Supporting information

Comparison of enhancement of anaerobic digestion of waste activated sludge through adding nano-zero valent iron and zero valent iron

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Text S1

NZVI was prepared by reducing Fe(III) with sodium borohydride ($NaBH_4$) as shown in Eq. 1. Briefly, 0.15 mol/L $NaBH_4$ was slowly added into 0.04 mol/L $FeCl_3 \cdot 6H_2O$ at room temperature within 30 min. The mixture was stirred for another 20 min, followed by standing for 1 h. Ethanol (99.9%) was used for washing and preservation of NZVI. The median diameter of the prepared NZVI was approximately 100 nm.

$$2Fe(H_2O)_6^{3+} + 6BH_4^- + 6H_2O \rightarrow 2Fe^0\,(s) + 6B(OH)_3 + 21H_2\,(g) \qquad (1)$$

Text S2

Lowry's method [1] was used to measure protein concentrations with bovine serum albumin as the standard, and the anthranone-sulfuric acid method [2] was used to measure the concentration of polysaccharides. The SS, VSS and COD were determined according to standard methods. The concentrations of VFAs, including acetate, propionate, butyrate and valerate, were determined using a gas chromatograph (GC, Agilent 6890N) equipped with a flame ionization detector.

For CH_4 and H_2 concentration (P_{CH4} and P_{H2}) analysis, 1-mL gas samples were collected and injected into a GC (9850T, FULI) with a thermal conductivity detector and a stainless-steel column packed with TDX-01 (1 m length). Nitrogen (99.999%) was used as the carrier gas. The operational temperatures of the injector, detector and column oven were 80, 100 and 80 °C respectively, and the bridge current was 80 mA. The cumulative volumes of CH_4 and H_2 (V_{CH4} and V_{H2}) were calculated through the equations: $V_{CH4} = P_{CH4} \times V_{biogas}$ and $V_{H2} = P_{H2} \times V_{biogas}$.

Text S3

SCOD is considered the main parameter to evaluate the sludge particulate material, which enables an evaluation of the maximum level of sludge solubilization [3]. VSS reduction is an indication of sludge stability, and is used for assessing the effectiveness of a process in stabilizing sludge [4]. In this study, SCOD was measured to calculate solubilization of WAS by Eq. 2.

$$Solubilization\ (\%) = \frac{SCOD_{en} - SCOD_{in}}{TCOD_{in} - SCOD_{in}} \quad (2)$$

where $SCOD_{en}$ is the soluble COD in the WAS on day 4 of hydrolysis-acidification, and $SCOD_{in}$ is the initial soluble COD in the WAS.

VSS reduction was calculated using Eq. 3.

$$VSS_{reduction}(\%) = \frac{VSS_{in} - VSS_{en}}{VSS_{in}} \times 100\% \quad (3)$$

where VSS_{in} is the initial content of VSS in the WAS, and VSS_{en} is the content of VSS in the WAS on day 4 of the hydrolysis-acidification.

Text S4

1. Sample collection, DNA extraction and PCR amplification

WAS samples were collected from each serum bottle at the end of anaerobic digestion (31 d), and stored at −20 °C until use. Genomic DNA was extracted triply from the mixed liquor sludge samples using a Power Soil DNA Isolation Kit (Sangon, China) according to the manufacturer's instructions. The three extractions were then pooled together and diluted to 10 ng/μL for the next experimental procedure.

Bacterial universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3 and V4 regions of the 16S rRNA gene with the reverse primers containing 6-bp barcodes tagging each sample (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai China). PCR amplifications were carried out in triplicate for each sample using 20-μL reaction mixtures containing 5× PCR buffer, 10 ng of template DNA, 0.2 μM of each primer, 0.25 mM of each dNTP, and 1 U Fast*Pfu* polymerase (TransGen, China). The PCRswere performed in the following conditions: 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and a final extension at 72 °C for 5 min. Reactions were performed in a GeneAmp 9700 thermocycler (ABI, USA). The triplicate amplicons were pooled together, electrophoresed on 2% (w/v) agarose gels, and recovered using an AxyPrep DNA Gel Extraction Kit (Axygen, USA).

2. 16S rRNA gene-based Illumina library preparation, sequencing and data

analysis

A QuantiFluor-ST Fluorometer (Promega, USA) was used to quantify the purified amplicons, and then, by combining equimolar ratios of amplicons from all samples, a composite sequencing library was constructed. The resulting library was sent for paired end sequencing (2 × 250 bp) on an Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co., Ltd. The 16S rRNA gene sequences obtained were compared with sequences in the GenBank database using the NCBI Blast search program (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

The sequencing data was then analyzed using Trimmomatic and FLASH software. Community estimators including richness estimator calculations (Ace and Chao indexes) and α-diversity estimator calculations (Simpson and Shannon indexes) were performed and analyzed using MOTHUR (version v. 1.30.1*; http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity).* The distance matrix between aligned DNA sequences was generated from these sequences. Subsequently, the Usearch program (v. 7.1) was used with the furthest neighbor algorithm to obtain the number of operational taxonomic units (OTUs); clone sequences with >97% similarity were grouped together and regarded as one OTU. Rarefaction curves were generated from the observed OTUs using R (v. 3.2.3). Based on the community composition and the environmental variable (i.e. NZVI concentration), redundancy analysis was performed with CANOCO 4.5 software.

Text S5

1. Bacterial variations in hydrolysis-acidification

A total of 48 phyla were detected by 16S rRNA high-throughput sequencing in the hydrolysis-acidification testsludge samples, mainly dominated by Proteobacteria, Aminicenantes,Bacteroidetes,Chloroflexi,Firmicutes,Spirochaetes, Actinobacteria and Acidobacteria. PhylaActinobacteria, Firmicutes, Bacteroidetes, Chloroflexi and Proteobacteriaare associated with WAS hydrolysis acidification, and are usually found in anaerobic digesters [5]. For example, Actinobacteria and Firmicutes can metabolize substrates such as proteins, lipids and celluloses by producing extracellular enzymes [6]; Bacteroidetes have the ability toconvert proteins and carbohydrates to propionate and acetate in anaerobic sludge fermentation [7].

The microbial populations changed significantly at the phylum level with various additions of NZVI and ZVI (Table S3 and Fig. S1a). The relative abundance of functional bacteria affiliated with Actinobacteria, Firmicutes, Bacteroidetes, Chloroflexi and Proteobacteria increased with increasing NZVI addition from 0.0 to 10.0 g/L; the sum of the relative abundance of these phyla was 52.01% of the total bacterial amount in the control and 73.85% at 10.0 g/L NZVI. This result indicates that NZVI was beneficial for the proliferation of microorganisms related to hydrolysis-acidification processes. The impact of ZVI addition on the microbial population was not so obvious. At 10.0 g/L ZVI, the relative abundance of functional bacteria of phyla Actinobacteria, Firmicutes, Bacteroidetes, Chloroflexi and

Proteobacteria was 56.30% of the total bacterial amount, while it was 52.01% at 0.0 g/L ZVI.

Twenty-nine classes were detected in sludge samples from the hydrolysis-acidification test, among which 14 (48.3%) were involved in hydrolysis-acidification (Fig. S1b). The class Betaproteobacteria, which includes chemoheterotrophic microorganisms that are responsible for the decomposition of organics [8], was the dominant bacterial class in the experimental systems (Table S1). The relative abundance of Betaproteobacteria increased with NZVI and ZVI addition, rising to 18.02% and 8.52% respectively at 10.0 g/L NZVI and ZVI (Table S1). Clostridia, Gammaproteobacteria and Bacteroidia were also abundant classes, the highest proportions of which were 13.47%, 10.40% and 10.70%, respectively. Apart from Bacteroidia, these classes increased with NZVI addition. Previous studies reported that Clostridia are the common acid-forming bacteria responsible for decomposing solid wastes and producing organic acids [9], and Gammaproteobacteria are widely present in anaerobic hydrolytic and acidification units for treatment of dyeing wastewater [10].

A total of 51 bacterial genera were classified among the test samples (Fig. S1c). As NZVI addition increased, the relative abundance of *Aminicenantes_norank* significantly decreased, from 21.20% (0.0 g/L NZVI) to 0.43% (4.0 g/L NZVI). Aminicenantesare frequently detected in anaerobic digestion systems [11]. In the present

study, it seems that *Aminicenantes_norank* could not survive high NZVI addition. The relative abundance of *Bacteroidetes_vadinHA17_norank* genus also decreased. *Bacteroidetes_vadinHA17_norank* are affiliated to Bacteroidetes [12], and they accounted for 5.10% of the bacterial genera in the control, but only 0.14% after 10.0 g/L NZVI addition. This observation was consistent with the relative abundance of Bacteroidetes decreasing at 4.0 and 10.0 g/L NZVI (Fig. S1a). *Candidate_division_WS6_norank* was the dominant bacterial genusin the anaerobic digestion system fed with 4.0 g/L NZVI (15.69%) andits relative abundance significantly increased after NZVI addition. A recent genome-wide study predicted Candidate phylum WS6 could be located in the anaerobic granule core and support a fermentative lifestyle [13]. We speculate that *Candidate_division_WS6_norank* might be beneficial for WAS fermentation.

The relative abundance of *WCHB1−60_norank* and *SC−I−84_norank* genera also increased with NZVI addition, reaching 6.06% and 5.14% respectively at 4.0 g/L NZVI. In addition, the relative abundance of *Gelria* increased in the 4.0 g/L NZVI-addition system, accounting for 3.96% of the total bacteria. *Gelria* is affiliated to Firmicutes, and contributes to anaerobic biodegradation and methane formation [14]. Our findings indicate that 4.0 g/L NZVI stimulated the proliferation of *Gelria*. This was in agreement with the increase in the relative abundance of Firmicutes with NZVI addition (Fig. S1a).

Taken together, NZVI addition stimulated proliferation of microorganisms responsible for hydrolysis-acidification, thus enhancing the hydrolysis and acidification processes in WAS.

2. Methanogenic archaeal variations

NZVI addition significantly influenced the microbial community structure of methanogenic archaea during the 31-day digestion process (Table S2, Table S4 and Fig. S2). As NZVI addition increased from 0.0 g/L to 10.0 g/L, the relative abundance of hydrogenotrophic methanogens rose from 20.59% to 83.99% of the total archaea, positively correlated to the NZVI dosage (Table S2). On the contrary, the relative abundance of aceticlastic methanogens first increased and then decreased with increasing NZVI addition. Specifically, it was maximal at 4.0 g/L NZVI addition, and dramatically declined on 10.0 g/L NZVI addition. Finally, hydrogenotrophic methanogens became the dominant populations at the NZVI dosage of 10.0 g/L (Table S2).

At the genus level, *Euryarchaeota_unclassified* occupied the highest percentage of the total methanogenic archaea in the control and dramatically decreased with increasing NZVI addition (Table S4 and Figure S3b). *Methanosaeta*, *Methanolinea*and *Methanobacterium*were the dominantgenera with NZVI addition. *Methanosaeta* are aceticlasticmethanoarchaea [15]; their relative abundance reached a maximum at 4.0 g/L NZVI, but decreased significantly at 10.0 g/L NZVI. This

phenomenon was consistent with the finding that hydrolysis-acidification was enhanced at 4.0 g/L NZVI. When NZVI addition increased further to 10.0 g/L, H_2 was accumulated in large amounts, and hydrogenotrophic methanogens proliferated. The amount of *Methanolinea*, a hydrogenotrophic methanogen [16], was roughly constant at lower levels of NZVI addition, but significantly increased at 10.0 g/L NZVI, which might be caused by the accumulation of H_2 in the system. *Methanobacterium*, which grows autotrophically with H_2 and CO_2 as sole sources of energy and carbon [17], substantially increased with NZVI addition. In the ZVI addition tests, *Methanolinea* and *Methanosaeta*were the dominant genera (Fig. S2b).

The relative abundances of methanogenic archaea at the order and genus levels with different NZVI/ZVI concentrations were detected(Fig. S2a and b). Seven orders of methanogenic archaea were detected, among whichMethanobacteriales, Methanomicrobiales and Methanosarcinales were previously found to be the main methanogens during anaerobic digestion in wastewater treatment [18].Methanobacteriales and Methanomicrobiales are hydrogenotrophic methanogens [19], and Methanosarcinales are aceticlastic methanogens [20].

It is clear that the methanogen distribution changed significantly after NZVI addition(Fig. S2a). With increasing concentration of NZVI, the relative abundance of Methanobacteriales, Methanomicrobiales and Methanosarcinales increased from 31.21% to 93.66% (Table S2). As NZVI addition increased from 0.0 to 10.0 g/L, the

relative abundance of Methanobacterialesgradually increased, reaching a maximum value of 36.13% at 4.0 g/L NZVI, far higher than that of 0.2% at 0.0 g/L NZVI. Also, the relative abundance of Methanomicrobiales increased with increasing NZVI dosage, and reached 51.61% of the totalarchaeaat 10.0 g/L NZVI. The proportion of Methanosarcinales increased then declined, maximizing at 4.0 g/L NZVI (40.90%) and falling to 9.67% when NZVI addition was increased to 10.0 g/L.

The distribution of methanogenic archaea also changed slightly with ZVI addition (Fig. S2a). The relative abundances of Methanobacteriales, Methanomicrobiales and Methanosarcinales increased by 29.52%, 76.5% and 53.48% respectively as ZVI addition increased from 0.0 to 10.0 g/L. It was clear that addition of NZVI and ZVI could promote the growth of methanogenic archaea, including hydrogenotrophic and aceticlastic methanogens.

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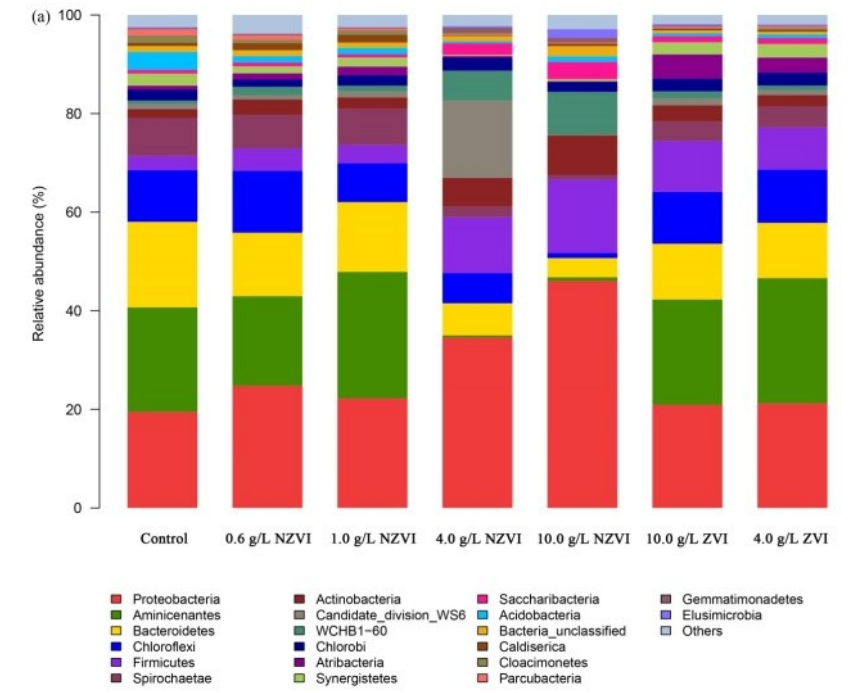
Table S1. Relative abundance of functional bacteria at the class level with various NZVI and ZVI additions (%)								
		0.0 g/L NZVI	0.6 g/L NZVI	1.0 g/L NZVI	4.0 g/L NZVI	10.0 g/L NZVI	10.0 g/L ZVI	4.0 g/L ZVI
Proteobacteria	Betaproteobacteria	6.02	7.03	6.27	14.21	18.02	8.52	8.14
	Gammaproteobacteria	4.41	7.27	5.96	8.99	10.40	5.59	4.77
	Alphaproteobacteria	2.99	5.08	3.36	7.69	11.55	3.55	3.57
	Deltaproteobacteria	5.92	5.14	6.54	3.55	5.66	3.19	4.61
	Betaproteobacteria	6.02	7.03	6.27	14.21	18.02	8.52	8.14
Firmicutes	Clostridia	2.86	4.43	3.50	11.00	13.47	10.18	8.47
	Negativicutes	0.017	0.004	0.017	0.14	1.34	0.03	0.02
Chloroflexi	Anaerolineae	3.67	6.33	3.58	3.78	0.28	5.32	6.49
	Chloroflexi_uncultured	2.56	3.76	2.39	1.71	0.30	4.05	3.04
	Caldisericia	0.66	1.40	1.57	0.14	0.49	0.35	0.41
	Chloroflexi_unclassified	1.03	1.20	0.92	0.17	0.03	0.34	0.54
Bacteroidetes	Bacteroidia	10.70	6.38	7.66	3.48	0.34	4.38	5.70
	Bacteroidetes_vadinHA17	5.10	5.49	5.36	0.52	0.13	6.16	4.68
	Sphingobacteriia	1.18	0.85	0.89	2.30	3.32	0.66	0.65
Actinobacteria	Actinobacteria	1.80	3.11	2.23	5.77	7.96	3.24	2.28

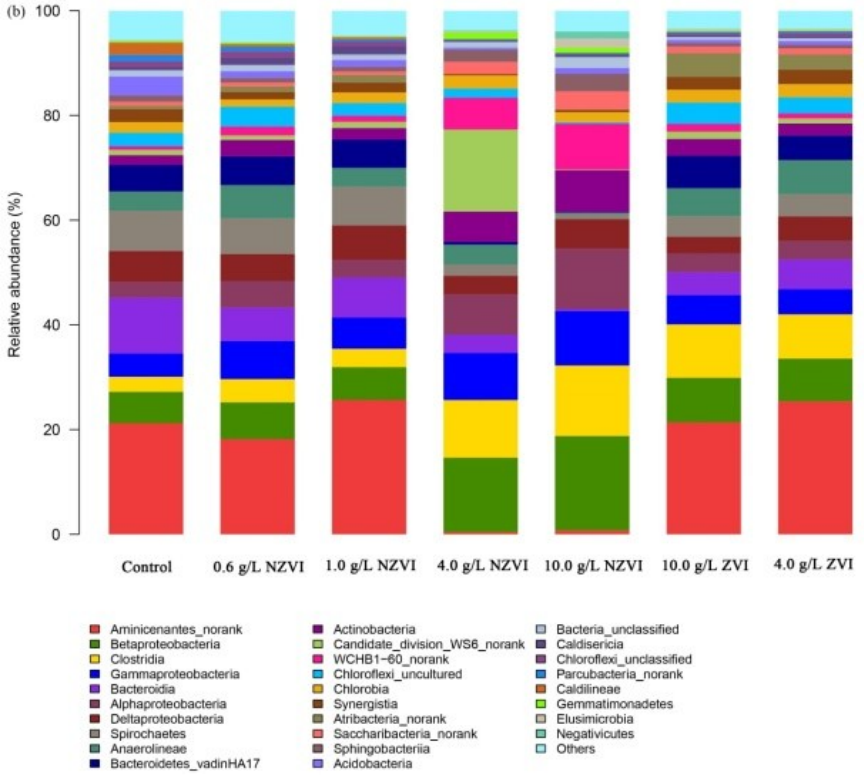
Table S2. Relative abundance of methanogens at the order level with various NZVI and ZVI additions (%)							
	0.0 g/L NZVI	0.6 g/L NZVI	1.0 g/L NZVI	4.0 g/L NZVI	10.0 g/L NZVI	10.0 g/L ZVI	4.0 g/L ZVI
Methanomicrobiales	20.39	19.28	30.01	16.37	51.61	26.41	37.26
Methanobacteriales	0.20	1.98	5.94	36.13	32.38	1.73	0.72
Hydrogenotrophic methanogens	20.59	21.26	35.95	52.5	83.99	28.14	37.98
Methanosarcinales	10.62	19.34	30.89	40.90	9.67	16.30	21.13
Methanogens	31.21	40.6	66.84	93.4	93.66	44.44	59.11
Methanomassiliicoccales	0.03	0.33	0.99	1.30	0.11	0.50	0.85
Euryarchaeota_unclassified	68.74	59.06	32.18	5.30	6.20	55.06	40.04
Others	0.03	0.33	0.99	1.30	0.11	0.50	0.85
Non-functional bacteria	68.76	59.07	32.18	5.30	6.23	55.06	40.04

Table S3. The relative abundance of bacteria at the phylum level following various NZVI and ZVI additions (%) in hydrolysis-acidification of WAS							
phylum	0.0 g/L NZVI	0.6 g/L NZVI	1.0 g/L NZVI	4.0 g/L NZVI	10.0 g/L NZVI	10.0 g/L ZVI	4.0 g/L ZVI
Proteobacteria	19.48	24.78	22.23	34.57	46.05	20.89	21.21
Bacteroidetes	17.35	12.87	14.15	6.514	3.885	11.311	11.21
Chloroflexi	10.43	12.56	7.84	6.118	1.016	10.50	10.79
Firmicutes	2.93	4.52	3.72	11.35	14.93	10.34	8.546
Actinobacteria	1.80	3.11	2.23	5.773	7.966	3.248	2.280
Percentage of functional bacteria	52.01	57.85	50.19	64.33	73.85	56.30	54.05
Aminicenantes	21.20	18.17	25.65	0.43	0.74	21.38	25.39
Spirochaetae	7.69	6.82	7.44	2.17	0.94	3.96	4.29
Candidate_division_WS6	1.05	0.87	1.19	15.69	0.17	1.39	0.98
WCHB1-60	0.67	1.69	1.17	6.06	8.69	1.47	0.96
Chlorobi	2.21	1.50	2.12	2.64	2.02	2.58	2.60
Atribacteria	0.79	1.23	1.71	0.30	0.09	4.87	3.04
Synergistetes	2.45	1.42	1.87	0.27	0.44	2.47	2.77
Saccharibacteria	0.77	0.76	0.66	2.32	3.49	1.25	1.18
Acidobacteria	3.63	1.29	1.28	0.41	1.11	0.59	0.71
Bacteria_unclassified	1.19	1.19	1.07	1.07	2.08	0.56	0.56
Caldiserica	0.66	1.408	1.57	0.14	0.49	0.35	0.41
Cloacimonetes	1.50	0.74	0.87	0.16	0.02	0.08	0.34
Parcubacteria	1.21	0.87	0.41	0.18	0.22	0.28	0.31
Gemmatimonadetes	0.28	0.30	0.16	1.21	0.94	0.22	0.17
Elusimicrobia	0.17	0.05	0.11	0.29	1.76	0.32	0.23
Others	2.45	3.77	2.44	2.25	2.86	1.85	1.92
Percentage of non-functional bacteria	47.98	42.14	49.80	35.66	26.14	43.69	45.94

Table S4. The relative abundance of methanogens at the genus level following various NZVI and ZVI additions (%) in whole anaerobic digestion

Order	Genus	0.0 g/L NZVI	0.6 g/L NZVI	1.0 g/L NZVI	4.0 g/L NZVI	10.0 g/L NZVI	10.0 g/L ZVI	4.0 g/L ZVI
Methanobacterias	Methanobacterium	0.18	1.91	5.59	35.21	30.43	1.67	0.66
	Methanobrevibacter	0.03	0.35	0.92	0.07	1.95	0.06	0.06
Methanomicrobiales	Methanolinea	17.49	13.78	17.70	13.67	36.80	23.29	26.17
	Methanospirillum	2.90	5.50	12.31	2.71	14.81	3.12	11.09
Methanosarcinales	Methanosaeta	10.55	19.02	30.23	37.54	9.32	16.20	20.72


(a)
Relative abundance (%)
0
20
40
60
80
100
Control
0.6 g/L NZVI
1.0 g/L NZVI
4.0 g/L NZVI
10.0 g/L NZVI
10.0 g/L ZVI
4.0 g/L ZVI
Proteobacteria
Aminicenantes
Bacteroidetes
Chloroflexi
Firmicutes
Spirochaetae
Actinobacteria
Candidate_division_WS6
WCHB1-60
Chlorobi
Atribacteria
Synergistetes
Saccharibacteria
Acidobacteria
Bacteria_unclassified
Caldiserica
Cloacimonetes
Parcubacteria
Gemmatimonadetes
Elusimicrobia
Others


(b)
Relative abundance (%)
0
20
40
60
80
100
Control
0.6 g/L NZVI
1.0 g/L NZVI
4.0 g/L NZVI
10.0 g/L NZVI
10.0 g/L ZVI
4.0 g/L ZVI
Aminicenantes_norank
Betaproteobacteria
Clostridia
Gammaproteobacteria
Bacteroidia
Alphaproteobacteria
Deltaproteobacteria
Spirochaetes
Anaerolineae
Bacteroidetes_vadinHA17
Actinobacteria
Candidate_division_WS6_norank
WCHB1-60_norank
Chloroflexi_uncultured
Chlorobia
Synergistia
Atribacteria_norank
Saccharibacteria_norank
Sphingobacteriia
Acidobacteria
Bacteria_unclassified
Caldisericia
Chloroflexi_unclassified
Parcubacteria_norank
Caldilineae
Gemmatimonadetes
Elusimicrobia
Negativicutes
Others

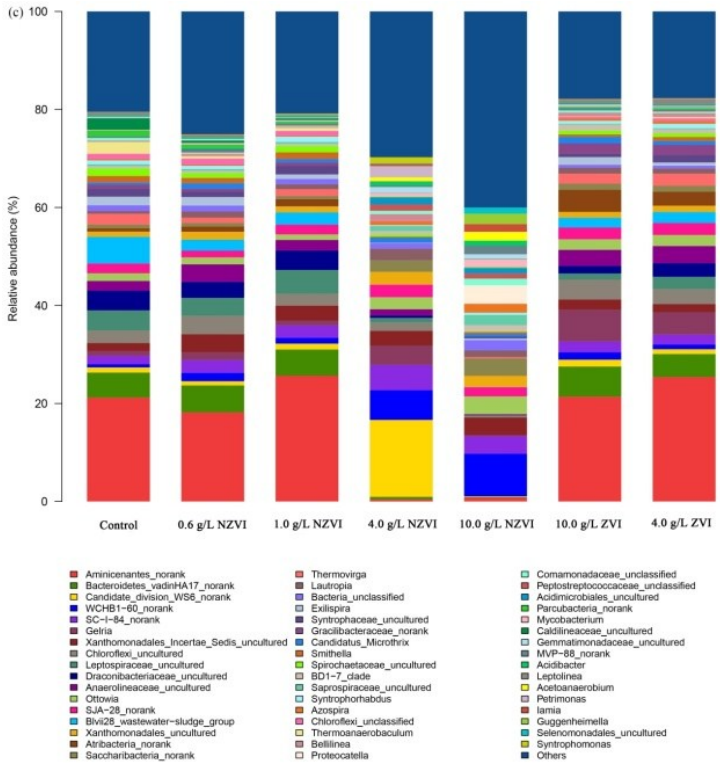


Fig. S1. (a) Bacterial distribution at the phylum level with various NZVI and ZVI additions; (b) bacterial distribution at the class level; (c) bacterial distribution at the genus level.

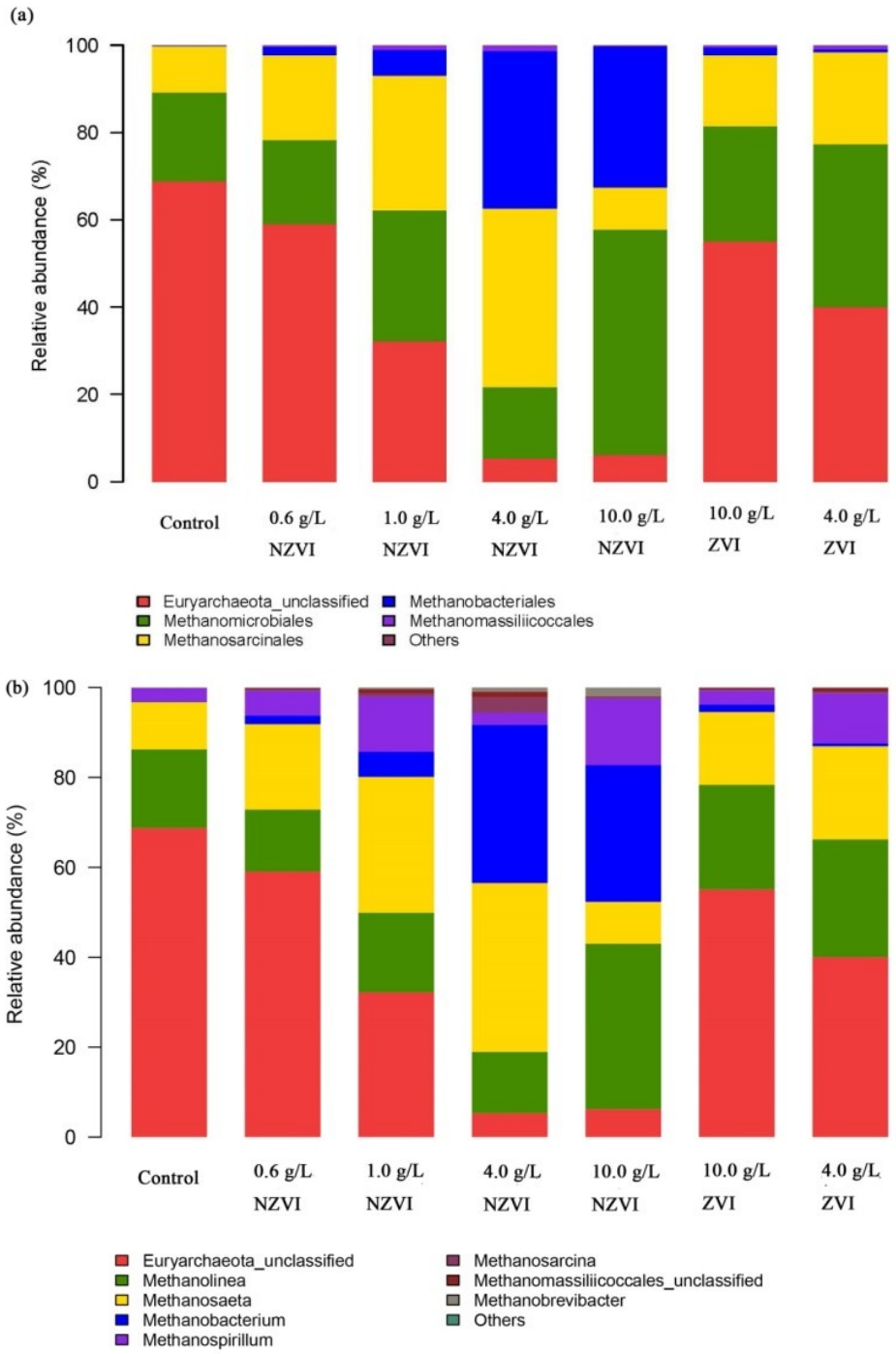


Fig. S2. (a) Methanogen distribution at the order level with various NZVI and ZVI additions; (b) methanogen distribution at the genus level with various NZVI and ZVI additions.