Enhanced Biogas Production from Nanoscale Zero Valent Iron-Amended Anaerobic Bioreactors

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Abstract

Addition of nanoscale zero valent iron (NZVI) to anaerobic batch reactors to enhance methanogenic activity is described. Two NZVI systems were tested: a commercially available NZVI (cNZVI) slurry and a freshly synthesized NZVI (sNZVI) suspension that was prepared immediately before addition to the reactors. In both systems, the addition of NZVI increased pH and decreased oxidation/reduction potential compared with unamended control reactors. Biodegradation of a model brewery wastewater was enhanced as indicated by an increase in chemical oxygen demand removal with both sNZVI and cNZVI amendments at all concentrations tested (1.25–5.0 g Fe/L). Methane production increased for all NZVI-amended bioreactors, with a maximum increase of 28% achieved on the addition of 2.5 and 5.0 g/L cNZVI. Addition of bulk zero-valent iron resulted in only a 5% increase in methane, indicating the advantage of using the nanoscale particles. NZVI amendments further improved produced biogas by decreasing the amount of CO₂ released from the bioreactor by approximately 58%. Overall, addition of cNZVI proved more beneficial than the sNZVI at equal iron concentrations, due to decreased colloidal stability and larger effective particle size of sNZVI. Although some have reported cytotoxicity of NZVI to anaerobic microorganisms, work presented here suggests that NZVI of a certain particle size and reactivity can serve as an amendment to anaerobic digesters to enhance degradation and increase the value of the produced biogas, yielding a more energy-efficient anaerobic method for wastewater treatment.

Key words: anaerobic bioreactor; anaerobic digestion; bioenergy; methanogenesis; nanoscale zero valent iron

Introduction

ANAEROBIC WASTEWATER TREATMENT offers many advantages compared with aerobic treatment, including decreased sludge production, lower energy requirements, and increased degradation of organic compounds (Batstone and Virdis, 2014). Moreover, the methane produced from anaerobic digestion may be used as an energy source for other processes. Anaerobic digestion, therefore, holds great promise as a sustainable technology for wastewater treatment and water reuse (Batstone and Virdis, 2014; Shah *et al.*, 2015).

Methanogenesis constitutes the final stage of a multistep sequence of microbial processes that occur during anaerobic digestion. Methanogens are typically characterized by slow metabolism and high vulnerability to disturbances (e.g., temperature and pH) in the system (Rajeshwari *et al.*, 2000). As a consequence of the slow growth rates of anaerobes, long residence times are required to achieve degradation. Thus, there is great interest in developing methods to boost the

methanogenic activity within anaerobic bioreactors through both biological and chemical amendments (Pobeheim *et al.*, 2010; Zhang *et al.*, 2011a; Park *et al.*, 2014; Teo and Wong, 2014). In particular, zero-valent iron (ZVI) has been observed to improve conditions for anaerobic degradation. For example, the addition of bulk ZVI (i.e., waste scrap metal) to an upflow anaerobic sludge blanket reactor resulted in a 21% increase in chemical oxygen demand (COD) removal and a 17% increase in methane production (Zhang *et al.*, 2011b). These observations were attributed to ZVI acting as an electron donor to decrease oxidation/reduction potential (ORP) and as a buffer to control pH through reduction of protons to hydrogen gas. Iron as an amendment is attractive due to its low cost and inert byproducts. Subsequent work showed that applying an electric field to the same ZVI-amended anaerobic reactor enhanced COD removal by 30% and increased the abundance of methanogens (Liu *et al.*, 2011). The applied voltage served to accelerate the oxidation rate of Fe⁰ to Fe²⁺, which, in turn, more effectively maintained optimal ORP and pH levels in the reactors.

While advantageous, the aforementioned method necessitated an additional energy requirement for achieving enhanced AnBR efficiency. The reactivity of ZVI instead can be greatly enhanced by reducing it to the nanoscale. Indeed, the high

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reductive capacity of nanoscale zero-valent iron (NZVI) has been exploited for its use in environmental remediation and treatment of wastewaters containing a wide variety of contaminants, including halogenated compounds, dyes, and toxic metals (Yan *et al.*, 2013). Some reports have shown toxicity of NZVI primarily to pure cultures (Auffan *et al.*, 2008; Lee *et al.*, 2008; Diao and Yao, 2009; Kim *et al.*, 2010). Generally, mechanisms of toxicity are oxidative stress induced by oxygenation of the reduced Fe species, disruption of membrane integrity, and disturbance of electron and ion transport channels after adhesion of the iron particles to the bacterial cell wall. The myriad possible redox mechanisms involving iron-based particles and their role in biotoxicity are outlined by Haohao *et al.* (2014). However, experiments in more complex mixed communities show low to no toxicity, although an influence on activity is observed (Diao and Yao, 2009; Xiu *et al.*, 2010; Fajardo *et al.*, 2012; Němeček *et al.*, 2014). A decreased direct interaction between NZVI and the microorganisms is likely the reason for the large discrepancies. While NZVI has been employed as a method to degrade complex contaminants in toxic wastewaters, few reports have investigated NZVI's ability to promote biodegradation by improving conditions for methanogenesis. One report investigating the impact of NZVI on a dechlorinating mixed culture observed an increase in methane production as a result of methanogens outcompeting the dechlorinating bacteria for produced H_2 (Xiu *et al.*, 2010). In contrast, Yang *et al.* (2013) found that the addition of freshly synthesized NZVI (sNZVI) negatively affected methanogenic activity due to the rapid over-production of H_2 (Yang *et al.*, 2013).

In the studies presented here, we provide evidence that NZVI can be used to increase overall efficiency of anaerobic batch reactors by enhancing degradation rates and increasing methane production. Two NZVI systems, including commercially available and freshly synthesized particles, were tested over a range of iron concentrations to determine optimal iron loading and particle size/reactivity. Effects on reactor chemistry (i.e., pH and ORP) were monitored, and anaerobic degradation was evaluated by monitoring COD removal and biogas production compared with unamended control reactors.

Experimental Protocol

Materials

Commercially available NZVI (cNZVI) was Nanofer 25, a slurry at 18 wt% Fe (NanoIron, s.r.o., Rajhrad, Czech Republic). To ensure maximum reactivity, cNZVI was used immediately after received. Bulk ZVI were iron filings purchased from Dowling Magnets (Elmhurst, IL). The iron filings were received as a solid stored in air. Ferrous sulfate heptahydrate was received from Fisher Scientific (Fairlawn, NJ). Sodium borohydride and HEPES were received from Sigma Aldrich (St. Louis, MO). COD digestion vials, high range, were received from Hach (Loveland, OH). All water was purified using a Barnstead NANOpure Diamond to a resistivity of $18.2 \text{ M}\Omega \cdot \text{cm}$ and autoclaved before use.

NZVI particles

All solutions were deoxygenated by autoclaving, then cooled to room temperature under a stream of nitrogen. This

method was proved effective at producing anoxic solutions by using resazurin as an indicator. All reactors and solutions were prepared in a controlled nitrogen atmosphere to preserve NZVI reactivity. HEPES buffer (50 mM, pH 7.0) was used to maintain a pH range that was amenable to methanogenesis without binding free iron. cNZVI suspensions were prepared by adding the appropriate amount of stock Nanofer 25 to HEPES buffer to make a total volume of 62.5 mL at concentrations of 1.25, 2.5, or 5.0 g Fe/L. A stock of sNZVI was prepared immediately before use by adding 100 mL of a 0.28 mM NaBH_4 solution drop-wise to 100 mL of a 0.18 mM FeSO_4 solution while mixing vigorously. After particle formation, 1.19 g of HEPES solid was added, and the pH was adjusted to 7.0. Aliquots (62.5 mL) of this stock sNZVI suspension were then added to the anaerobic batch reactors to achieve a final concentration of 1.25 g Fe/L without further processing or clean-up. Particles were imaged on an FEI Tecnai G² Twin transmission electron microscope (TEM). Brunauer-Emmett-Teller (BET) surface area was performed on lyophilized samples using a Beckman Coulter SA3100 Surface Area and a Pore Size Analyzer.

Anaerobic batch reactor set-up

Biomass used in these experiments was obtained from a pilot-scale anaerobic membrane bioreactor that was used to treat brewery wastewater. The biomass was blended to homogenize and diluted by half to reach a total suspended solids of 18.38 mg/L. The model feed (i.e., wastewater) was prepared to mimic the brewery wastewater to which the biomass was acclimated. The aqueous solution (500 mL) consisted of beer (218.75 mL), glucose (2.9 g), whey protein powder (2.7 g), vegetable oil (1.3 mL), and micronutrients (0.35 mL of a 100 $\times$ diluted stock of Metsource AN; River Bend Labs, St. Charles, MO). Batch reactors were 500 mL serum bottles containing 125 mL biomass, 12.5 mL feed, and 62.5 mL cNZVI or sNZVI suspension at appropriate concentrations prepared as described earlier. Unamended control reactors (i.e., 0 g/L) contained 125 mL biomass, 12.5 mL feed, and 62.5 mL HEPES buffer without particles. All reactors were prepared in an anaerobic chamber (Coy Labs, Grass Lake, MI) with an atmosphere composed of 5% CO_2 , 5% H_2 , and 90% N_2 , sealed with a butyl rubber septum and crimp cap, and incubated at 30°C with shaking. Each reactor was run in triplicate.

Sampling and measurements

A pressure gauge (Parr Instrument Company, Moline, IL) outfitted with an adapter and needle was used to measure pressure changes within the batch reactor through the septa on day 1, 3, 7, and 10. After the pressure readings, the pressure in the reactor was relieved to 1 atm. Samples of the headspace were then taken using a gas-tight syringe, and composition was determined on an SRI 8610C GC (SRI Instruments, Torrance, CA) with a thermal conductivity detector (TCD) (valve temp = 65°C, detector temp = 160°C, TCD cell = 150°C, oven = 80°C, carrier gas = He). Standard curves were constructed using pure H_2 , CH_4 , and CO_2 (balance N_2) to calibrate the peak areas to percent composition. The volume of H_2 , CH_4 , and CO_2 produced from each bioreactor was then calculated from the concentrations

measured by gas chromatography (GC) and the total pressure readings of the bioreactors using Boyle's gas law:

$$P_1 V_1 = P_2 V_2$$

where P_1 is the measured pressure in the sealed reactor before release, V_1 is the calculated volume of biogas produced from the bioreactor, P_2 is the pressure after release (i.e., 1 atm), and V_2 is the measured volume of gas in the headspace at 1 atm as determined from the GC measurement and standard curves.

Aliquots (8 mL) were removed from the reactors on days 0, 1, 3, 7, and 10 for the remaining analyses. ORP and pH were measured under anaerobic conditions using an Accumet Excel XL20 conductivity and pH meter (Fisher Scientific). COD concentrations were determined using the reactor digestion method (Hach Method 8000). Solids were removed before COD measurements by centrifugation (15872 *ref*, 5 min).

NZVI in saturated CO₂ solutions

Aqueous solutions of saturated with CO₂ (Perrier, Nestlé Waters, France) were used immediately on opening at room temperature. NZVI suspensions (5 mL) in nanopure water were added to 10 mL CO_{2(aq)} with particle concentrations of 1.25 g/L (cNZVI and sNZVI) and 2.5 g/L (cNZVI). A blank was prepared by adding pure nanopure water in place of the NZVI suspension. The serum bottles were sealed anaerobically with septum and crimp cap and incubated at 30°C with shaking. After 7 days, the amount and composition of the gas in the headspace was measured as described earlier. The dark gray solids were collected by centrifugation, washed with nanopure water and ethanol, and dried under vacuum overnight. X-ray photoelectron spectroscopy (XPS) was performed on the dried samples using a Kratos Analytical Axis Ultra with a monochromatic Al K α X-ray source (150 W). The pass energy was set to 20 eV for scanning the Fe 2p and C 1s regions. All spectra were calibrated to the main C 1s peak at 284.5 eV and are provided in Supplementary Fig. S2.

Results and Discussion

NZVI particle systems

In field studies, NZVI concentrations as high as 20 g/L have been employed (Mueller *et al.*, 2013). However, in-

hibitory effects with NZVI concentrations as low as 1.6 g/L have been reported in lab-scale experiments (Yang *et al.*, 2013). Thus, a preliminary test was run with cNZVI at concentrations ranging from 0 to 80 g Fe/L to determine the optimal NZVI dose for the anaerobic batch reactors. Biogas production and composition were monitored and revealed that the optimal range for NZVI concentrations was 0–5 g Fe/L as indicated by increased methane production without complete inhibition of cellular respiration (i.e., CO₂ production) (Supplementary Fig. S1).

Composition of NZVI reported in the literature can vary widely in terms of particle size, as well as the presence/absence and type of stabilizer. In this study, we elected to compare cNZVI with NZVI that was synthesized immediately before loading into the batch reactor (sNZVI). The cNZVI was received as a slurry (18 wt% Fe) in water and is described by the manufacturer being stabilized only by an “inorganic modifier.” We observed with TEM that cNZVI is composed of aggregates of primary particles with diameter $d = 119 \pm 42$ nm (Fig. 1A). The needle- and sheet-like structures surrounding the iron particles that are visible in the TEM image are FeOOH and Fe_xO_y species (Cirtiu *et al.*, 2011). Charge transfer through the oxides occurs easily and freely due to its semiconductive nature and noncrystalline structure, thus preserving the high reactivity of the Fe⁰ core (Yan *et al.*, 2013). These iron oxide surface modifiers aid in stabilizing the particle in aqueous suspension. However, cNZVI was characterized by an aggregate size of 3.6 ± 0.1 μ m in solution, indicating that the oxide layer did not completely inhibit particle aggregation (Table 1).

The sNZVI was characterized by primary particles of sizes similar to those of cNZVI ($d = 123 \pm 51$ nm) with a high degree of agglomeration (Fig. 1B, Table 1). Of note, the method of synthesizing sNZVI without a stabilizer used in these experiments resulted in NZVI that was not colloidal stable, as indicated by the large aggregate size (Table 1). Often carbohydrate-based polymers, such as carboxymethyl cellulose and xanthan gum, are employed to prevent particle aggregation and delay iron oxidation. However, these stabilizers were not used, as they would be potential carbon sources in the bioreactors of these experiments that would affect methane production and COD measurements, inhibiting a direct comparison between sNZVI and cNZVI. However, a thin oxide

FIG. 1. Transmission electron microscopy images of (A) commercially available nanoscale zero-valent iron (cNZVI) ($d = 119 \pm 42$ nm) and (B) synthesized NZVI (sNZVI) ($d = 123 \pm 51$ nm). cNZVI has undergone greater oxidation as shown by the higher concentration of amorphous, lower-density oxide layers on the dark circular iron particles.

TABLE 1. PARTICLE SIZE AND SURFACE CHARACTERIZATION OF sNZVI, cNZVI AND ZVI USED IN THIS STUDY

	Primary particle size (nm) ^a	Aggregate size (μm) ^b	XPS % peak area						BET surface area (m ² /g)
			Fe 2p		O 1s				
			Fe-Fe (705.8–706.1 eV)	Fe-O (709–711.4 eV)	O-Fe (529.3–529.7 eV)	O-H (531–532.5 eV)	O-C (531.2 eV)	S-O (535.2 eV)	
sNZVI	123 ± 51	3.6 ± 0.1	19.53	80.47	4.42	84.15	—	11.43	2.433
cNZVI	119 ± 42	270 ± 29	9.32	90.68	8.9	91.1	—	—	16.671
ZVI	177 μm ^c		0	100	22.82	33.92	43.26	—	0.47

^aSize measured from TEM.^bDv50 measured by laser diffraction.^cSize reported by supplier.

BET, Brunauer-Emmett-Teller; NZVI, nanoscale zero valent iron; cNZVI, commercially available NZVI; sNZVI, synthesized NZVI; ZVI, zero valent iron; TEM, transmission electron microscope; XPS, X-ray photoelectron spectroscopy.

shell is inevitably present on the surface of sNZVI as a result of slow oxidation by water (Yan *et al.*, 2013). Since this thin oxide layer does not serve as a stabilizer to prevent against aggregation, sNZVI underwent gelation and gravitational sedimentation to a greater extent than did cNZVI, as is expected for an aqueous dispersion of NZVI that does not have a stabilizer (Phenrat *et al.*, 2007). There was also evidence of core dissolution occurring in sNZVI (Fig. 1B), which occurs as the Fe⁰ cores dissolve away, leaving behind the more stable iron oxide shell (Yan *et al.*, 2012).

XPS analysis proved that cNZVI had undergone a greater extent of oxidation than sNZVI (Table 1). Both systems contained Fe⁰ as indicated by an Fe 2p peak at 706 eV associated with Fe-Fe bonding. cNZVI is manufactured in a manner that generates an oxide shell on the particle surface to serve as a stabilizer. Alternatively, the sNZVI has a thin oxide shell resulting from a reaction with water in the short time between formation and addition to the reactor. Electrons can transport across the oxide layer, thus reactivity is not inhibited by this oxidation. Assuming the cores are mostly Fe⁰, the ratio of Fe⁰:Fe^{2+/3+} is likely higher than what is shown in Table 1 as XPS is limited to a probing depth of 10 nm. No Fe⁰ was found in the top 10 nm of the bulk ZVI, as is expected with iron filings that are stored in air as a solid. As shown in Figure 1, oxidation also results in changes in surface area as the iron oxides deposit on the particle surface. The BET surface areas of each particle system are shown in Table 1. As expected, bulk ZVI had the lowest surface area. sNZVI had a BET surface area of 2.433 m²/g, while cNZVI was 16.671 m²/g. The strong magnetic forces between iron particles and agglomeration that occur on drying often result in NZVI particles with BET surface areas less than 37 m²/g (Wang *et al.*, 2009).

Effect of NZVI on reactor solution chemistry

While anaerobic digestion offers a less energy-intensive alternative to aerobic digestion, the microbial processes required are much more sensitive to perturbations in the system and decrease pH to a point outside of the optimal range for methanogenesis. Thus, changes in solution chemistry, including pH and ORP, were measured along the course of the experiment.

As shown in Figure 2A, the addition of NZVI resulted in a slight increase in the initial pH compared with the un-

amended reactors. An increase in pH was observed for all reactors with or without NZVI amendment between days 0 and 3, after which pH levels decreased slightly. There was no significant difference in the pH changes between reactors amended with cNZVI, regardless of concentration. The reactor that amended with sNZVI exhibited pH values that were slightly lower than those observed in the cNZVI-amended reactors but higher than those of the unamended reactors.

Reaction of NZVI with available protons can drive pH up according to the following reaction:

This reaction can balance the decrease in pH in the bio-reactors that results naturally from the acetogens. The smaller change in pH on the addition of sNZVI compared with cNZVI at an equal iron concentration (1.25 g/L) suggests that sNZVI had lower reactivity. As mentioned earlier, sNZVI was not colloiddally stable. Although composed of nanoscale primary particles, the large aggregates of sNZVI resulted in decreased reactivity. Loss in reactivity is further supported by the lower change in ORP after the addition of sNZVI compared with cNZVI. As shown in Figure 2B, all doses of NZVI resulted in decreased ORP compared with the unamended control as a result of the oxidation of Fe⁰. The addition of 5 g/L NZVI resulted in the greatest decrease, with 2.5 g/L addition resulting in equally lowered ORP levels with the exception of day 3. All reactors had a similar ORP at day 10, suggesting that after this time period the surfaces of all NZVI particles had significantly reduced in reactivity. Soluble Fe²⁺ concentrations were measured using ICP-OES; however, there was no significant difference among the various NZVI treatments (data not shown). It is likely that any Fe²⁺ was bound to solid particles and/or precipitated out in insoluble iron oxide forms.

Effect of NZVI on microbial activity

COD is a measure of oxidizable organic compounds present in a sample and is often used to assess wastewater quality. As the microbes degrade organic compounds in the feed, the COD decreases. In these experiments, COD was measured as a way to monitor degradation of the feed in the

FIG. 2. Changes in (A) pH and (B) oxidation/reduction potential (ORP) in bioreactors with addition of sNZVI (1.25 g/L), cNZVI (1.25, 2.5, or 5.0 g/L) or unamended (0 g/L) over 10 days.

anaerobic reactors. Figure 3 shows the percentage of decrease in COD compared with unamended reactors. A decrease in COD was measured at day 0 and was generally proportional to NZVI dose. As no biodegradation had occurred at $t=0$, these results suggest that some organic compounds likely sorbed to the particles and were removed during the centrifugation step performed before COD analysis. Over the first 3 days, 5.0 g/L cNZVI resulted in the greatest changes in COD removal compared with the unamended control. On days 7 and 10, 2.5 g/L cNZVI resulted in the greatest COD removal. No specific trend was observed for the sNZVI versus cNZVI. While some variability in COD measurements is likely due to sorption onto the solid particles, overall the addition of NZVI to the reactors was found to increase COD removal, indicating the enhancement of anaerobic activity. Indeed, previous reports of adding bulk iron to anaerobic reactors also reported an increase in COD removal as a result of ZVI enriching bioreactor chemistry (Liu *et al.*, 2011; Zhang *et al.*, 2011b). Moreover, the overall enhancement of biodegradation by addition of NZVI is supported by the increased biogas production, discussed in the next section.

Three key microbial groups are involved in the conversion of organic materials to methane. Hydrolyzing and fermenting bacteria convert organics to fatty acids, alcohols, carbon dioxide, ammonia, and hydrogen. Acetogenic bacteria then convert the fatty acids and alcohols into hydrogen, carbon dioxide, and acetic acid. Lastly, the methanogenic bacteria convert hydrogen, carbon dioxide, and acetate into methane. Based on these reactions, monitoring biogas production (i.e., methane and carbon dioxide) is an accurate measure of anaerobe health and metabolic activity. In terms of anaerobic digestion of wastewaters, methane production is of importance not only as a measure of microbial health but also because methane is a valuable byproduct and potential energy source.

Direct effects of NZVI on reactor chemistry (i.e., increasing pH and lowering ORP) can contribute to favorable conditions for methanogenesis. The cumulative volume of methane and carbon dioxide produced are shown in Figure 4. Reactors dosed with 2.5 and 5.0 g/L cNZVI resulted in equally increased methane production (182 mL). The addition of 1.25 g/L NZVI, both cNZVI and sNZVI, resulted in 158 mL of total produced methane. These were a 28.3% and

FIG. 3. Percentage of decrease in chemical oxygen demand (COD) in bioreactors amended with sNZVI (1.25 g/L) or cNZVI (1.25, 2.5, or 5.0 g/L) over 10 days compared with unamended (0 g/L).

10.7% increase in methane production compared with unamended reactors, respectively. Although sNZVI had greater Fe^0 content than cNZVI (at least within 10 nm of the surface according to XPS, Table 1), both systems had an equivalent effect on methane production. The large aggregate size and

low BET surface area of sNZVI (Table 1) suggest that the available surface area of sNZVI for the reaction was limited. The importance of particle size is further shown in Figure 5A, where the addition of bulk ZVI resulted in only a 5% increase in methane production. By reducing the size of the ZVI down

FIG. 4. Cumulative volume of (A) methane and (B) carbon dioxide produced from bioreactors amended with sNZVI (1.25 g/L), cNZVI (1.25, 2.5, or 5.0 g/L) or unamended (0 g/L) over 10 days.

FIG. 5. Cumulative volume of (A) methane and (B) carbon dioxide produced from bioreactors amended with 1.25 g Fe/L cNZVI, 1.25 g Fe/L bulk zero-valent iron (ZVI) or unamended (0 g/L) over 10 days.

to the nanoscale, methane production increased by an additional 26% compared with bulk ZVI at equal iron loadings. These results indicate that NZVI can be added to anaerobic bioreactors to achieve greater improvements in methanogenic activity while using less material than bulk ZVI amendments. In addition, the NZVI amendment does not need to be prepared immediately before addition to see sufficient reactivity.

Toxicity of nanoscale iron to methanogens previously reported by Yang *et al.* (2013) was not observed in these experiments. In this previous study, reactors amended with 30 mM (1.675 g/L) NZVI resulted in a 69% decrease in methane production along with a significant increase in COD and volatile fatty acids (VFA) concentrations. However, this toxicity was not observed when a ZVI powder with a particle size of approximately $\sim 200 \mu\text{m}$ was used. Of note, Yang *et al.* (2013) also observed elevated levels of H_2 production in their NZVI-amended reactors, while H_2 levels remained below the limit of detection in our studies. Moreover, increased concentrations of VFAs other than acetic acid were not observed in our NZVI-amended reactors, indicating stable microbial health and feed degradation (Supplementary Table S1). The NZVI systems used in this study had negligible toxicity in the bioreactors. The advantage of reducing ZVI to the nanoscale and the accompanying increased surface area to volume ratio compared with bulk iron was clearly beneficial in the reactors presented here. However, this advantage was lost for stable

suspension of particles with diameters near 100 nm and below, which appear to have deleterious effects on anaerobic digestion (Yang *et al.*, 2013). Taken together, the results presented here along with those of Yang *et al.* (2013) indicate that aggregate size plays a vital role in whether ZVI amendments will promote or inhibit methanogenic activity.

NZVI-amended reactors in this study also released less CO_2 (Fig. 4B). Reactors amended with 1.25, 2.5, and 5 g/L cNZVI released 51%, 57%, and 58% less CO_2 , respectively, than reactors without NZVI amendment. Reactors amended with sNZVI showed a 6% decrease in volume of CO_2 measured. Bulk ZVI amendments did not affect the amount of CO_2 released compared with the control (Fig. 5B) due to the low surface area and minimal Fe^0 species near the bulk ZVI surface. Carbon dioxide can be removed biotically as hydrogenotrophic methanogens convert CO_2 to methane using H_2 as a reducing agent. NZVI can also undergo an oxidation/reduction reaction with CO_2 and water to produce iron carbonate and hydrogen according to the reaction:

To confirm the ability of NZVI to remove CO_2 from solution, batch reactors were prepared where cNZVI and sNZVI were added to saturated CO_2 solutions. As shown in Table 2, the addition of 1.25 g/L cNZVI resulted in a decrease in CO_2 by 47% compared with the reactors without NZVI added. The

TABLE 2. REMOVAL OF CO₂ FROM SOLUTION BY NZVI COMPARED WITH CONTROL (NO PARTICLES)

Particle system	Dose (g/L)	% decrease in CO ₂ in headspace
cNZVI	1.25	47 ± 13
	2.5	61 ± 16
sNZVI	1.25	56 ± 10

removal of CO₂ increased to 61% as the dose of cNZVI was doubled to 2.5 g/L. Of note, the addition of 1.25 g/L sNZVI also resulted in a significant decrease in CO₂ (58%), whereas in the bioreactors containing biomass a less significant removal of CO₂ was observed. It is likely that heteroaggregation between the unstable sNZVI and the solid biomass would have inhibited a reaction with CO₂ in the bioreactors. As indicated by XPS, bulk ZVI had minimal Fe⁰ near the surface, thus it was unable to scavenge any CO₂.

Solids from these reactors were collected, dried, and analyzed using XPS to identify iron and carbon species present. Iron carbonates were found in both cNZVI and sNZVI as indicated by C 1s peak at 289 eV, Fe 2p_{1/2} peak at 710 eV, and Fe 2p_{3/2} at 724 eV (Supplementary Fig. S2) (Heuer and Stubbins, 1999). These results indicate that the addition of NZVI to anaerobic bioreactors is not only advantageous in terms of improving methanogenesis but also improves the quality of the produced biogas by decreasing the CO₂ content.

Conclusions

Addition of NZVI to anaerobic batch reactors achieved a 28% increase in methane production and improved COD removal compared with unamended control reactors. Addition of bulk ZVI only resulted in a 5% increase in methane production, indicating the advantage of reducing iron to the nanoscale. The beneficial influence of NZVI was attributed to direct effects on reactor chemistry (i.e., pH and ORP) that promoted methanogenesis. While the use of NZVI is advantageous as an amendment for anaerobic digestion compared with bulk ZVI, particle size and reactivity must be optimized. Reducing particle size to below 100 nm may result in deleterious effects due to high reactivity and overproduction of H₂, as was previously reported (Yang *et al.*, 2013). Moreover, although sNZVI had a higher Fe⁰ content than cNZVI, the effects on methane production were similar to cNZVI as a result of the significant aggregation of the unstabilized sNZVI. These results indicate the importance of employing a surfactant to preserve nanoscale particle size and colloidal stability. Of note, the addition of surface-bound stabilizers can also block reactivity of Fe⁰ with the surrounding media and limit reactivity of the NZVI system (Phenrat *et al.*, 2009). Our work suggests that an oxide layer provides sufficient stability while still allowing electron transfer from the Fe⁰ to the surrounding environment to promote methanogenic activity. Future work will investigate the use of biodegradable surfactants that can stabilize NZVI during addition and are then degraded in the reactor to enable optimal NZVI reactivity.

The NZVI amendments employed in this study were also found to decrease the amount of CO₂ released from the

bioreactors by 58%. These results indicate that NZVI can be employed as an amendment to anaerobic bioreactors to not only improve degradation efficiency but also render the produced biogas more valuable. While these results are promising, the benefits of NZVI addition are limited by the short reactive lifetime of NZVI. Future work is focused on engineering methods for removal of used NZVI and reapplication of fresh NZVI to the anaerobic reactors to yield longer-term benefits.

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Supplementary Data

Biogas production from preliminary dose experiment; VFA concentrations; XPS spectra; reactor chemistry with bulk ZVI amendments.

Author Disclosure Statement

No competing financial interests exist.

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