Low Temperature Anaerobic Digestion of Pre-ozonated Waste Activated Sludge: Impact of Zero-Valent Iron Addition and Process Staging

Presented by

Xuan Wu

Department of Civil Engineering and Applied Mechanics

McGill University, Montreal

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ABSTRACT

Waste activated sludge is commonly treated through anaerobic digestion (AD) in wastewater treatment plants to reduce the amount of solids waste to handle and recover energy. AD reactors are typically operated at a temperature higher than ambient to increase the kinetics of the rate-limiting step in AD process. However, since heating takes up a considerable proportion of the recovered energy, and waste disposal accounts for a substantial portion the operation budget, many pretreatment and operational methods have been developed to enhance the biosolids reduction and potentially improve the energy recovery. One of the least investigated strategy to improve energy recovery is to reduce the operation temperature of AD once the pretreatment is applied to the waste activated sludge. This is the topic of the study in this thesis.

This thesis investigated the improvement in the performance of the digestion of ozonated waste activated sludge at 15°C and room temperature (22.6±0.3°C) with the addition of zero-valent iron (ZVI) and the separation of the digester in a two-stage process. The experimental period was divided into two phases. In Phase I, a high-rate single-stage process was applied to three 4-L reactors at two different temperatures (one at 15°C and two at room temperature). ZVI powder was added to the 15°C and one of the two reactors at room temperature. By comparing the performance of the ZVI-free reactor (control), the effect of ZVI on pre-ozonated AD was evaluated. In Phase II, both reactors receiving ZVI in Phase I were transformed into two-stage digestion systems (i.e., fermentation reactor followed by a methanogenic reactor), while the other operation parameters (ZVI dosage, temperature, and ozone dose to the WAS) remained the same. Results obtained over 278 days of operation revealed that the addition of ZVI in Phase I increased the biogas production by 24% when operating at room temperature and increased the proportion of biomethane in the biogas from 65% to 74%. These improvements were associated with the more efficient conversion of hydrolyzed chemical oxygen demand (COD) into biomethane because the volatile suspended solids (VSS) destruction did not significantly change. In Phase II, ZVI-dosed two-stage anaerobic digestion showed an increase in specific methane production of 5% at 15°C and 14% at room temperature compared to the same reactors in Phases I. These improvements were only slightly higher than the changes in the average performance of the control reactor (single-stage ZVI-free reactor at room temperature), which recorded an increase in specific methane production of 3%. In conclusion, ZVI addition improved the biomethane production of anaerobic digestion at low temperatures, but its effect on solids reduction was limited. Conversely, staging did not appear to

provide clear additional benefits to the process. This work will contribute to the optimization of energy recovery from anaerobic digestion, and may provide new avenues to enhance the process performance for small processes that are more sensitive to the impacts of environmental conditions.

RÉSUMÉ

Les boues activées en excès produites par le traitement des eaux usées sont souvent traitées par digestion anaérobie afin de réduire la quantité de déchets solides et de valoriser leur contenu énergétique. Dans l'industrie, la performance de la digestion est maintenue en chauffant le digestat à des températures supérieures à celles ambiantes pour augmenter la cinétique de l'hydrolyse qui est souvent l'étape déterminant la vitesse de dégradation. Cependant, étant donné que le chauffage requiert une grande partie de l'énergie produite et que les coûts de traitement des déchets augmentent continuellement, de nombreuses méthodes de prétraitement et d'opération ont été développées pour réduire la quantité de biosolides produits et pour augmenter la récupération d'énergie. Une voie additionnelle et peu étudier pour augmenter la récupération de l'énergie et d'opérer les digesteurs à des températures plus basses que celle actuellement en pratique, ce qui pourrait maximiser l'avantage du prétraitement. Cette approche est le sujet de ce mémoire.

Le mémoire étudie les bénéfices de l'ajout de fer zérovalent (acronyme anglais ZVI) et de la séparation du procédé de digestion en deux étapes (réacteurs en séries de fermentation et méthanisation) sur la performance de la digestion anaérobie à 15°C et à température ambiante des boues activées ozonées. La période expérimentale a été divisée en deux phases. Dans la phase I de l'étude, un procédé à une seule étape a été appliquée à trois réacteurs opérés soit à 15°C (un réacteur) ou à température ambiante (deux réacteurs). L'un des deux réacteurs opérés à température ambiante ont reçu du ZVI et l'autre n'en a pas reçu pour servir de réacteur contrôle. Dans la phase II, les deux réacteurs recevant du ZVI ont été transformer en procédés de digestion en deux étapes tout en conservant l'addition de ZVI au réacteur méthanogénique. Les autres variables (dosage de ZVI, température, dose d'ozone) sont restées les mêmes que dans la première phase, et le réacteur contrôle fut aussi opéré de manière similaire à la première phase. Les résultats de l'opération des réacteurs sur 278 jours ont révélé que l'ajout de ZVI en phase I augmentait la production de biogaz de 24% pour le réacteur à température ambiante, et a augmenté la proportion de biométhane dans le biogaz de 65% à 74%. Ces améliorations étaient associées à une conversion plus efficace de la demande chimique en oxygène (DCO) hydrolysée en biométhane, car la destruction des solides volatils en suspension (VSS) n'a pas changé de manière significative. Dans la phase II, en comparant les performances d'un même réacteur fonctionnant dans les deux phases, la conversion à un procédé en deux étapes n'a montré qu'une augmentation de la production de méthane de 5% à 15 °C

et de 14% à température ambiante, ce qui est modeste considérant que la production de méthane a aussi augmenté de 3% en moyenne pour le réacteur contrôle. En conclusion, l'ajout de ZVI a amélioré la production de biométhane de la digestion anaérobie à basses températures, mais son effet sur la réduction des solides n'était pas significatif. Par ailleurs, la conversion du procédé à une étape en procédé à deux étapes n'a pas produit de bénéfice supplémentaire. Ces travaux permettront d'optimiser la récupération d'énergie de la digestion anaérobie. Cela peut offrir de nouvelles voies pour améliorer la performance des petits procédés qui sont plus sensibles aux impacts des conditions environnementales.

CONTRIBUTION OF AUTHORS

The work in this thesis will be the topic of an article in preparation. Important part of the text will be reused in this article. Thus, the contributions to the text presented herein and that will be followed in the article. Xuan Wu designed the details and conducted the experiments, analyzed the data, and led the writing of the thesis manuscript. Chenxiao Liu assisted with analysis of VFA, and microbial community composition, and with writing. Xuan Lin helped with the 16S rRNA amplicon data processing. Alexandra Tsitouras helped revising the thesis manuscript. Dominic Frigon obtained the funding, designed the major lines of inquiry, supervised the research, and reviewed the manuscript.

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Chapter 1 Introduction

1.1 Challenges of sludge disposal

Anaerobic digestion (AD) is adopted as an essential and efficient method to deal with the problem of sludge disposal when treating municipal wastewater. AD can further consume organic matter and produce biogas (methane content: 60-70% per volume), while reducing the volume of final waste solids. At the same time, AD sanitize the waste activated sludge (WAS) by destroying most of the pathogens, and reducing nauseous odors (Appels et al., 2008). When treating municipal wastewater, WAS is an unavoidable by-product that needs to be handled properly. Usually, the WAS is thickened and dewatered first, and then transported to landfill or incineration facilities (Appels et al., 2008). However, concerns about sludge disposal are rising rapidly, and strict regulations towards waste disposal have been promoted. It is stated by *Québec Residual Materials Management Policy* that 'the ultimate goal is to ensure that no sludge is landfilled until it has been demonstrated that recovery is not an economically viable option' (Éditeur Officiel du Québec, 2021). In addition, action plans are continuously being released to quantify the goals of recycling. Therefore, wastewater treatment plants (WWTPs) are obliged to minimize their biosolids production and add new values to the organic waste. Waste biosolids contain abundant hydrocarbons, phosphorous and nitrogen, and can be treated to produce biogas and fertilizers. Thus, AD is performed in modern WWTPs to optimize costs and enhance resource recovery.

1.2 Pre-ozonated anaerobic digestion at low temperature

Anaerobic digestion includes four steps: hydrolysis, acidogenesis, acetogenesis and hydrogenotrophic/aceticlastic methanogenesis (Batstone & Jensen, 2011). Among the four steps of AD, hydrolysis is usually considered to be the rate-limiting step because of the recalcitrant bacterial cell components leading to low biodegradability and solubility, such as cell walls and extracellular polymeric substances (Mottet, 2013). To better degrade WAS and keep the solids retention time (SRT) relatively low, a large amount of energy is consumed to maintain operation conditions of AD reactors in mesophilic (30-38°C) or thermophilic (50-57°C) temperature ranges. According to Appels et al. (2008), an SRT of approximately 20 days can be achieved when operating AD at

temperatures over 30℃, while lower temperatures would significantly decrease AD efficiency and increase the required SRT. For example, it is known that AD can be operated at 20℃ (Dolejs et al., 2018; Keating et al., 2018), but the SRT of these reactors are usually much longer to achieve similar solids reduction performance to the ones of the 35℃ digesters (Rittmann & McCarty, 2001).

Despite the biochemical challenges in reducing the operation temperature of AD while maintaining a relatively low SRT, there is a high interest in reducing the operation temperature of AD to maximize energy recovery. In one approach, Keating et al. (2018) enriched at 12℃ and 19℃ a methanogenic microbial community for almost 2.5 years starting from a 37℃ inoculum. Their procedure enhanced the biochemical rates of the hydrolysis and methanogenesis at 12℃ by 20-30 folds between the first and 500th day of their experiment. Many pretreatment and co-treatment methods have been developed to enhance the rate and efficiency of AD (Tian et al, 2019; Carrere et al., 2010). Xie et al. (2007) managed to apply ultrasonication pre-treatment to a 5,000-m^3 egg-shape digester at 29-33℃ with an HRT of 22.5 days and achieved 45% more biogas production compared to the untreated control group. Weemaes et al. (2000) adopted pre-ozonation in their batch reactors with a 30-day SRT at 33℃ and increased methane production by 100% compared to the non-ozonated group. Given that hydrolysis is typically the rate limiting step, it is possible to use pre-treatment and co-treatment methods to reduce the temperature of AD, and hence further improve the energy recovery of the process.

The novel combinations of physical, chemical, and biological pretreatment methods to improve biodegradability of WAS have been studied at high mesophilic and thermophilic temperature ranges, and they have been shown to benefit down-stream AD processes (Ariunbaatar et al., 2014). Among the various pretreatment methods, pre-ozonation is commonly used to enhance disinfection and biodegradability. Ozonation is advantageous since it can be easily combined with the treatment facilities in WWTP, and there is little harmful or toxic residual accumulation after ozonation (Zhen et al., 2017). Therefore, previous research investigated the effects of pre-ozonation on enhancing AD at high mesophilic and thermophilic temperatures (Carrere et al., 2010). Although successful, there remains concern for the use of pre-ozonation of WAS before AD, since the process has a high energy demand. A study conducted by Bakhshi et al. (2018), which used en-

ergy balancing, suggested that energy recovered from the enhanced pre-ozonated AD could approximately compensate for the energy consumption of ozonation. The findings from these earlier studies revealed the potential of ozonation-combined AD to enhance energy recovery.

1.3 Opportunities of zero-valent iron addition and two-stage processes

Considering the potential limitations of acidogenesis and methanogenesis steps of the methanogenic food-web, an increasing number of studies investigated the zero-valent iron (ZVI) addition to AD. Detailed mechanisms behind these improvements remain unknown. Nonetheless, here are a few hypotheses. First, ZVI can function as an electron donor as it is reduced from Fe^0 to Fe^{2+}, producing hydrogen, and thus positively affect hydrogenotrophic methanogenesis (Dinh et al., 2004). Consequently, ZVI reduces oxidation-reduction potential (ORP), and can provide a favorable environment for methane production (Yamanaka & Ounuki, 1974; Zerkle et al., 2005). Second, ZVI can also enhance certain enzyme activities related to acidogenesis and methanogenesis (Choong et al., 2016). In addition to the initial interests in the coupling of acidogenesis and methanogenesis, Liu et al. (2012) found that fermentation could also be enhanced through ZVI addition. Irrespective of the mechanisms, adding ZVI appears to have a positive effect on the hydrolysis, organic acid formation and methanogenesis, which results in higher conversion of WAS organics into volatile fatty acids (VFAs) and thus increase methane production.

The potential of psychrophilic AD was studied by Lin (2018). Lin operated four 4-L cylinder digesters with pre-ozonation and suggested that the moderately low temperature (15℃) of the ozonated WAS was limited by methanogenesis. For the current thesis, it was hypothesized that ZVI could enhance methanogenesis and help reduce the VFA accumulation at 15℃. Thus, ZVI was added to high-rate single-stage digesters in a first set of experiments. Then, the operation these reactors was changed to a two-stage process, with ZVI dosing remaining in the methanogenetic reactors. The influence of ZVI on both operation status performances was evaluated, attempting to show the potential of ZVI additive in improving low-temperature AD applications.

Two-stage AD processes have been used for a long time to improve AD by optimizing the conditions associated with each step of the methanogenic food-web. The development of microbial ac-

tivities can be affected by many operational parameters, such as pH, and hydrogen and VFA concentrations. The purpose of a two-stage AD process is to operate hydrolysis/acid formation and methanogenesis in two separate reactors. The low pH produced in the former reactor results in an unfavorable environment for methanogens, but can enhance hydrolysis (Sung & Tao, 2003; Cirne et al., 2007). It was reported that a two-stage AD process improves AD performance by increasing the organic loading rate (OLR), reducing reactor volume, providing more gas production and better stability of the digestion process (Lindner et al., 2015). Aslanzadeh et al. (2014) revealed that compared to single-stage reactors, the two-stage reactors increased the OLR from 2 gVS/L/d to 14 gVS/L/d.

In the current thesis, it was hypothesized that AD staging would have a synergistic effect with the ozone pretreatment of WAS. This hypothesis was building on previous reports that partial ozonation of the digestate (i.e., a fraction of the content of the methanogenic reactor was ozonated and then returned to the reactor) can increase the biogas conversion by up to 80%, compared to only ozonating the incoming municipal sludge (Yasui et al., 2005). This capacity to improve biomethane production by ozonation of the digestate was also observed in a previous study conducted by Bakhshijooybari (2020). However, the ozonation also kills methanogens, which is detrimental to AD. As a solution, partially ozonating the fermentate (i.e., the content of the fermentation reactor) instead of the digestate in a two-stage AD process could provide similar benefits without affecting the methanogens. Furthermore, Subha & Mythukumar (2012) stated that ozone performances could be improved at low pH due to the higher solubility of ozone molecule. Finally, Wilde et al. (2014) suggested that Fe(II) and Fe(III) catalyzed ozonation would result in higher organic compounds removal, suggesting that ZVI could be added to the first stage for additional benefits.

Considering all these elements, it was resolved to test our hypothesis of the possible interactions between all these reactor configurations by operating two-stage digesters at 15°C and room temperature. The goal was to compare the reactor performances for the ozonation of the first stage (i.e., fermentate), ozonation of the one-stage digestate, or the ozonation of the feed WAS. Although we hoped to test all these possible synergies, restrictions to lab access due to COVID-19 and the loss of one of our bench-scale AD reactors limited the scope of the study and only WAS ozonation was tested (see the description of experiment objectives in 1.4). In all experiments, ZVI was dosed

to the second stage (i.e., methanogenic) reactors, based on the results of a preliminary WAS solubilization study (Chapter 3).

1.4 Objectives

This study was conducted to:

1) Determine the effect of ZVI addition on AD performance with pre-ozonation under 15℃ and room temperature (22.6±0.3℃);
2) Determine the effects of combining two-stage processes and ZVI addition with pre-ozonation under 15℃ and room temperature;
3) Assess the effects of ZVI presence and sludge pH on pre-ozonation performance by conducting WAS solubilization tests

1.5 Organization of the thesis

The introduction states the background and basic knowledge of AD, as well as the pre-treatment and co-treatment methods adopted in this thesis, including pre-ozonation, ZVI addition and two-stage processes.

The second chapter is a literature review of relevant studies on anaerobic digestion combined with ozone pretreatment, ZVI addition or two-stage processes. The chapter also describes the basic principles underlying the microbial food-web supporting AD, and the known mechanisms leading to AD enhancement by pretreatment or co-treatment methods.

The third chapter reports on the results of an ozone solubilization study to determine the impact of pH and ZVI addition on the reaction of sludge with ozone. This study was conducted for an early assessment of the potential benefits of ozonating the fermentate (i.e., first-stage solids in a two-stage process) instead of the WAS or the single-stage digestate. Furthermore, this study tested the interaction between ozone and ZVI during the ozonation pre-treatment that was suggested in the literature.

The fourth chapter summarizes the performances of anaerobic digesters fed with ozonated WAS. Firstly, digesters were operated at 15℃ and room temperature with ZVI additive as a variable to

determine the influence of ZVI on anaerobic digestion. Then, a subsequent experiment was done by changing certain single-stage digesters to two-stage ones, aiming to evaluate the additional benefits of staged AD process with ZVI addition at low temperatures.

Finally, the fifth chapter presents the overall conclusions of the current thesis and possible work to be done in the future.

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Chapter 2 Literature review

2.1 Introduction

As a commonly utilized method of WAS treatment, the theory of anaerobic digestion is studied to get better performance of biogas production and solids reduction (Zhou et al., 2017). Thus, various pre-treatment and co-treatment approaches have been investigated. In this chapter, the microbial food-web of AD process is firstly reviewed and presented. The effects of ozonation, ZVI additive and two-stage process on AD are then summarized from literatures to establish a theoretical basis for the analysis in Chapter 3 and 4.

2.2 Anaerobic digestion food-web

WAS is a major by-product of the activated sludge wastewater treatment process, consisting of large amounts of biomass generated by cell growth and decay, as well as other particulate organic compounds (Wei et al., 2013). The investment of treating and managing excess WAS accounts for 25-60% of the costs in WWTPs (Zhou et al., 2017). To reduce this cost and recover energy, AD is often used to digest WAS before final disposal. Conventional AD practice has the disadvantage of long retention times, low gas production and an overall low solids degradation rate (Rittmann & McCarty, 2001). These are due to limited hydrolysis rate resulting from the slow degradation of insoluble and bio-recalcitrant proteinaceous cell walls and extracellular polymeric substances (Zhang et al., 2009). Therefore, extra energy investment is required to assist the hydrolysis process to better solubilize WAS and make it more biodegradable.

AD is a complex multistep process which involves complicated microbial community and requires strict anaerobic condition. According to Batstone and Jensen (2011), the four stages of AD are hydrolysis, acidogenesis, acetogenesis and hydrogenotrophic/aceticlastic methanogenesis (Fig. 2.1). In hydrolysis complex organic matters are disintegrated into polymers like proteins, lipids and polysaccharides and then further converted into their monomers by extracellular enzymes. These monomers are subsequently fermented into VFAs and alcohol during acidogenesis process. Further transformation is done by syntrophic bacteria that convert VFAs and alcohol into acetate,

hydrogen and carbon dioxide in the acetogenesis process. Finally, during methanogenesis, methane is produced utilizing the substrate provided in the previous step. The methanogenesis stage consists of two main pathways, one of which is performed by aceticlastic methanogens that consume half of the acetic acid molecule as electron donor and the other half as electron acceptor. The other group of methanogens is hydrogenotrophic methanogens that reduce carbon dioxide and oxidize hydrogen.

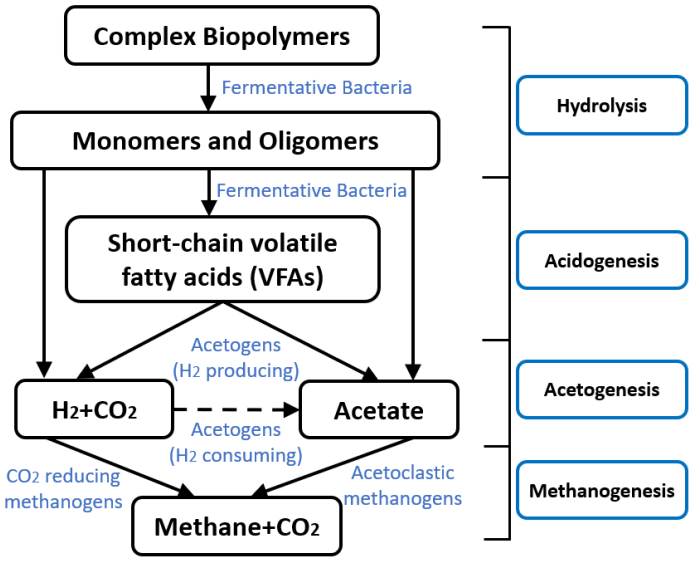

Fig. 2.1 Microbial stages of anaerobic digestion process

Hydrolysis is usually considered to be the rate-limiting step of AD because of the low biodegradability and solubility of WAS caused by the recalcitrant bacterial cells components, such as cell walls and extracellular polymeric substances (Mottet, 2013). This slow biodegradation requires AD to be operated under the mesophilic condition at 35-37℃ to accelerate the hydrolysis process, even though the SRT is still 20-30 days at 35℃ (Rittmann & McCarty, 2001). Thus, various pretreatment methods have been studied to improve the biodegradability of WAS to achieve shorter the SRT and reduce the operating temperature.

2.3 Ozone pretreatment of anaerobic digestion

Pre-ozonation is a method applied to improve WAS biodegradability that received increasing attention in the last two decades. Otieno et al. (2009) reported that pre-ozonation of municipal

WAS led to a 50% decrease in TSS and a 25% increase in soluble chemical oxygen demand (sCOD) value. Silvestre et al. (2014) evaluated the effect of ozone dose on biogas production, and suggested that lower doses of ozone led to faster increases in biogas production while higher doses resulted in slower conversion due to inhibition by long-chain fatty acids reaction products (i.e. aldehydes).

2.3.1 Effects on the characteristics of WAS

Among all the pretreatment methods used to solubilize WAS, ozonation shows high efficiency and is the most widely used peroxidation process (Zhen et al., 2017). Scheminski et al. (2000) suggested that ozone could increase the osmosis of cell membranes, alter cell membrane permeability, damage cell walls, and release intracellular substances. After ozone pretreatment, a decrease in the volatile suspended solid (VSS)/total suspended solid (TSS) ratio and pH value can be observed (Bougrier et al., 2006; Zhao et al., 2007), caused by the cell lysis and the formation of easily biodegradable acidic intermediate compounds such as acetic acids and humic substances (Otieno et al., 2019). Furthermore, Zhang et al. (2009) reported that an ozone dose less than 0.5 g-O_3/g-TS has limited influence on the particle size in WAS, but according to Park et al. (2004), higher ozone doses can result in increasing of small particle percentage, which is due to the biomass disruption during the pretreatment process.

2.3.2 Effects on the microbial activity of WAS

The loss of biomass activity can be observed after ozone pretreatment. Studies have been conducted to reveal the significant inactivation of different microbial groups such as general heterotrophic bacteria (Saktaywin et al., 2005) and nitrifying bacteria (Kobayashi et al., 2001). Moreover, the experiment conducted by Yan et al. (2009) showed through PCR-DGGE that with increasing ozone dose less bands were detected in the DGGE fingerprint, indicating that ozonation caused cell lysis, releasing and degrading DNA from the bacterial cells. Finally, the total loss of protease enzyme activity could be observed with the ozone dose of 0.10 g-O_3/g-TSS or more.

2.3.3 Improvement of biodegradability of WAS

It is reported that the biodegradability of WAS can be improved after ozone pretreatment. Saktaywin et al. (2005) revealed that although 40% of the sCOD formed by ozonation remained refractory, the remaining sCOD was biodegradable. Yeom et al. (2002) found that WAS ozonated with the dose of 0.1 g-O_3/g-TSS was 2-3 times more biodegradable than the non-ozonated sludge incubated under both aerobic and anaerobic conditions for five days. However, when the ozone dose increased to a certain level, the ozone mineralized the biodegradable products instead of oxidizing the refractory portion. Yeom et al. (2002) tested the effects of ozone dose on the ozonated sludge, and reported that the mineralization became greater with an ozone dose higher than 0.1 g-O_3/g-TSS. In addition, Bougrier et al. (2006) pointed out that mineralization occurred at the dose of 0.15 g-O_3/g-TS. Therefore, in practical applications, the ozonation process should be monitored to determine the optimal ozone dose applied to different sludge types.

2.4 Zero-valent iron combined with anaerobic digestion

The effect of ZVI on AD practices has been the topic of a number of studies, aiming to provide a secure and effective method to accelerate and enhance AD process. Zhang et al. (2020) reviewed the main progress and possible mechanics in utilizing ZVI to promote in-situ methane production, accelerate electron transportation and to enhance degradation of certain pollutants. According to this study, ZVI addition method was considered to be economical, efficient and carbon-neutral in AD treatment. It is reported that by adding ZVI, the degradation of protein increased by 21.9%, VFA reduction increased by 37.3%, the methane yield increased by 43.5%, and certain critical enzymes in hydrolysis and acidification increased by 60% (Feng et al., 2014). Zhao et al. (2018) compared the effects of ZVI and Fe_3O_4 in AD process, showing that ZVI additive increased the methane production from hydrogenothrophic methanogenesis process by 70% compared to the blank group, while Fe_3O_4 had a slight inhibitory effect on syntrophic methane production. There is no consensus on how ZVI reacts in different microbial stages, but theories and kinetics have been discussed to explain the function of ZVI in AD applications.

2.4.1 Anaerobic iron corrosion

It was suggested by Dinh et al. (2004) that anaerobic corrosion of iron is a biochemical process and is usually linked to sulphate-reducing bacteria, which acts upon iron by consuming 'cathodic hydrogen' generated through the reaction between iron and water ($4Fe + SO_4^{2-} + 4H_2O \rightarrow FeS + 3Fe^{2+} + H_2 + 8HO^-$). The sulphate-reducing bacteria also oxidize iron indirectly by producing hydrogen sulfide as corrosive agent ($6[CH_2O] + Fe + SO_4^{2-} + 2H^+ \rightarrow 2HCO_3^- + FeS + H_2O$). It is typically believed that hydrogenotrophic methanogenesis pathway produces less biomethane than aceticlastic methanogenesis in the digestion of primary and secondary solids form wastewater treatment (Grady et al., 2011). The hydrogen generated in anaerobic iron corrosion can be utilized by hydrogenotrophic methanogens to benefit methane production. In addition, since iron corrosion happens with the consumption of H^+, the acid produced in the previous AD steps can be then buffered, which favors the methanogen growth (Wei et al., 2018).

2.4.2 Iron impacts on oxidation-reduction potential

ZVI can also be dosed in fermentation reactors of two-stage AD systems, because the oxidation-reduction potential (ORP) strongly influences fermentation types (Ren et al.,2007). It is suggested in the same study that the overall rate of methanogenic metabolism is influenced by fermentation products, because acetate and butyrate are more favorable substrates than propionate for downstream methanogenesis. Among the three types of fermentation (butyric acid type, propionic acid type and ethanol type fermentation) in AD, butyric and ethanol type both can convert organic matter to acetate, and these two fermentation types can be enhanced by ZVI, which provides a reductive environment and at the same time declines propionate production, providing a favorable substrate environment for methane production (Wang et al., 2006).

2.4.3 Iron impacts on microorganisms

Iron is one of the trace elements that are essential for both microbial metabolism and anabolism. In AD, iron is indispensable in the synthesis of cytochromes and oxidases of methanogens (Yamanaka & Ounuki, 1974; Zerkle et al., 2005). As well as being an important part of the microbes, iron can also work as extracellular electron donors in the respiration processes of methanogens (Newman & Kolter, 2000). Furthermore, Xu et al. (2019) suggested that (semi)conductive iron

oxides could serve as electron conduits to stimulate direct interspecies electron transfer and work as electron carriers through Fe(II/III) redox cycle to mediate interspecies electron transfer.

ZVI is considered to positively impact certain enzyme activities, such as the crucial pyruvate-ferredoxin oxidoreductase in acidogenesis, which contains Fe-S clusters. All the four steps of AD are catalyzed by complex enzyme networks, and the enzymatic activities in methanogenesis involve more metals than those in the other steps (Zerkle et al., 2005). The study done by Choong et al. (2016) shows that iron can activate various enzymes relevant to not only methanogenesis but also acidogenesis, serving as active sites, cofactor and metalloenzymes constituent. Hao et al. (2017) also pointed out that ZVI could increase the activities of hydrolysis-acidification enzymes. Additionally, iron can provide low ORP, which enhance methanogens enzymatically (Zhang, 2014). Thus, ZVI is believed to enhance the microbial activity of all the AD steps with different mechanisms.

2.5 Two-stage anaerobic digestion

Two-stage anaerobic digestion refers to the configuration separating the four biochemical tesps of the anaerobic digesting process into two individual reactors, through which the optimal conditions for the unique biomasses in both reactors can be developed (Baldi et al., 2019). These two main stages are known as ‘acid fermentation’ and ‘methanogenesis’. The former stage involves the first two steps of anaerobic digestion: hydrolysis and acidogenesis, producing VFAs. Then, methanogenesis in the second reactor either consumes the VFAs by converting them into methane and carbon dioxide, or utilizes hydrogen for biomethane yield. The two groups of microorganisms can be divided because of their different physiological and nutritional requirements (Azbar & Speece, 2001; Baldi et al., 2019). Pohland and Ghosh (1971) revealed that a shorter SRT favored the enrichment of dominant fermentative bacteria group in the first stage, while a longer SRT promoted the growth of methanogens in the second stage and thus could encourage VFA consumption. Finally, the separation in two stages improved performance compared to single-stage anaerobic digestion.

Zhang and Noike (1991) presented that, compared to the single-stage system, the propionate concentrations were 30-50% higher, and the growth of homoacetogens was 2-10 times faster than in

the two-stage reactors. It was pointed out by Komatsu et al. (1991) that certain aromatic compounds were inhibitory to methanogens, but acidification could saturate double bonds so the wastewater would be more amenable to methanogenesis. Compared to single-stage AD, lipids degradation in a two-stage system tends to be more efficient. In addition, two-stage systems have advantages in the selection and enrichment of microbial groups, making the AD process more stable and providing the methanogenesis with enhanced buffering capacity (Aslanzadeh et al., 2014). As a result, two-stage systems usually can be operated with a higher organic loading rate (OLR) and less reactor volume (resulting in smaller footprint).

2.6 Conclusion

Iron additive and two-stage process are both reported to benefit AD process, and thereby promote biomethane yield and reduce biosolids production. Although detailed mechanism behind the improvement caused by ZVI remains unclear, iron addition has been found effective in accelerating hydrolysis and methanogenesis processes and can increase the microbial activity in AD reactors. The two-stage process separates the two groups of unique biomasses in AD applications and provide favorable environment for them to proliferate, through which the overall AD operation becomes more stable and efficient. However, their effects on AD are different based on the various types of WAS adopted in practice. Therefore, both techniques and their interaction were tested and evaluated under low-temperature conditions in this project (Chapter 4).

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Chapter 3 Solubilization tests of waste activated sludge

3.1 Introduction

Waste activated sludge (WAS) produced by biological wastewater treatment is a complex mixture of organic compounds, including rich phosphorus and nitrogen (Wei et al., 2013). AD is a commonly used method to partially recover the energy remained in WAS. However, the poor biodegradability of WAS results in low rates of hydrolysis (first step in methanogenic food-web), and low yield of methane. To hydrolyze WAS more efficiently, ozone pretreatment is applied and shows great potential in improving AD performance (Carrere et al., 2010). However, several strategies of WAS ozonation application remain to be investigated to optimize AD performances. Two of these strategies were evaluated in this chapter: operating ozonation under different pH and applying ZVI additive in the system. Together, these strategies provide new possibilities for optimization of the ozone treatment.

Literature reports that the solubility of ozone is readily influenced by operation pH, and this effect is caused by the relationship between ozone decomposition behavior and oxidation potential (Subha & Mythukumar, 2012). Ozone exists in acidic pH as its molecular form, and it decomposes into secondary oxidants (OH·, HO_2 ·, HO_3 ·, and HO_4 ·) under alkaline conditions. In these functional groups, hydroxy-based free radicals (OH·) has the highest oxidation potential of 2.8 V. However, with pH increasing, this potential decreases to 1.4 V in alkaline conditions (Hoigne & Bader, 1976). An ozonation study with synthetic wastewater was conducted by Hausler et al. (1993), attempting to evaluate the performance of ozone in different pH conditions, and the ozonation rates increased significantly when changing the pH condition from basic to acidic. Martín Santos et al. (2003) applied ozone to Vinasse wastewater, and higher COD removal was achieved at acidic pH. These studies suggest that ozonation performance would be better at low pH.

Yasui et al. (2005) conducted a full-scale AD application with partial ozonation (part of the digestate was ozonated and fed back to the reactor) and achieved high VSS degradation rate of 88%. However, this approach also inactivates methanogens in the digestate, which may result in a reduction in COD conversion to methane. The potential deleterious effects of digestate ozonation on methanogens and the observations in the literature that certain ozone reactions are more potent at

low pH together suggest a possible optimization path by applying ozonation treatment to the first stage (i.e., fermentate) of a two-stage AD process. The fermentate of first stage is usually at low pH due to the production of organic acid. And it can also avoid the destruction of methanogens which only enrich in the second stage. Therefore, WAS ozonation was tested at different pH by measuring the resulting COD solubilization, and the result was presented and discussed in this chapter.

Previous studies further suggested the feasibility of iron catalytic ozonation in some conditions. Malik et al. (2008) studied the catalytic ability of Fe(II) and nanoscale ZVI to improve degrading complex textile effluent. In this study, the iron-treated wastewater gained 47% improvement in COD removal. Nawrocki and Kasprzyk-Hordern (2010) stated the complexity of catalytic ozonation, which could result from combined kinetics instead of only hydroxyl radical formation. Based on these earlier studies, ZVI could have the potential to positively affect WAS solubilization.

In order to test the possible impacts of pH and ZVI addition, the performance of COD solubilization was evaluated by calcualting the solubilization efficiency. This study provided relevant information for the final design of the AD reactor study presented in Chapter 4. In the current chapter, ozonation tests were conducted in different pH conditions (pH=2, 7 and 10), with and without the presence of ZVI.

3.2 Material and methods

3.2.1 Experimental setup

The WAS was collected in the Régie de l'Assainissement des Eaux du Bassin La Prairie (St-Catherine, Quebec, Canada). Raw WAS was sent to the laboratory overnight and was preserved at room temperature with aeration. Subsamples were taken before ozonation, and the TSS of each subsample was adjusted to 2.1-2.8 g/L. In the ozonation process, pure oxygen gas (Praxair, Canada) was converted to ozone by the OZOMAX generator (OzoMax Inc., USA), with a steady flow rate of 6.5 SCFH (0.19 m^3/h). For the ozonation experiment, 0.2 L of WAS was introduced in a 0.5-L ozone contactor with two ports for ozone gas inlet and outlet (Fig. 3.1). The vessel was then sealed and connected to the ozone generator. The ozone outlet was connected to either the MINI-HICON Ozone Analyzer (IN-USA, USA, used in batch 1 and 2) or the Model 106-H Ozone Monitor (2B

Technologies, USA, used in batch 3 and 4) with a datalogger. The outlet ozone concentration was recorded automatically with a specified time interval (4 sec for MINI-HICON Ozone Analyzer and 2 sec for Model 106-H Ozone Monitor), and the dose was calculated based on the difference between the transferred ozone mass (integration over time of concentration × gas flow rate), which was the mass fed to the contactor minus the mass in the effluent, divided by the sludge volume (0.2 L).

Four batches of experiment were conducted, and each batch contained 6 sub-experiments (3 pH × 2 ZVI addition [no ZVI, 2 g/L]). Before ozonation started, the pH was adjusted to 2, 7 or 10 with 2 mol/L HCL or 10 mol/L NaOH, and 2 ml of antifoam (Antifoam 204, SIGMA-ALDRICH, Canada; Antifoam A, SIGMA-ALDRICH, Canada) was added to the WAS. Antifoam reduced the formation of foams in the contactor. The entire ozonation process was conducted at room temperature and separated in cycles of 10 minutes to allow for sampling of the contactor content to determine the evolving physicochemical properties of the ozonated WAS. This operation cycle was conducted repeatedly until the total ozonation time reached 120 min (batch 1 and 2) or 100 min (batch 3 and 4). 2g/L of ZVI was added to all the dosed reactors. Samples were taken both before and during the experiment to determine the initial and solubilized sCOD (Standard Method 5220D; APHA et al., 2012). The solubilized sCOD was the sCOD difference before and after ozonation. The initial TSS and VSS were tested as well (Standard Method 2540G; APHA et al., 2012). In addition to the test reactor, a non-ozonated control reactor was set up for each sub-experiment. These control reactors were set up with beakers and operated under the same conditions as the corresponding test reactors except that air instead of ozone was bubbled during the experiment. Such control reactors were used to assess any change in sCOD brought by the addition of antifoams or volatilization,

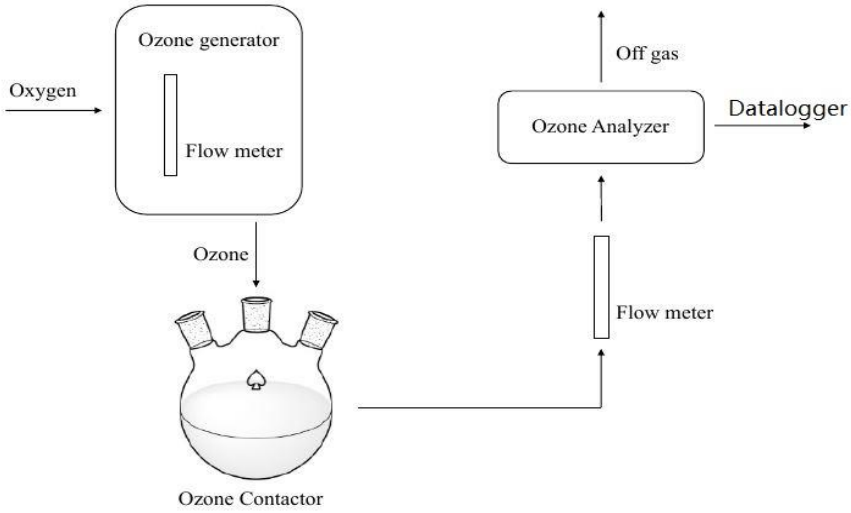

Fig. 3.1 Diagram of the solubilization unit set up

3.2.2 Statistical method

Two-way ANOVA was analyzed to test the significance of the effects of pH and ZVI addition on COD solubilization efficiency using EXCEL (version 2107) Data Analysis tool (Zaiontz, 2014). The solubilization tests were conducted in four replicates (four batches).

3.3 Results and discussion

The sCOD increasing was linear correlated to ozone dose before the rates of increase slowed down and plateaued (Fig. 3.2). The solubilization efficiency was defined as the mass of increased sCOD per mass of ozone consumed. In these experiments, the overall effect of pH cannot be defined as statistically significant at a critical P=0.05 (Table 3.1). However, pH had a significant impact on solubilization efficiency with no ZVI presence ($P<0.05$), and the efficiency decreased under acidic condition. In the same pH condition, ZVI-dosed WAS consumed more ozone than the iron-free groups, and ZVI showed a significant impact on the solubilization efficiency ($P<0.05$), suggesting that adding ZVI to ozone pretreatment decreased the rate of sludge solubilization (Table 3.1). The difference in pH effect with and without ZVI addition (i.e., interaction between main treatments) were not found to be significant (Table 3.1).

Table 3.1 Results of solubilization test

Solubilization Efficiency [a] (g-sCOD/g-O_3)			
Parameter	pH=2	pH=7	pH=10
ZVI: 0g/L	4.01±2.38	4.28±1.09	5.08±1.61
ZVI: 2g/L	2.98±0.27	2.57±0.53	3.11±0.25

[a]± Standard deviation of 4 independent experiments of solubilization measurements (n=4)

The results showed that iron addition had negative effects on WAS solubilization, and alkaline environment favored WAS solubilization without adding ZVI. This result is in concordance with the WAS solubilization study conducted by Cosgun & Semerci (2019). They summarized the results of 26 batches of experiment with or without acidic, alkaline treatments and with different doses of ozone. It revealed that with ozone pretreatment, both sludge solubilization and phosphorus releasing were improved the most by adopting alkaline treatment (increased by 23.9% and 152.6%, respectively), while acidic treatment reduced COD solubilization during ozonation, with only 10% increased COD release. This result indicated that compared to the improved solubility of molecular ozone in acidic conditions, the effect of ozone decomposition product existing in high pH (mostly OH·) played a more important role in improving solubilization performance.

Studies have been conducted to investigate the catalyst that enhances reactive hydroxyl radical generation and ozone decomposition, which can be applied to wastewater treatment (Nawrocki & Kasprzyk-Hordern, 2010). Malik et al. (2008) investigated the influence of catalytic pre-ozonation via Fe(II) and nanoscale zero-valent iron on improving biodegradability of complex textile effluent, achieving 134.6% enhancement on biodegradability index (BI=BOD_5/COD). The main reaction of Fe(II) and Fe(III) catalyzed ozonation is to enhance metal oxalate complexation or to assist the generation of hydroxyl radicals and thus advance the removal of organic compounds (Wilde et al., 2014), which can be expressed as in eq. 3-1 to eq. 3-4:

$$Fe^{2+} + O_3 \rightarrow Fe^{3+} + \cdot O_3^- \quad \text{eq. 3-1}$$

$$\cdot O_3^- + H^+ \rightarrow O_2 + OH\cdot \quad \text{eq. 3-2}$$

$$Fe^{2+} + O_3 \rightarrow FeO_2^+ + O_2 \quad \text{eq. 3-3}$$

$$FeO_2+ + H_2O \rightarrow Fe^{3+} + OH\cdot + OH^- \quad \text{eq. 3-4}$$

It is shown in Fig. 3.3, the concentration of dissolved iron increases with operation time, whereas not catalyzing the ozonation process. One explanation could be the consumption of Fe(II) to generate other ion groups (such as Fe^{3+} and $H_2O_2/HO_2\cdot$ pair), instead of forming hydroxyl radicals (Zhang et al., 2013). In addition, compared to the Fe(II) dose in the study of Malik et al. (2008), which ranged from 1 to 6 g-Fe(II)/L, dissolved iron concentration used in this experiment were lower (<1 g-Fe(II) /L). The lack of enough Fe(II) could also lead to less efficient catalytic ozonation. One possible solution would be adding extra Fe(II) ion or nZVI as presented in the literature, and further studies are needed to investigate their effects on WAS ozonation.

3.4 Conclusion

WAS solubilization was tested at pH 2, 7 and 10, with or without ZVI and the best efficiency was found in the condition of pH=10 without ZVI. Although acidic ozonation and catalytic ozonation are verified to improve solids destruction, sCOD and nutrients release in some conditions, they affected WAS solubilization negatively according to the analysis in this chapter. Since the solubilization test was performed to preliminarily assess the ozonation of fermentate (from fermentation reactors) and digestate (from methanogenetic reactors), results shown in this chapter suggest the highest solubilization efficiency would be achieved with ozonating digestate, which has higher pH. Moreover, it also reveals that adding ZVI to fermentate would further reduce its solubilization performance. Therefore, ZVI was only added to methanogenetic reactors in Phase II experiment presented in Chapter 4.

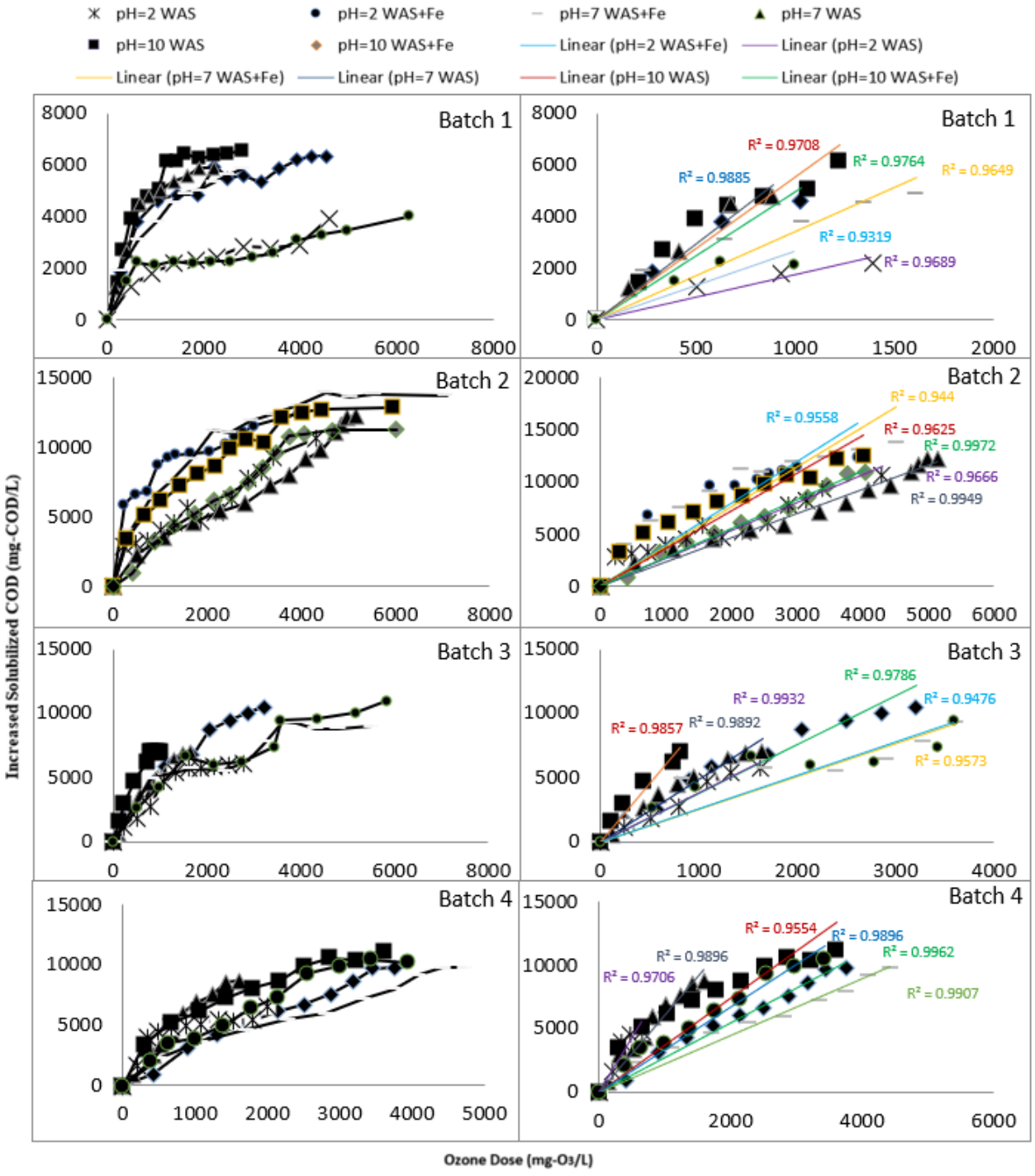

Fig. 3.2 WAS solubilization curves and linear correlation analysis results

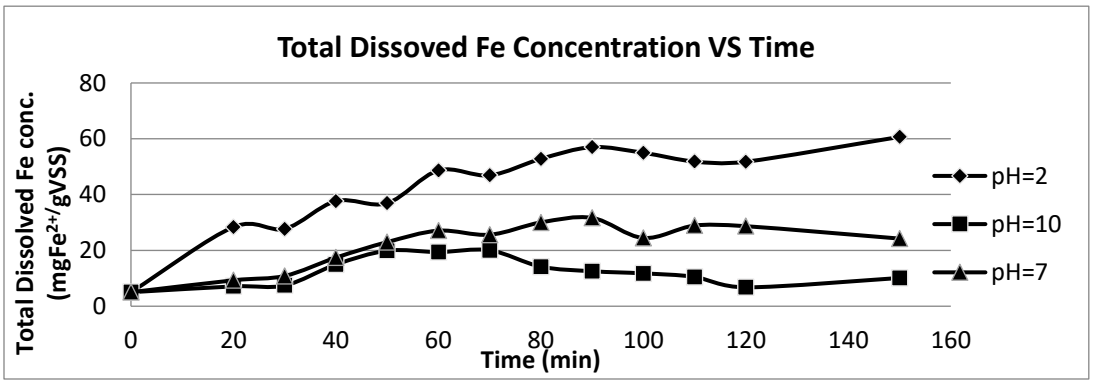

Fig. 3.3 Concentration change of total dissolved iron (as ferrous) during the ozonation process

3.5 Reference

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Chapter 4 High-rate anaerobic digestion of pre-ozonated waste activated sludge combined with zero-valent iron additive and two-stage process

4.1 Introduction

It was reported that the operation of AD at 20°C fed with pre-ozonated WAS was able to maintain a relatively high solids destruction and methane production at SRTs of 15 and 20 days (Bakhshi et al., 2018; Lin, 2018). However, volatile fatty acid (VFA) accumulated at 20°C, while this situation was not observed at 35°C. The VFA accumulation was exacerbated with the further decrease in operation temperature at 15°C, suggesting a limitation with methanogenesis. This previous work also reported that the ozonation of a return stream of digestate provided slight benefits in solids destruction over the ozonation of the feed WAS, but also led to additional VFA accumulation likely due to the inactivation of methanogens in the returned sludge (Bakhshi et al., 2018). Therefore, the current study aimed at finding approaches that could improve the performance in these conditions.

Zero valent iron (ZVI) addition and the split of the AD process into two-stages (i.e., low pH fermentation reactor and methanogenic reactor) are both reported to have a positive influence on solids destruction and biogas production (Aslanzadeh et al., 2014; Dinh et al., 2004; Daniels et al., 1987). For the current process improvement study, it was hypothesized that ZVI addition and two-stage processes would provide additional benefits through specific mechanisms. First, ZVI addition is believed to serve as direct electron donor for methanogenesis and consequently improving electron transfer rate (Daniels et al., 1987), a property that was hypothesized to improve the efficiency of VFA transformation to methane. Second, splitting the AD process into two stages could provide the possibility of ozonating the fermentate (i.e., partially degraded solids) to focus the oxidative power on the most recalcitrant fraction of the solids, while limiting inactivation of methanogens. Slow-growing methanogens in a single-stage reactor were partially killed by ozonating recycled digestate in previous studies (Saktaywin et al., 2005; Yan et al., 2009), which would be solved by applying a two-stage process. Finally, it was initially hypothesized that performing ozonation of solids at low pH with the addition of ZVI could result in further benefits based on literature observations (section 3.1). The study presented in Chapter 3 did not support

this last hypothesis, and they even suggested that ZVI could reduce the transformation efficiency of ozone on the solids. Therefore, it was decided to only add ZVI to the methanogenic reactor.

The reactors were operated in two phases. Phase I was the single-stage phase when all the digesters were operated as single-stage process with pre-ozonated WAS feed. ZVI was added to certain reactors to test the effects of ZVI alone, while a group of single-stage and ZVI-free control reactor was kept (due to equipment limitations the control reactors were only operated at room temperture). Phase II was the two-stage phase when all the test groups (reactors with ZVI) were changed to be two-stage digesters with ZVI dosed in the second-stage methanogenetic reactors. The impacts of ZVI addition and staging would be evaluated in this phase. The control group was operated in the same condition as that in Phase I. An additional study was planned in Phase II to investigate the benefits of ozonating the fermentate and return digestate flow over feeding with ozonated WAS alone. However, this part in Phase II study was abandoned due to lab-access restrictions during the COVID-19 pandemic. Therefore, this chapter presents the results of Phase I and the first half of Phase II. In addition to reactor performances, microbial community compositions were analyzed by 16S rRNA gene amplicon sequencing to assess the potential of ZVI for enriching specific microbial population, which could be involve in direct interspecies electron transfer.

4.2 Material and methods

4.2.1 WAS feed and ozonation

Feed WAS was collected in Régie de l'Assainissement des Eaux du Bassin La Prairie (St. Catherine, Québec, Canada) every week throughout the experiment, and was sent to the laboratory overnight. In Phase I, the feed was added directly to the single-stage reactor. Alternatively, in Phase II, the feed was added to the primary bottle reactors (except for the control reactor which remained a single stage), and then the fermentates were extracted with a syringe and fed to corresponding 4-L methanogenic reactors. Details are presented below in Section 4.2.2 (Fig. 4.1).

All WAS feeds were ozonated by bubbling ozone gas generated by an OZOMAX generator (OZO 2VTT, OzoMax Inc., USA). To do so, pure oxygen gas (Praxair, Canada) flowed through the generator at a steady rate of 6.5 SCFH (0.19 m^3/h). Ozone reacted with WAS in a 5-L bottle contactor, which was sealed and connected to the MINI-HICON Ozone Analyzer (IN-USA, USA) or Model

106-H Ozone Monitor (2B Technologies, USA) equipped with a datalogger. The outlet ozone concentration was recorded automatically with a specific time interval (4 sec for MINI-HICON Ozone Analyzer and 2 sec for Model 106-H Ozone Monitor), and the dose was calculated based on the difference between the transferred ozone mass (integration over time of concentration × gas flow rate), which was the mass fed to the contactor minus the mass in the effluent, divided by the sludge volume (1.5 L).

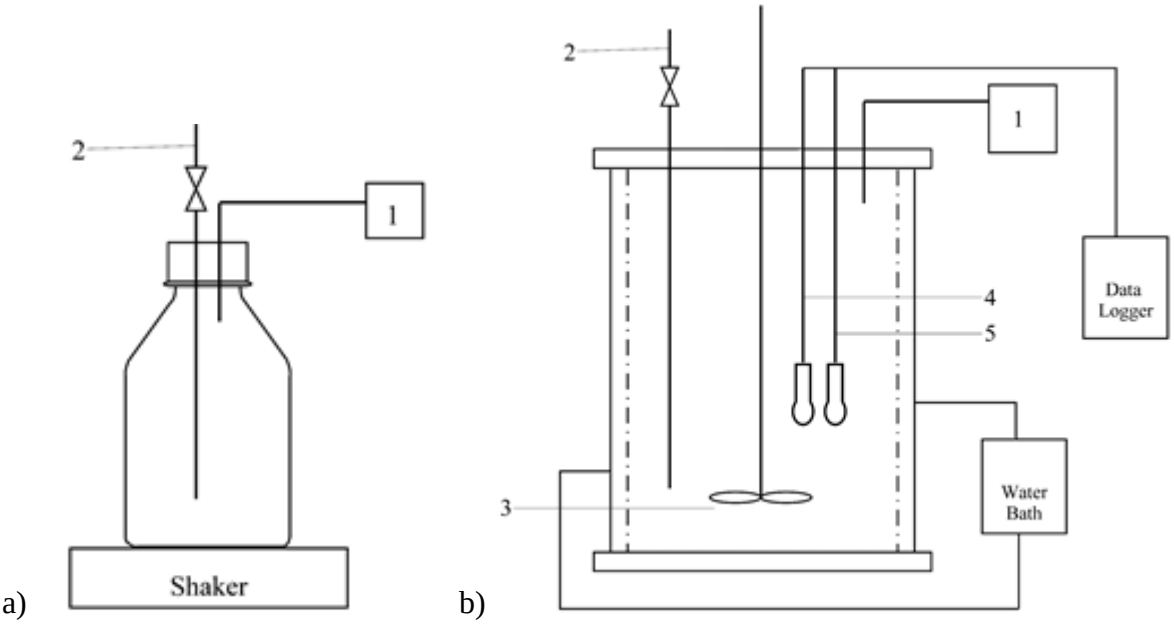

Fig. 4.1 Diagrams of reactors. a) 1-L hydrolysis-acidification bottle reactor in Phase II (working volume: 0.5 L); b) 4-L anaerobic digester (working volume: 2 L). The shown components are: 1. Tedlar® gas sampling bag; 2. Feeding & wasting ports; 3. Mechanical mixer; 4. Temperature probe; 5. pH probe.

Ozonation pretreatment was continuously conducted for 1 hour, treating 1.5 L WAS per batch, and then was stopped for sampling. By using the same ozonating strategy, the ozone dose of feeding WAS was maintained at an average of 470 ±105 mg-O_3/L or 27.2±6.1 mg-O_3/g-VSS. WAS before and after ozonation treatment was sampled for sCOD (Standard Method 5220D, APHA et al., 2012), TSS and VSS (Standard Method 2540G, APHA et al., 2012) measurements. After ozonation, the treated WAS was stored at 4℃ until fed to the reactor within the following 7 days.

4.2.2 Reactor set-up and operation

Three 4-L cylinder reactors were used in the experiment (with the working volume of 2 L), which were mixed using overhead mechanical mixers with 100 rpm (Fig. 4.1). One of the reactors was operated at 15℃, and the other two reactors were maintained at room temperature. A circulating water bath (NESLAB RTE740, Thermo Fisher Scientific, USA) was utilized to cool down the 15-℃ digester. The temperature and pH of each reactor were monitored by two sets of online build-in probes (Apex Classic and Apex EL, Neptune systems, Canada), and both of temperature and pH were checked offline in the waste digestate every two days using a pH meter (Orion Star™ A2110, Thermo Fisher Scientific, USA) and a glass thermometer (Thermo Fisher Scientific, USA). The total HRT and SRT of all the reactors were set at 20 days with feeding and wasting performed every two days. Before extracting the digestate from the reactors, the speed of mechanical agitators was adjusted to 180 rpm for 30 min to improve the mixing of ZVI powder within the entire volume of the digester. Then, sludge digestate was extracted using syringes through a tube extending to half of the height of working volume to minimize oxygen penetration of the digester, and feed was added through the same tube. Results suggested that relatively dense ZVI powder particles did not leave the reactor homogeneously with the digestate. Consequently, it was resolved to maintain the initial ZVI concentration (2 g/L) throughout the experiment by measuring the ZVI in the waste and simply replace the removed ZVI. Powder ZVI was separated from the digestate using a powerful magnet (Neodymium Disc Magnets, Fisher Scientific) before any other physicochemical analysis. The extracted ZVI powder was washed with distilled water, then desiccated at room temperature and weighed. The extracted powder was kept at -80℃ for 16S rRNA analysis. On average the amount of ZVI waste by this procedure resulted in a nominal ZVI retention time of 84 ± 1 days (15℃) and 88 ± 2 days (room temperature).

Biogas was collected using SupelTM-Inert Multi-Layer Foil Screw Cap Valve sampling bag (ThermoFisher Sci., Canada), and the volume was measured by a 200-mL syringe. The measurement was done when the gas bag reached 80% full. Because of the low gas production at 15℃, the gas measurement was conducted less frequently at this temperature (usually every 10 days) than at room temperature.

The experiment lasted 278 days and included two phases (i.e., time periods):120 days for Phase I and 178 days for Phase 2. In Phase I, all reactors were operated as single-stage anaerobic digesters. Four gram of microscale ZVI powder (99% Iron Powder, ACROS Organics, USA) were added to the 15℃ reactor and to one of the room-temperature reactors. The second room-temperature reactor was kept as a ZVI-free control. A second 15℃ control reactor was planned, but equipment failure led to the permanent loss of this reactor system.

In Phase II, the two ZVI-dosed 4-L reactors were changed to be two-stage digesters with the fermenter stage operated in 1-L bottles with 0.5-L working volume (Fig. 4.1), while the ZVI-free reactor remained to be single-stage (negative control with same operation as Phase I). The two bottle fermenters were shaken at 175 rpm to ensure complete mixing.

4.2.3 Offline measurements

Standard methods (APHA et al., 2012) were used to measure total suspended solids (TSS), volatile suspended solids (VSS) (Standard Method 2540G for both TSS and VSS) and soluble COD (sCOD, filtered through 0.45μm filter, Standard Method 5220 D) and these tests were conducted once per week. For the reactors dosed with ZVI, the iron mixed with sludge sample was removed by using a strong magnet to ensure the accuracy of the solids test. The total iron concentration was analyzed based on the Ferrozine method suggested by Lovley and Phillips (1986), and the absorbance was measured in 96-well assay plates with the SpectraMax microplate reader (Molecular Devices LLC, USA).

The biogas composition was analyzed using the Agilent® 7820 A gas chromatography system (Agilent Technologies, USA), which adopted the Agilent® PoraPLOT Q (25 m × 0.32 mm × 10 μm) capillary column (Agilent Technologies, USA) and a TCD detector. The carrier gas used was helium with a flow rate of 6.5 L/min. Gas samples were injected manually with a dose of 50 μL and a split ratio of 15:1. The total analysis time was 2.5 min.

VFAs were also measured with the Agilent® 7820 A gas chromatography system (Agilent Technologies, USA), with a different column CP7485 (Agilent Technologies, USA) and an FID detector. The carrier gas was helium, and the flow rate was 6.5 L/min. A G4513A auto-sampler (Agilent Technologies, USA) was exploited to inject VFA samples. The total analyzing time of each sample

was 17 min. This analysis was based on the method recommended by Falk et al. (2014). Samples were centrifuged for 10 min with 20×g using a microcentrifuge (Thermo Scientific™ Sorvall™ ST 8 small benchtop centrifuge, Thermo Fisher Scientific, USA), and the supernatant was collected and then acidified with phosphoric acid.

4.2.4 Statistical analysis

Two-way ANOVA was used to evaluate the significance of the effects of ZVI and staging on methane production, sCOD and VSS concentration using EXCEL (version 2107) Data Analysis tool (Zaiontz, 2014). The average values of each SRT in steady state of the same phase (≥ 2 SRTs) were adopted as time replicates in the ANOVA analysis. Given that a single reactor was operated per conditions, true independent replication was not possible. However, taking the average of an SRT as a measurement provides datapoints that are 20 days apart representing samples with approximate 65% turnover with respect to the bulk sludge. Considering the turnover and the variation over time in the feed, these datapoints are approaching independence. Furthermore, by taking the averages over an entire SRT, the degrees of freedom remain low and likely more realistic with respect to the true replication.

4.2.5 DNA extration,16S rRNA Sequencing and Bioinformatics

Biomass sample was collected from each reactor at the end of Phase II steady-state operation. The collected samples were centrifuged at 20×g for 10 minutes, and the solid pellet was stored at -80°C before DNA extraction.

DNA was extracted in duplicate from WAS sample, ZVI surface (i.e., magnetically recovered and washed ZVI powder), fermentate and digestate samples using DNeasy PowerSoil Kit (Qiagen, Germantown, MD, USA). Total extracted DNAs were quantified with Quant-iT PicoGreen dsDNA Reagent and SpectraMax Fluorescence Microplate Reader (Molecular Devices LLC, USA), following the device procedure (Molecular Devices, 2005). The bacterial and archaeal 16S rRNA genes were amplified through PCR amplification using 515F and 806R primer sets for 16S rRNA genes (Caporaso et al., 2012) following protocol developed by Hales et al. (1996). Length of amplicons were checked with 1% agarose gel electrophoresis (B2 Mini Gel Electrophoresis Systems, Thermo Scientific) after PCR amplification. Amplicons were sequenced on Illumina

MiSeq PE250 platform at McGill University and Genome Quebec Innovation center (Montreal, QC, Canada).

Sequencing data were analyzed as described in the study of Jiang et al. (2020). In short, DNA sequences were paired, trimmed and filtered with QIIME2 (v2020.8) (Bolyen et al. 2019) through the q2-demux plugin and then denoised with DATA2 (Callahan et al. 2016). Quality control was conducted through a secondary filtration with 98.7% Actual Sequence Variants (ASV) threshold. Taxonomic assignment was conducted with MiDAS 3 database (Dueholm et al., 2020; Nierychlo et al., 2020). Classification of read was performed at genus level when possible. The differentiation of growing and non-growing microorganisms was performed based on the ratio between their relative abundance in digester and in the feed according to the 16S rRNA gene amplicon data (Kirkegaard et al., 2017). This ratio was defined as 20 according to Jiang et al. (2020), who analyzed the data of 46 digesters from 22 wastewater treatment plants during 2011-2016. Furthermore, they verified the final ratio by comparison with the growth rate calculated based on mass balance. Therefore, the ratio of growing differentiation was also adopted as 20 in the current study, and ASVs with a ratio over 20 were determined as growing ones in the reactor (Fig. A4.1).

4.3 Results

4.3.1 Preliminary test on zero-valent iron recovery

A trial reactor operation was conducted before the main experiment. Sludge samples were taken at different depths of the digester and the ZVI mass at each depth was measured. It revealed that ZVI powder particles were not homogeneously suspended in the reactor, and it was difficult to remove them without a complex procedure involving completely opening the reactor. To determine the ZVI management procedure, two sets of data were considered. First, a series of ZVI recovery tests (Table 4.1) were conducted using parallel 1-L reactors containing 200 mL of pristine digestate dosed with 2 g/L of ZVI (i.e., the same concentration as the 4-L reactors). These reactors were purged with nitrogen to render them anaerobic, and they were incubated for 7 days. Three replicate experiments were performed under the conditions of 15°C and room temperature. After incubation, ZVI was recovered using a strong magnet and weighed after desiccation. These experiments showed that over 90% of ZVI could be retrieved after 7 days of reaction in the digesters.

These results indicated that the transformation of ZVI to non-magnetic form of iron (e.g., Fe^{2+} precipitates) in the digesters was limited, and that the ZVI in the digestate could be efficiently retrieved by magnetic separation.

The second set of data considered were the concentrations of Fe^{2+} (Fig A4.1) and ZVI in the digestate. On average, the iron concentrations measured in the digestate were 133±13 mg/L and 112±16 mg/L, respectively, for these two forms. Ferrous and ferric were detected existing in the feed WAS as well, thus the total iron concentration in digesters, which was regarded as ZVI corrosion, was considered conservative (overestimated). Because 2 g/L of ZVI had been initially added to the digester, it was concluded that most of the ZVI remained in the reactors untransformed. Therefore, to control the amount of ZVI in the reactor, it was elected to simply measure the concentration of magnetic ZVI recovered from the wasted digestate and to replace this amount by fresh ZVI. This procedure resulted in an average nominal ZVI retention time of 84 ± 1 days at 15°C and 88 ± 2 days at room temperature.

Table 4.1 Summary of Zero Valent Iron (ZVI) recovery tests

Temperature (°C)	ZVI dose (g/L)	Recovery [a] (%)
15	2	94.8±1.5
Room temperature	2	90.9±2.0

[a]± Standard deviation of three parallel experiments (n=3)

4.3.2 Pre-ozonation

All reactors in the current study were fed with ozonated WAS. The overall ozone dose was adjusted to 472±41 mg-O_3/L or 27±6 mg-O_3/g-VSSin (± standard deviation of 22 batches), which corresponded to a high WAS solubilization efficiency before the solubilization curves plateaued (Chapter 3, Fig. 3.2). After ozone pretreatment, the average sCOD concentration increased by 3,886±322 mg-sCOD/L, resulting in an average COD solubilization efficiency of 8.2±1.3 g-sCOD/g-O_3 (Table 4.2). No significant mineralization was observed.

Table 4.2 WAS characterization and pre-ozonation performance

Parameter	Phase I		Phase II	
	Fresh WAS[a]	After Ozonation[a]	Fresh WAS[b]	After Ozonation[b]
sCOD (mg/L)	1520±65	4160±203	1347±44	4950±646
pH	5.94-6.32	5.71-6.29	5.97-6.44	5.83-6.40
Total Suspended Solids (g/L)	15.5-29.3	13.3-27.4	23.5-28.4	22.2-28.3
Volatile Suspended Solids (% TSS)	75-77	75-78	68-76	69-79
Ozone dose (mg-O_3/g-VSSin)	29.4±10.6		27.3±6.2	
Ozone Solulibilization Efficiency (g-sCOD/g-O_3)	7.1±1.3		8.8±0.5	

[a]± Standard deviation of 10 batches of ozonation in Phase I
[b]± Standard deviation of 12 batches of ozonation in Phase II

4.3.3 Performance of the anaerobic digesters

The reactor runs were divided in two phases (i.e., two time periods) (Fig. A4.2 & Fig. A4.3). Throughout the experiment, three systems were operated either at 15°C (1 system) or room temperature (2 systems). The system at 15°C and the one at room temperature (i.e., test system) received ZVI. In Phase I (120 days), all systems were operated as single-stage reactors, whereas in Phase II (178 days) the systems receiving ZVI were operated as two-stage reactors with ZVI addition to the methanogenic reactor; the operation of control room-temperature reactor remained the same during the entire experiment.

In Phase I, the VSS destruction was between 31% and 35% among all systems, and differences were not statistically significant ($p>0.05$; Table 4.3). However, the ZVI-dosed systems at room temperature showed significant ($p<0.05$) increase in specific methane production by 24% over the ZVI-free group. This increase in methane production was accompanied by a significant ($p<0.05$) increase in the proportion of methane in the biogas from 65% to 75% (Table 4.3). Unexpectedly, ZVI addition did not significantly ($p>0.05$) change sCOD concentrations. Similarly, the average VFA concentration was not significantly affected by ZVI addition ($p>0.05$), but it was in agreement with the increase in methane production (Table 4.3). The ZVI addition did not appear to modify the composition of the VFA (Fig. 4.3 and Fig A4.2). Finally, in the iron-dosed 15°C reactor, the average sCOD concentration was 78% higher, and the specific methane production (volume

per VSSin) was 50% lower than those in the ZVI-dosed room temperature system. Altogether, these observations indicate that the lower temperature exhibites a kinetic limitation in the conversion of sCOD to methane, but not in the VSS destruction.

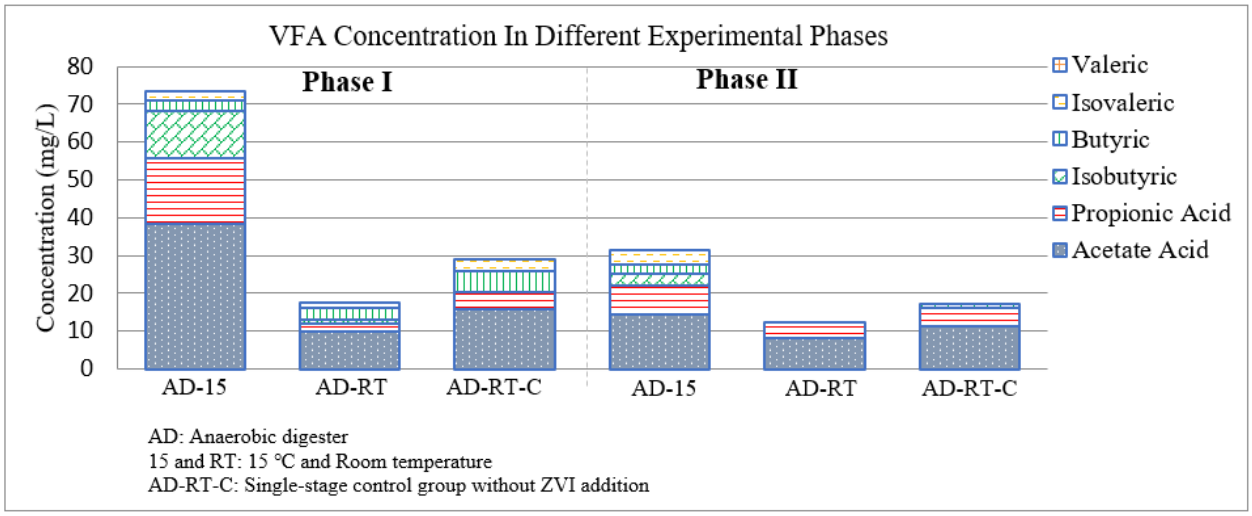

Fig. 4.3 Comparison of the VFA concentration with and without ZVI addition at 15°C and room temperature in two experimental phases

Table 4.3 Summary of anaerobic digester performances in steady state

Parameter	15°C Reactor (ZVI)		Room Temperature Reactor (ZVI)		Room Temperature Reactor (ZVI-free control)	
Experimental Phase	Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
Operation Temperature (°C)	15.0±0.0[a]	15.0±0.0	22.5±0.1	22.5±0.2	22.5±0.2	22.7±0.3
pH in fermentation reactor	N/A	6.03±0.09	N/A	6.01±0.08	N/A	N/A
pH in methanogenetic reactor	7.20±0.18	7.13±0.12	7.23±0.13	7.15±0.08	7.22±0.13	7.16±0.14
Volatile Suspended Solids (g/L)	12±1	11±1	12±1	10±1	12±1	10±1
Total Suspended Solids (g/L)	17±2	16±2	17±1	15±1	18±2	15±2
sCOD (mg/L)	1603±205	1819±201	899±63	958±81	829±180	1044±59
VSS Destruction (%)	32±9	39±10	35±6	41±6	31±6	39±9
CH_4%	71±3	74±4	74±5	75±3	65±2	69±1
Specific Methane Production (mL CH_4/g-VSSin)	123±20	128±23	245±52	279±41	198±33	204±29
Methane Production (mL CH_4/g-VSSdestroyed)	364±32	313±52	644±4	715±100	609±5	517±34
VFAs (%sCOD)	4.3±0.1	1.7±0.1	1.3±0.0	1.3±0.0	2.4±0.1	1.7±0.0
Total Iron Concentration (mg-Fe^{2+}/g-VSS)	13.7±2.2	10.7±0.6	13.3±2.4	8.3±0.2	N/A	N/A

[a]± Standard deviation of the average of measured data in each SRT in steady state for each reactor (n⩾2SRT)

Table 4.4 Summary of two-stage anaerobic digester performances with different stage SRTs in Phase II

Parameter	15℃ Reactor (ZVI)		Room Temperature Reactor (ZVI)		Room Temperature Reactor (ZVI-free control)
SRT of the fermentation reactor (day)	4	6	4	6	N/A
SRT of the methanogenetic reactor (day)	16	14	16	14	
Operation Temperature (℃)	15.0±0.0[a]	15.0±0.0	22.5±0.3	22.5±0.3	22.7±0.3
pH in fermentation reactor	6.03±0.09	6.07±0.05	6.01±0.08	6.18±0.05	N/A
pH in methanogenetic reactor	7.13±0.12	7.07±0.08	7.15±0.08	7.11±0.10	7.16±0.09
Volatile Suspended Solids (g/L)	11±1	11±1	10±1	12±1	10±1
sCOD (mg/L)	1819±201	1727±132	958±81	837±127	773±113
VSS Destruction (%)	39±10	37±9	41±6	40±8	39±9
CH_4%	74±2	70±5	75±5	75±3	65±2
Specific Methane Production (mL CH_4/g-VSSin)	128±23	133±42	279±41	233±55	204±29
Methane Production (mL CH_4/g-VSSdestroyed)	313±52	305±52	715±100	519±100	517±34
VFAs (%sCOD)	1.7±0.1	4.6±0.1	1.3±0.0	1.3±0.0	1.7±0.0
Total Iron Concentration (mg-Fe^{2+}/g-VSS)	10.7±0.6	11.3±0.4	8.3±0.2	7.4±0.4	N/A

[a]± Standard deviation of the average of measured data in each SRT in steady state for each reactor (n⩾2SRT)

In Phase II, the two ZVI-dosed reactors (15℃ and room temperature) were changed to two-stage processes. The SRT of the fermentation reactors was initially set as 4 days while the second-stage methanogenesis reactor SRT was 16 days, and the operation time was 80 days. The pH of both fermentation reactors was around 6. However, the optimal pH for the process is recommended to be 4.5-5.5 to achieve a better methane yield (Tapia-Venegas et al., 2013). Therefore, the fermentation SRT was then extended to 6 days, and operated for 78 days. While the pH did not change with the longer fermentation SRT, the specific methane production decreased from 279 mL-CH_4/g-VSSin to 233 mL-CH_4/g-VSSin at room temperature (Table 4.4). Thus, the first steady state with a 4-day/16-day SRT was adopted as the overall Phase II performance (Table 4.3). When comparing the two-stage digesters to themselves in Phase I (single-stage phase), the specific methane production increased by 5% at 15℃ and 14% at room temperature. However, little change was observed in methane proportion, VFA and iron concentration after applying the two-stage process. The 15℃ reactor with ZVI had 90% and 74% higher sCOD than the ones at room temperature, with 56% and 40% less specific methane production per VSSin, while the difference of VSS destruction and dissolved iron concentrations were not significant. VFA concentrations were low inside all the digesters (Fig. 4.3). At room temperature, the methane fraction of ZVI-dosed two-stage reactor (74%) was 14% higher than the control group (65%), with the specific methane production increased by 37%

4.3.4 16S rRNA Sequencing

The potential mechanisms of improved methane production caused by ZVI was also investigated by analyzing the microbial community composition. Differentiation could be found among feed WAS, fermenters, methanogenic reactors and ZVI surface, with genera often being dominant in only one compartment. According to the ratio-based method described in 4.2.5, a bimodal distribution of the ratios (Fig. A4.1) could be observed, indicating those microbial groups with high ratios were significantly enriched in the digesters compared to the influent streams. Therefore, genera with a ratio over 20 was determined as the growing microbial group in the reactor. Specific enrichment of genera was found on the surface of ZVI, including *Methanothrix* (Domain *Archaea*, main methanogen population detected), *Acetobacterium* (phylum *Firmicutes*, family *Eubacteriaceae*) and midas_g_3337 (phylum *f_Bacteroidetes*, family vadinHA17). Conversely, some genera were mainly in the bulk of the digestate, including *DMER64* (phylum *Bacteroidetes*, family *Rikenellaceae*), *LNR_A2-18* (phylum *Cloacimonadota*, family *Cloacimonadaceae*) and *Christensenellaceae_R-7_group* (phylum *Firmicutes*, family *Christensenellaceae*) (Fig. 4.4).

According to the results of 16S rRNA amplicon analysis (Fig. 4.4), *Rikenellaceae DMER64* was abundant in all digesters, and was the dominant bacteria in two ZVI-dosed reactors, with the relative abundance of 37.5% at 15℃, 35.7% in room-temperature test group and 20.7% in the room-temperature control group. Its concentration in ZVI-dosed reactors increased by 17% (at 15℃) and 15% (at room temperature) compared to the room-temperature control group. *Methanothrix* was the dominant archaea on the ZVI surfaces, with the relative abundance of 13.3% (15℃) and 18.7% (room temperature), respectively. Furthermore, the specific reads of *Methanothrix* on ZVI surface were 40 (15℃) and 20 (room temperature) times more than those in the corresponding bulk digestate. This enrichment of *Methanothrix* on ZVI is consistent with the enhanced conversion from VSS to methane in ZVI-dosed reactors, and could suggest two potential underlying mechanisms: longer SRT of ZVI powder favoring microbial growth and direct interspecies electron transfer (DIET).

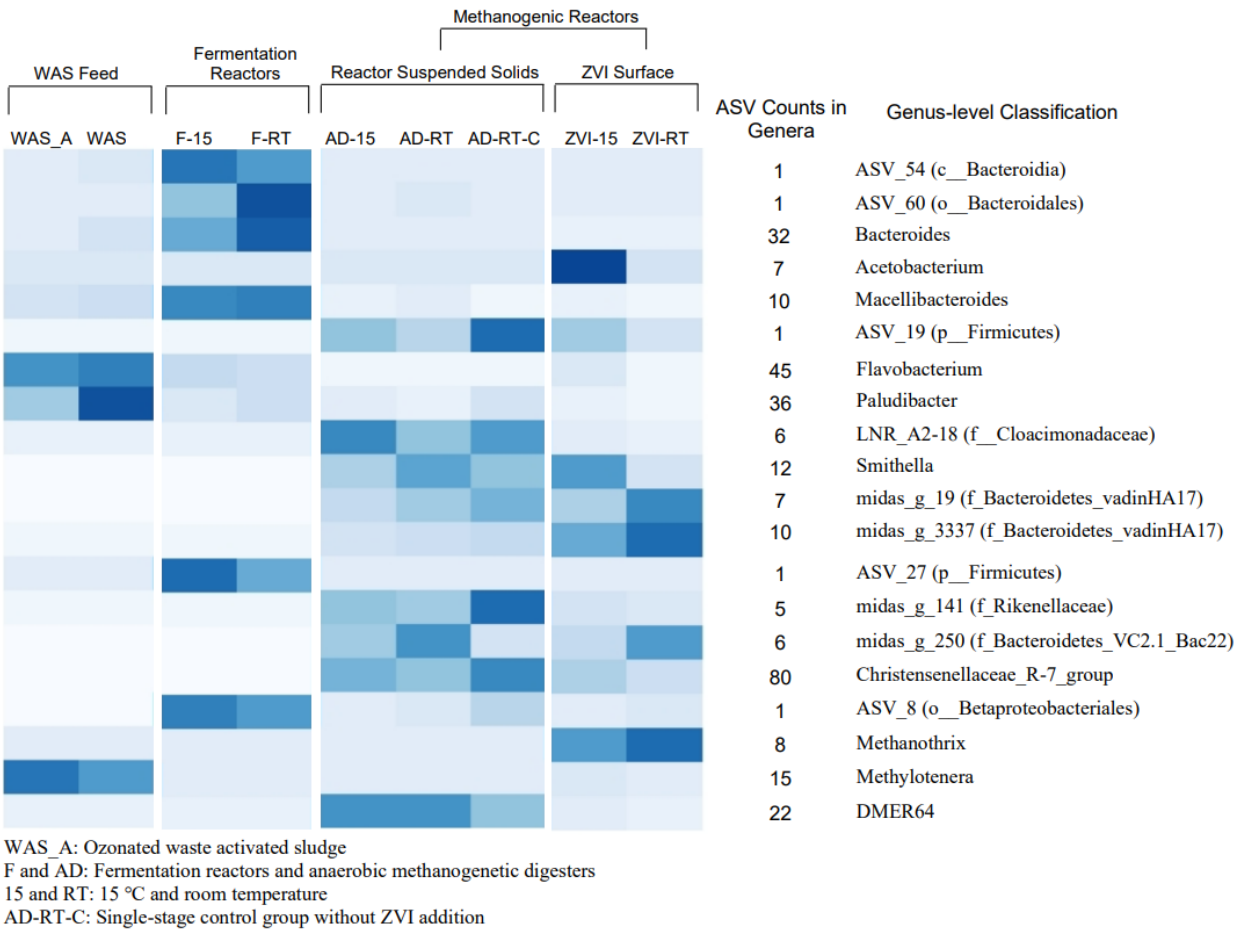

Fig. 4.4 Average heatmap (n=2) of the top 20 most abundant genera at the end of Phase II. Two steady-state samples were collected in different SRTs (sampled on day 249 and 270), and the standard deviation of these genera readings were less than 20% between the time replicates for all the samples. ASV (Actual Sequence Variants) are sequences observed in the current reactors, but not reported in the MIDAS or Genebank databases. MIDAS designation are ASVs observed only with molecular approaches and grouped together as a genus in the MIDAS database.

4.4 Discussion

4.4.1 Improvement of methane production by zero-valent iron addition

Methane production was increased by 24% by ZVI addition in Phase I, while the differences in VSS destruction were not found to be significant ($P>0.05$). In addition, the concentrations of VFA were lower in the room temperature methanogenic reactors receiving ZVI. All these results implied that the efficiency of converting hydrolyzed COD to methane was improved by ZVI addition. The observations remained similar in Phase II, when the systems receiving ZVI were transformed to two-stage systems.

The increase in methane production rate reported here is consistent with previous studies. Hao et al. (2017) found a 21% increase in CH_4 production upon the addition of 10 g/L waste iron scraps in two-phase AD process. Their results suggested that iron mainly enhanced hydrolytic enzyme activities and improved the electron transport rate. Low VFA residue indicated high substrate consumption rates of down-stream methanogens for both reactors. Moreover, higher sCOD residue represented less efficient fermentation ability.

In order to support the long-term development of the technology, it is important to understand the mechanisms responsible for the increase in methane production upon the addition of ZVI. One mechanism was explained by some researchers as associated with higher H_2 generation, which was the electron donor for hydrogenotrophic methanogenesis (Wang et al., 2013; Kim et al., 2013; Luo & Angelidaki, 2012). Wei et al. (2018) summarized studies regarding iron-enhanced hydrogen evolution and suggested significant anaerobic iron corrosion stoichiometry as presented in reaction (4-2) and (4-3). Moreover, ZVI could also serve as direct electron donor to enhance methane yield through hydrogenotrophic methanogenesis (Feng et al., 2014). The reactions can be described as:

ZVI directly serving as electron donor: $CO_2 + 4Fe^0 + 8H^+ \rightarrow CH_4 + 4Fe^{2+} + 2H_2O$ eq. 4-1

Anaerobic iron corrosion: $Fe + 2H_2O \rightarrow Fe(OH)_2 + H_2$ eq. 4-2

$3Fe(OH)_2 \rightarrow Fe_3O_4 + H_2 + 2H_2O$ eq. 4-3

Hydrogenotrophic methanogenesis: $CO_2+4H_2 \rightarrow CH_4+2H_2O$ eq. 4-4

To evaluate the importance of ZVI oxidation in the increase of methane production, the total iron concentration (as Fe^{2+}) in the digestate was measured after the magnetic removal of ZVI from the sample. For the room-temperature digester receiving ZVI during Phase I, the average total Fe^{2+} concentration was 13.3 ± 2.4 mg-Fe^{2+}/g-VSS or 161.1 ± 26.7 mg-Fe^{2+}/L (Table 4.3 and Fig. A4.2), representing a molar flux of 0.21 mmol/d. If all the Fe^{2+} was produced by ZVI oxidation (eq. 4-1), the maximum methane produced by this kinetic would have been 1.19 mL/d. If all the Fe^{2+} was oxidized through stoichiometry of anaerobic iron corrosion, the hydrogen evolution could be calculated through reactions (eq. 4-2) and (eq. 4-3), which would have resulted in a methane increase of 0.38 mL/d according to reaction (eq. 4-4). Both results were small compared to the daily methane production increment of 79 mL/d. This low Fe^{2+} concentration was consistent with the low dissolution of ZVI powder shown in the recovery test (section 4.3.1). Therefore, ZVI oxidation does not appear to be the leading cause of enhanced methane yield.

Beyond oxidation of ZVI that could lead to the formation of methane, ZVI particles could impact the activity of microorganisms present in the digesters. Consequently, a second mechanism that could explain the increased methane production is that the long SRT of ZVI in the reactor favored the growth and retention of methanogenic populations by forming biofilms on the surface of the particles. This is supported by the enrichment of genus *Methanotrix* on ZVI surfaces at both temperatures (Fig. 4.4). *Mehanothrix spp.* were reported to be able to produce biomethane through both hydrogenotrophic and aceticlastic pathways (Liu et al., 2019). This is in concordance with the enhanced conversion from organic solids to methane. Genus *Acetobacterium* was also detected growing on ZVI surfaces, which is known to perform fermentation and only produce acetate in AD reactors (Bengelsdorf et al., 2018). *Acetobacterium* could grow by using H_2 + CO_2 as well as organic compounds (i.e., fructose, glucose, lactate, glycerate, formate, and O-methylated aromatic compounds) as substrate (Bengelsdorf et al., 2018). The hydrogen gas produced on ZVI surface could have provided substrate for this bacterial genus and thus encouraged its enrichment. Additionally, the long SRT is also a better niche for these organisms to proliferate. The population of midas_g_3337 is another enriched microbial group on ZVI surface. While information regarding this specific bacterial population is limited in literature, it is a member in bacterial family lineage vadinHA17 within the phylum *Bacteroidetes*. Mei et al. (2020) pointed out that this family lineage

was related to protein hydrolyzing and amino acid degradation. However, no evidence could be found regarding *Acetobacterium* and midas_g_3337 involvement in electroactive direct interspecies electron transfer. Consequently, the exact metabolic pathways operating on the surface of ZVI remain to be probed more deeply. More research needs to be conducted to investigate the meaning of the enrichment of *Acetobacterium* and midas_g_3337 on ZVI surface.

Beyond enrichment of some populations in biofilm on ZVI surfaces, ZVI may have changed the bulk microbial community also. The population DMER64 (family *Rikenellaceae*) grew in the bulk digestate. Its abundance in ZVI-dosed reactors was 15% to 17% higher than in the ZVI-free control reactor (Fig. 4.4). It is an active fermentative anaerobic microorganism widely existing in AD systems, which is reported to have potential in establishing magnetite-mediated direct electron transfer with methanogens (Lee et al., 2019). This finding could support the role of ZVI in enhancing direct interspecies electron transfer in these reactors.

4.4.2 Effect of two-stage process on anaerobic digestion

Comparing the two-stage systems in Phase II to the same systems operated as single stage in Phase I, the specific methane production was found to increase by 5% and 14% at 15℃ and room temperature, respectively. The same comparison for the ZVI-free room-temperature system (control) that was kept as single-stage in Phases I and II revealed an increase of 3% in specific methane production (from 198 mL-CH_4/g-VSSin to 204 mL-CH_4/g-VSSin). The two-stage system showed limited improvement in terms of methane content, VSS destruction and reducing propionic acid fermentation. Therefore, the conversion from one-stage to two-stage reactors only produced a modest but significant increase ($P<0.05$) in specific methane production.

This lack of improvement in the overall performance of two-stage system may have a few explanations in the context of the current study. It is possible that the high pH in the fermentation reactors (around pH 6) was not as optimal as the typical values between 4.5-5.5 (Tapia-Venegas et al., 2013). The exact reason for this situation remains unknown, but it could be affected by low degradability of the WAS. Lindner et al. (2015) reported that substrates with high organic loads, such as sugar beet substrate, could lead to lower pH value in the fermentation reactor and result in six-time higher hydrogen production with more methane yield. This points out the importance of

hydrogen concentration measurement in the headspace of the fermentation reactors, which should be included in future studies. It is possible that a certain amount of sCOD could have been transformed to hydrogen instead of methane. However, this situation does not account for the similar VSS destruction observed in Phase I and Phase II.

4.5 Conclusion

This study investigated the effects of ZVI addition and two-stage process on anaerobic digestion receiving pre-ozonated WAS and operated at temperatures lower than the typical 30-35°C. ZVI addition to single-stage AD was shown to enhance conversion of destroyed VSS to methane. A possible mechanism behind this enhancement is the enrichment of methanogenic biomass (genus *Methanothrix*) on the surface of ZVI powder. The much higher SRT of ZVI than the bulk of the digestate likely provided a favorable environment for biofilm formation and retention of the important populations. Furthermore, ZVI could have the potential to encourage the growth of certain microorganisms (*Rikenellaceae DMER64*) through promoting the interspecies electron transfer. However, the observed syntrophic populations (genus *Acetobacter*) and genus midas_g_337 enriched on the surfaces of ZVI particles were not reported to participate in direct interspecies electron transfer. More work is necessary to fully explain the improved conversion of destroyed VSS to methane. The two-stage AD system configuration showed a modest improvement in specific methane production over the single-stage, but no additional enhancement was observed on VSS destruction.

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Chapter 5 Conclusion

This study was conducted to investigate the effects of ZVI additive and two-stage process on anaerobic digestion receiving pre-ozonated WAS in low-temperature conditions. ZVI powder showed great potential in increasing biomethane production (increased by 24%) and also increased the methane content from 65% to 75% at room temperature. The enhanced conversion efficiency of destroyed VSS to methane was the main factor resulted in the improvement of methane production, because the VSS destruction rates showed limited increase with ZVI addition. Mechanism behind the improvements in efficiency of conversion to methane remained to be fully elucidated, but analysis was conducted suggesting that iron oxidation was not the main cause. However, ZVI could potentially benefit the biofilm growth on its surface and some bacteria growth in the digestate, thus increasing the overall microbial activity. More data needed to be collected to explore the effects of ZVI on VFA production, microbial development and enzymatic activities. The two-stage process with ZVI showed some improvement in biogas production compared to the results of single-stage operation. However, the improvement in methane content was limited. This lack of efficiency was at least partially caused by the relatively high pH environment in the acidification reactors compared to the operation conditions typically reported in literatures, but detailed reasons remain unknown because of the lack of data regarding hydrogen concentration and WAS characterization. An experiment was also planned to test the effects of ozonating return digestate and fermentate in two-stage systems, but not completed due to the COVID-19 pandemic and the related restrictions to laboratory access. Nevertheless, it was suggested to investigate the effects of ozonation on returned digestate without ZVI in future two-stage AD research, because the preliminary solubilization tests showed negative effects of ZVI and low pH (without ZVI) on WAS ozonation. Finally, this thesis documented the potentials and challenges of applying ZVI-dosed two-stage anaerobic digestion to treat pre-ozonated WAS. It is crucial to continue the study and solve the engineering problems of standardizing ozonation and ZVI applications.

Appendix

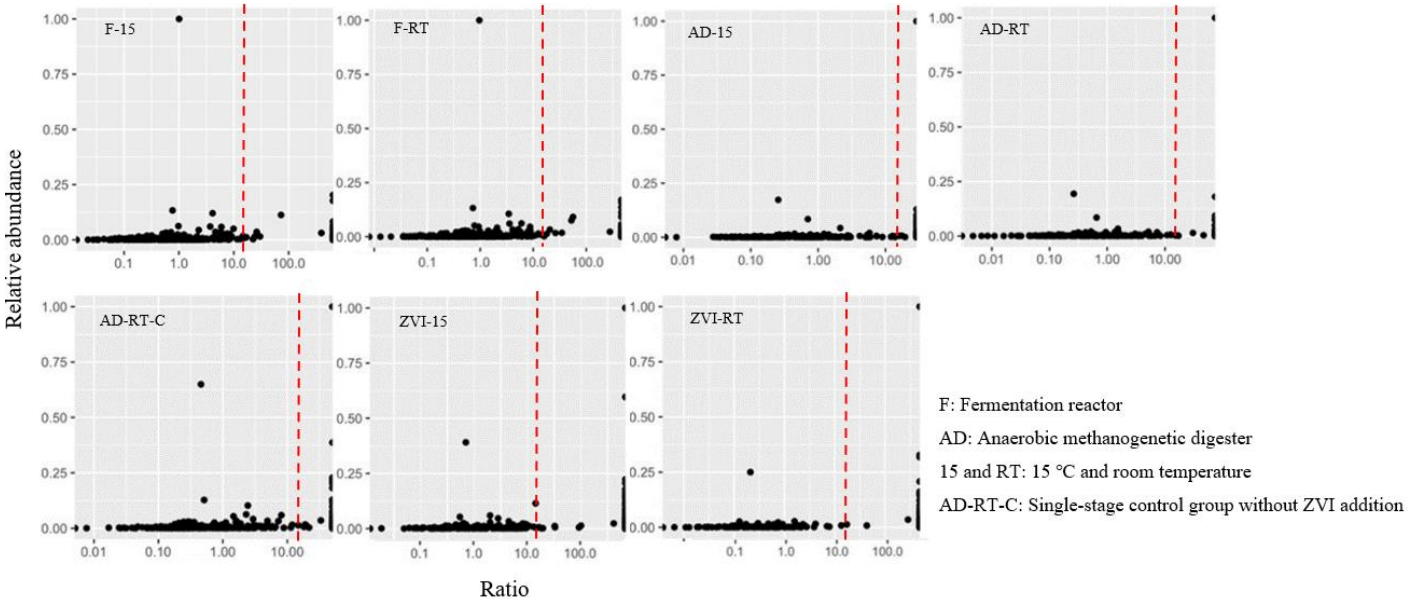

Fig. A4.1 Relative abundance vs. ratios for growing/non-growing ASV differentiation. The data points located at the graph boundary (bottom-right) had an infinite classification ratio, since their relative abundance in the influent stream was 0.

Temporal profiles of anaerobic digesters

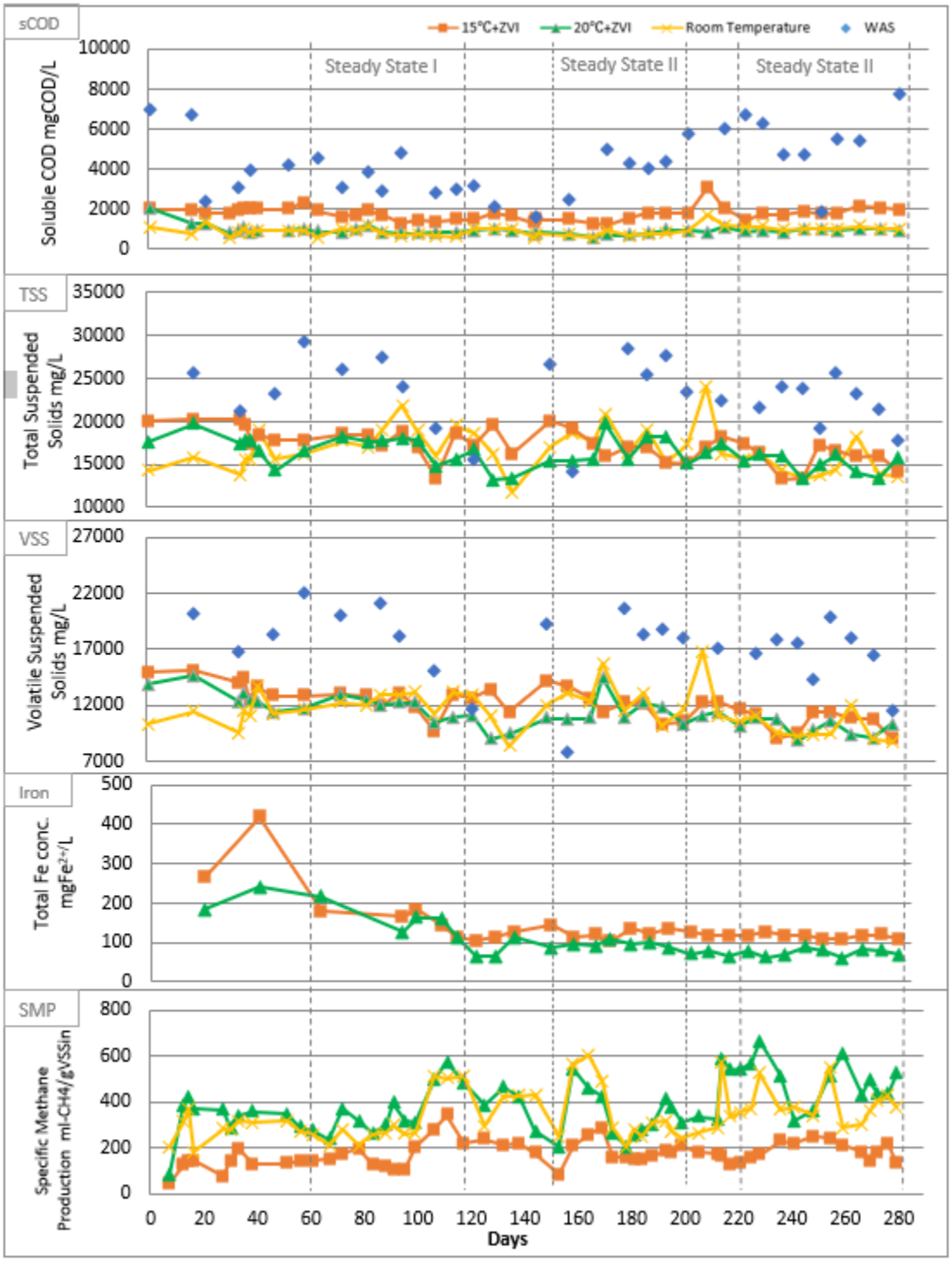

Fig. A4.2 Temporal profile of reactors. Phase I (single stage) ranges from day 1 to 120 and Phase II (two stage) ranges from day 121 to 280. The steady state of Phase I started from day 58 to 120, and the two of Phase II steady states started from 150 to 200 and 221 to 278.

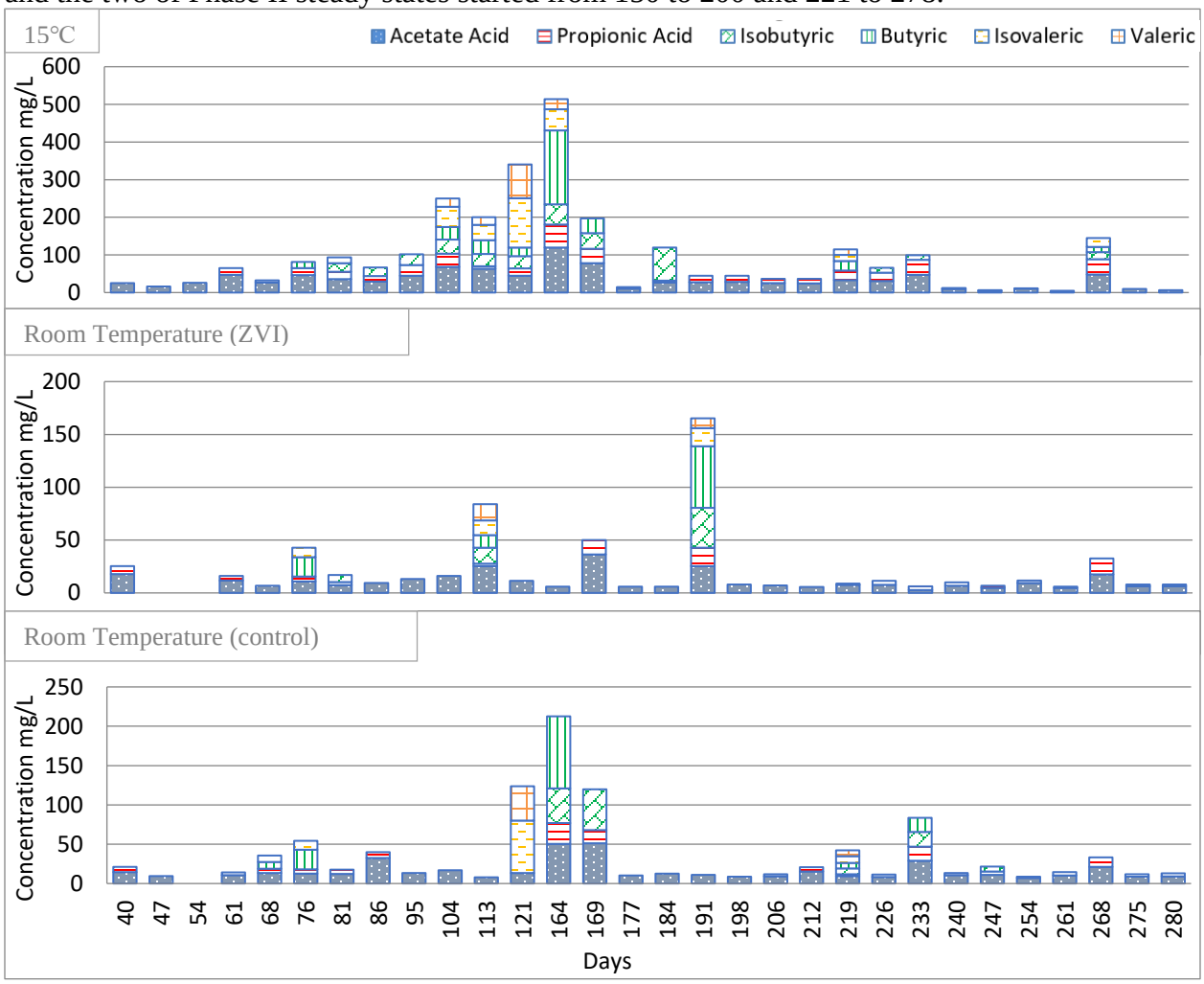

Fig. A4.3 Temporal profile of VFAs in different anaerobic digesters. Phase I (single stage) ranges from day 1 to 120 and Phase II (two stage) ranges from day 121 to 320. The steady state of Phase I started from day 58 to 120, and the two of Phase II steady states started from 150 to 200 and 221 to 278.

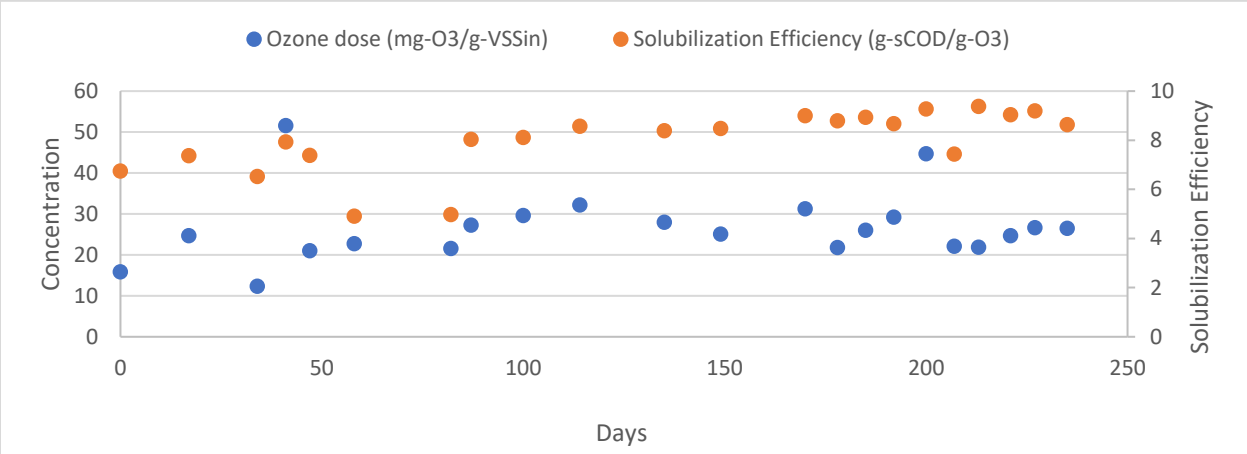

Fig. A4.4 Performance of WAS pre-ozonation during the experiment