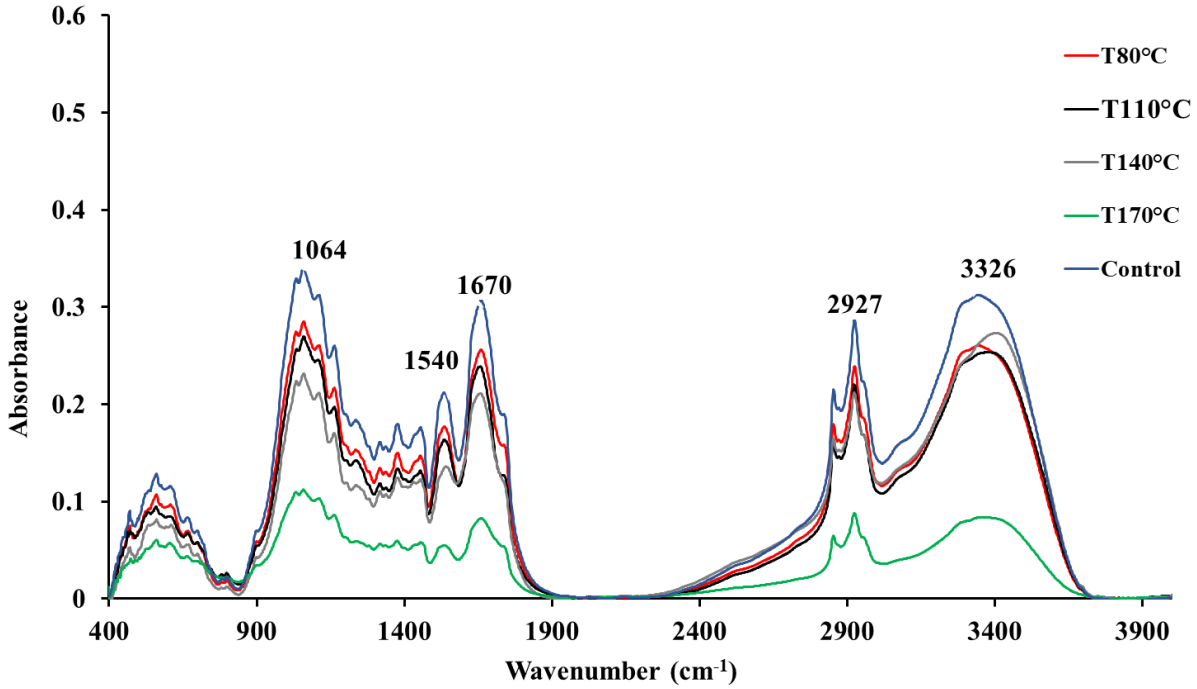

Figure A 3. FTIR spectrum of raw and pretreated samples.

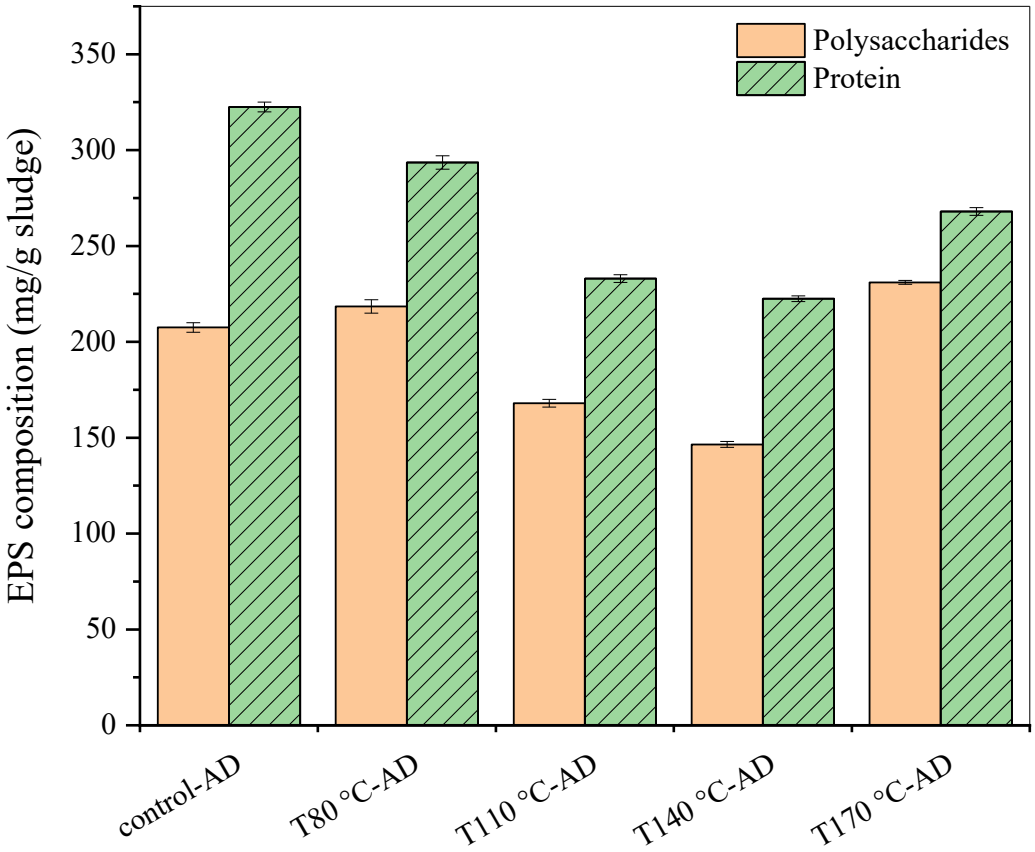

Figure A 4. Extracellular polymeric substances (polysaccharides and proteins) after AD.

Table A 3. The diversity and richness of the microbial communities.

	Chao 1	Pielou	OTUs	Shannon	Coverage
Control	171	0.87	170	6.4	1
Control-AD[a]	35	0.64	35	3.3	1
THP-80°C	179	0.83	174	6.2	1
THP-80 °C-AD[a]	75	0.77	74	4.8	1
THP-110°C	120	0.81	117	5.6	1
THP-110°C-AD[a]	83	0.75	83	4.8	1
THP-140°C	126	0.81	126	5.6	1
THP-140°C-AD[a]	91	0.78	91	5.1	1
THP-170°C	95	0.79	95	5.2	1
THP-170 °C-AD[a]	93	0.84	92	5.5	1

[a]Digestate from BMP test

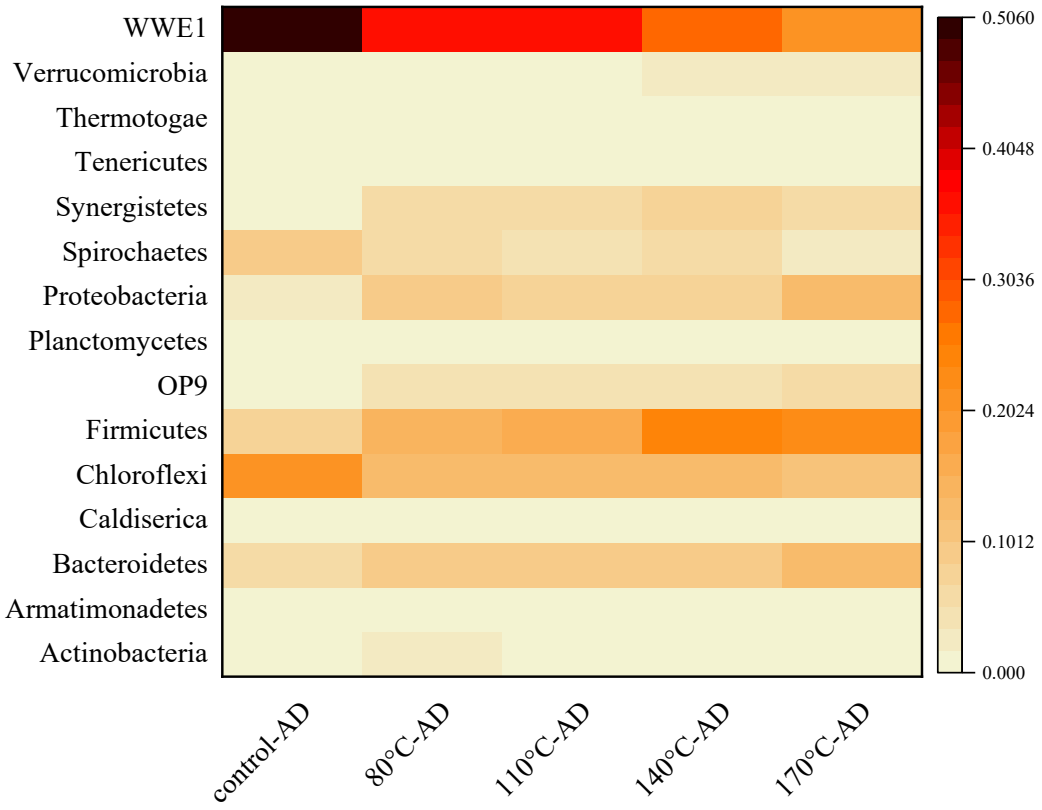

Figure A 5. Relative abundance of bacterial communities at the phylum level

Appendix B

Supplementary Information for Chapter 4

Table B 1. Characteristics of substrate and inoculum.

Parameters	**Substrate (TWAS[a])**	**Inoculum (Digested sludge)**
TSS (mg/L)	34125±125	15625±125
VSS (mg/L)	31750±1250	14625±129
TCOD (mg/L)	35891±331	23064±1061
SCOD (mg/L)	3705±218	4664±325
TVFA (mg/L)	108±2	158±0.5
TAN (mg/L)	211±31	1181±67
Alkalinity (mg/L)	2954±56	6655±55
pH	6.7±0.005	7.9±0.01

[a] Thickened waste activated sludge (TWAS)

Table B 2. Primers used for studying ARGs and 16S rRNA

	Forward (5'-3')	**Reverse (5'-3')**
*tet*A	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG
*tet*B	GGCAGGAAGAATAGCCACTAA	AGCGATCCCACCACCAG
*tet*W	GAGAGCCTGCTATATGCCAGC	GGGCGTATCCACAATGTTAAC
*tet*M	ACAGAAAGCTTATTATATAAC	TGGCGTGTCTATGATGTTCAC
*tet*Q	AGAATCTGCTGTTTGCCAGTG	CGGAGTGTCAATGATATTGCA
*tet*X	CAATAATTGGTGGTGGACCC	TTCTTACCTTGGACATCCCG
*sul*1	CGCACCGGAAACATCGCTGCAC	TGAAGTTCCGCCGCAAGGCTCG
*sul*2	TCCGGTGGAGGCCGGTATCTGG	CGGGAATGCCATCTGCCTTGAG
*erm*B	GATACCGTTTACGAAATTGG	GAATCGAGACTTGAGTGTGC
*erm*C	TTTGAAATCGGCTCAGGAAAA	ATGGTCTATTTCAATGGCAGTTACG
bla_{AOX}	ATATCTCTACTGTTGCATCTCC	AAACCCTTCAAACCATCC
bla_{TEM}	ATCAGCAATAAACCAGC	CCCCGAAGAACGTTTTC
*intl*1	CCTCCCGCACGATGATC	TCCACGCATCGTCAGGC
*intl*2	GTTATTTTATTGCTGGGATTAGGC	TTTTACGCTGCTGTATGGTGC
16S rRNA	CCTACGGGNGGCWGCAG	GACTACHVGGGTATCTAATCC

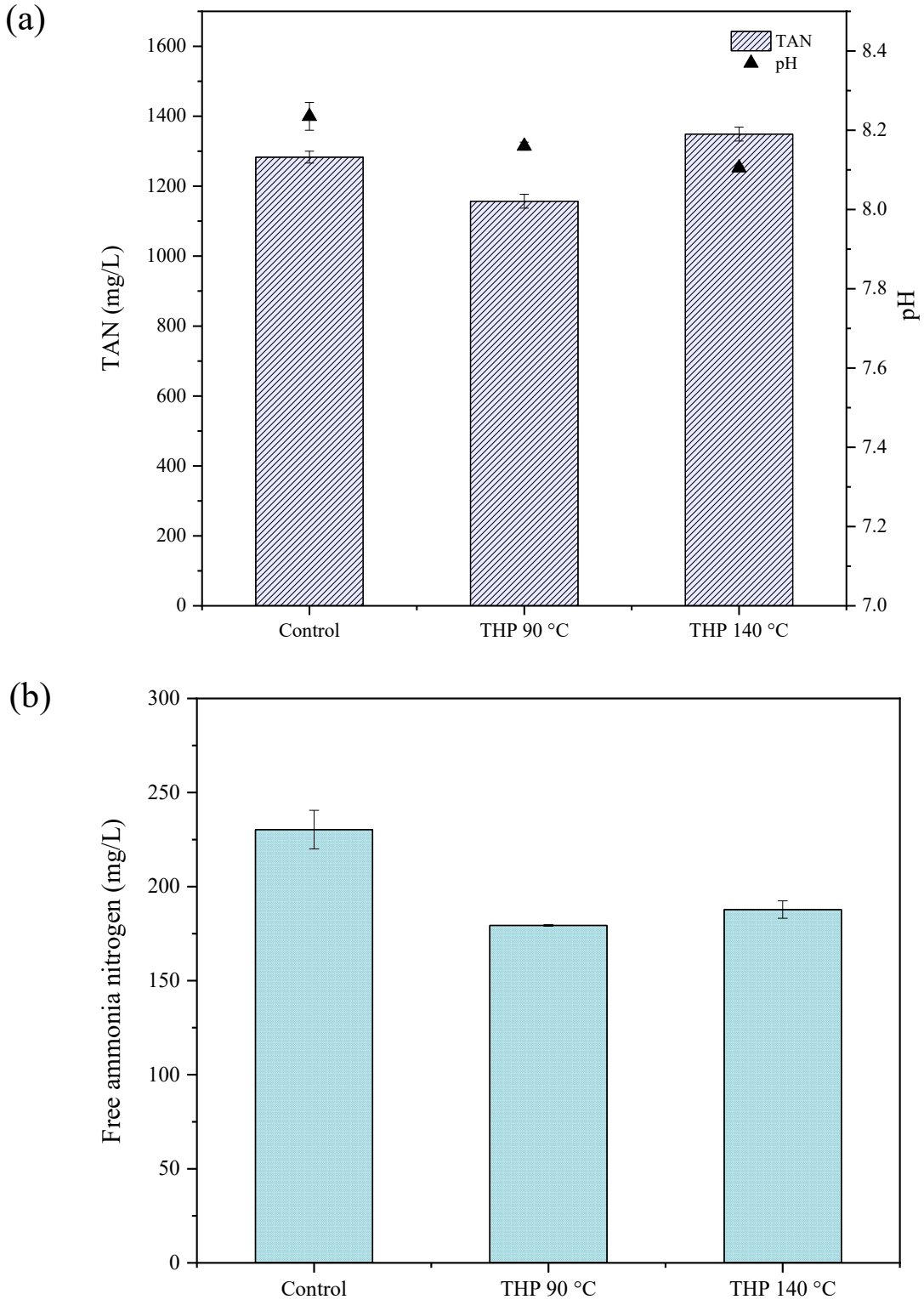

Figure B 1. (a) TAN, (b) free ammonia nitrogen (FAN) in digested sludge.

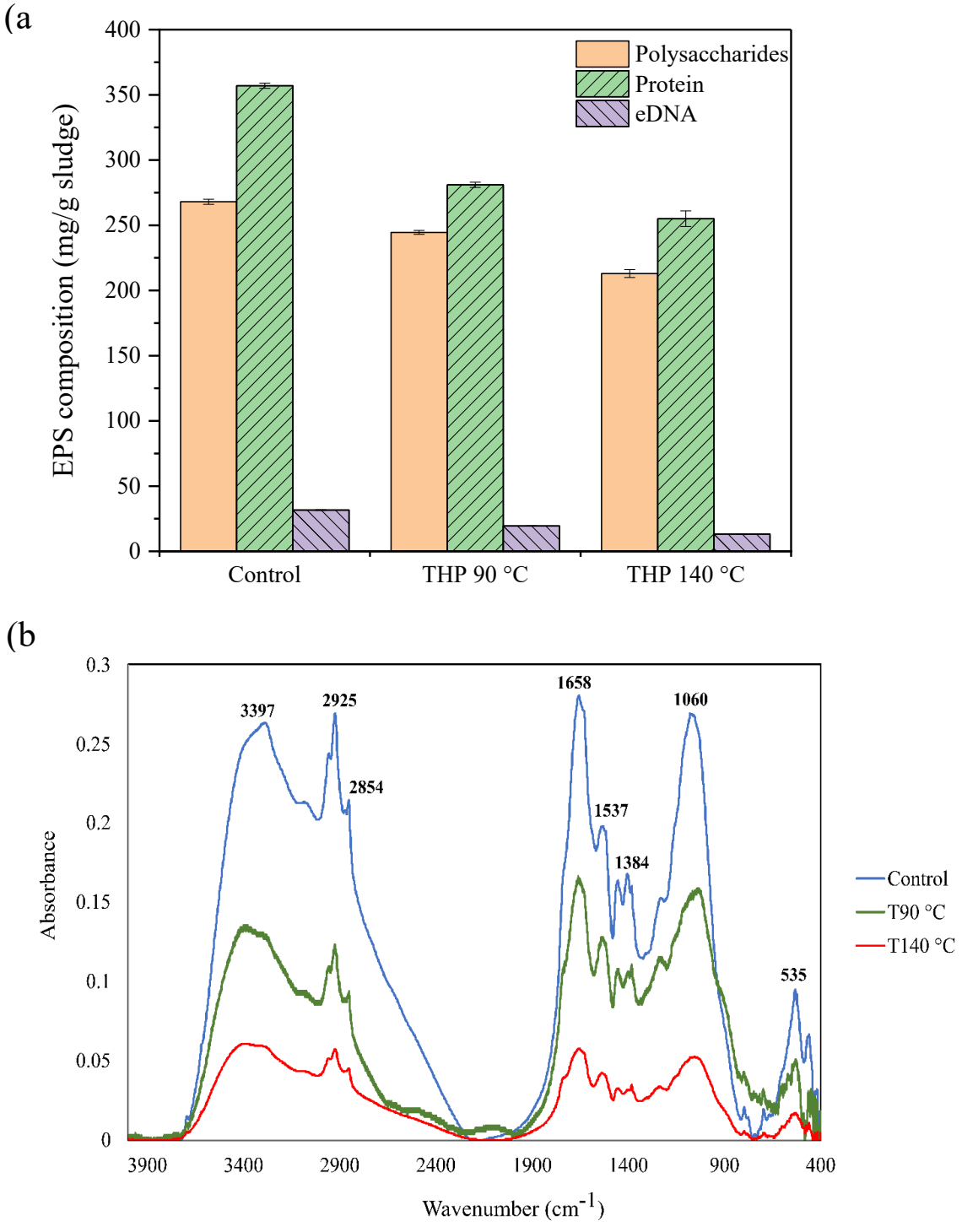

Figure B 2. (a) EPS composition and (b) FTIR spectrum in digested sludge.