Environmental Science & Technology

Article

pubs.acs.org/est

How Does Poly(hydroxyalkanoate) Affect Methane Production from the Anaerobic Digestion of Waste-Activated Sludge?

Dongbo Wang,[†,‡,§] Jianwei Zhao,[†,‡] Guangming Zeng,[*,†,‡] Yinguang Chen,[*,§] Philip L Bond,[∥] and Xiaoming Li[†,‡]

[†]College of Environmental Science and Engineering, Hunan University, Changsha 410082, China

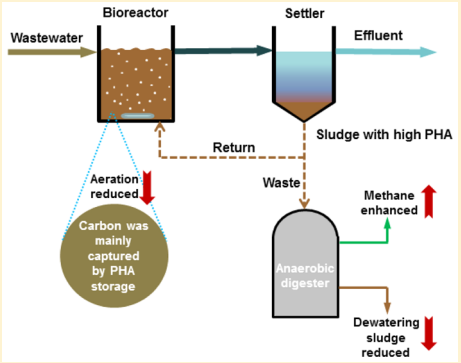
[‡]Key Laboratory of Environmental Biology and Pollution Control, Hunan University, Ministry of Education, Changsha 410082, China

[§]State Key Laboratory of Pollution Control and Resources Reuse, School of Environmental Science and Engineering, Tongji University, 1239 Siping Road, Shanghai 200092, China

[∥]Advanced Water Management Centre, The University of Queensland, St. Lucia, Brisbane, Queensland 4072, Australia

Supporting Information

ABSTRACT: Recent studies demonstrate that, besides being used for production of biodegradable plastics, poly(hydroxyalkanoate) (PHA) that is accumulated in heterotrophic microorganisms during wastewater treatment has another novel application direction, i.e., being utilized for enhancing methane yield during the anaerobic digestion of waste-activated sludge (WAS). To date, however, the underlying mechanism of how PHA affects methane production remains largely unknown, and this limits optimization and application of the strategy. This study therefore aims to fill this knowledge gap. Experimental results showed that with the increase of sludge PHA levels from 21 to 184 mg/g of volatile suspended solids (VSS) the methane yield linearly increased from 168.0 to 246.1 mL/g of VSS (R^2 = 0.9834). Compared with protein and carbohydrate (the main components of a cell), PHA exhibited a higher biochemical methane potential on a unit VSS basis. It was also found that the increased PHA not only enhanced cell disruption of PHA cells but also benefited the soluble protein conversion of both PHA- and non-PHA cells. Moreover, the reactor fed with higher PHA sludge showed greater sludge hydrolysis and acidification than those fed with the lower PHA sludges. Further investigations using fluorescence in situ hybridization and enzyme analysis revealed that the increased PHA enhanced the abundance of methanogenic Archaea and increased the activities of protease, acetate kinase, and coenzyme F420, which were consistent with the observed methane yield. This work provides insights into PHA-involved WAS digestion systems and may have important implications for future operation of wastewater treatment plants.



INTRODUCTION

As a byproduct of biological wastewater treatment, large amounts of waste-activated sludge (WAS) are inevitably produced,[1−3] which is a big problem faced by wastewater treatment plants (WWTPs) nowadays.[4,5] WAS will cause secondary pollution, if it is not treated and disposed of appropriately. On the other hand, WAS contains high levels of organic matter such as protein and carbohydrate, which makes it appealing as a renewable bioenergy resource.[6−9] As a technology for preventing pollution and for recovery of sustainable energy, the anaerobic digestion of WAS therefore attracts great interest, by which the amount of sludge is reduced, pathogenic microorganisms are killed, and energy biogas methane is also produced.[10,11] With the increasing concerns of energy supply, strategies to enhance methane yields from WAS anaerobic digestion are gaining much attention.[11−14]

In principle, there are two main parameters that influence methane production from WAS anaerobic digestion; these are the operational conditions of the digester and the characteristics of the WAS. However, most research to date has focused on the former. Several operational factors such as the pretreatment methods of sludge, the type of digester, and the microbial ecology within the digester are reported to affect methane yields.[11−16] The corresponding strategies developed on the basis of these factors enhance methane yields either by accelerating the disruption of extracellular polymeric substances (EPSs)/the cell envelope or by enriching the abundance of methane-producing Archaea.[10,15] For example, WAS solubilization was substantially improved, which thereby caused a 27% increase in methane production, when it was pretreated with 2.13 mg/L free nitrous acid (FNA) for 24 h.[13] By using a

Received: June 27, 2015
Revised: September 8, 2015
Accepted: September 18, 2015
Published: September 18, 2015

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262

combination of an alkaline pretreatment with an expanded granule sludge bed digester, Zhang et al. demonstrated that Methanosarcinaceae populations and methane yields were much higher than those in a continuous stirred tank reactor.[15] Apart from the operation conditions of the digester, the characteristics of WAS can also affect methane generation. However, little attention has been paid to this topic so far.

Poly(hydroxyalkanoate) (PHA), which is an energy and carbon storage polymer, is readily accumulated in heterotrophic microorganisms during wastewater treatment, particularly under substrate feast−famine conditions.[17−20] By modifying the wastewater treatment processes and/or optimizing the operational parameters, it has been verified that WAS wasted from WWTPs can contain high levels of PHA.[6,7,21−23] The PHA-rich sludge is usually used as a source for producing biodegradable plastics,[24−26] but Huda and co-workers recently pointed out another novel direction: being a substrate for methane production.[27] It was found that most of the PHA accumulated in a sludge was degraded only within the initial 2 d of digestion, and the methane yield produced from sludge with PHA was 25% higher than that without PHA.[27] This finding provides opportunities to enhance methane production from anaerobic digestion of WAS by focusing on the sludge characteristics. Thus, by combining the tactic of PHA accumulation in WAS with other strategies of digester control, methane production would likely be further enhanced. Considering the huge quantities of WAS treated daily, this PHA-based method should have significant economic and ecological consequences.

The discovery that WAS enriched with PHA enhances methane yield provides important hints for the development of both wastewater treatment and WAS anaerobic digestion.[27] However, details of how the PHA accumulated in the cells enhances methane production remain largely unknown, which makes this process a "black box" with limited understanding. It is known that PHA is mostly located inside the cell, while other main cell components such as protein and carbohydrate are located intracellularly and externally. With an increase of the sludge PHA content, other sludge components such as protein and carbohydrate on a volatile suspended solids (VSS) basis will inevitably decrease.[7] These changes have led us to ask several questions.

Compared with protein and carbohydrate (the main cell components), does PHA have a higher biochemical methane potential on a unit VSS basis? How does PHA affect the anaerobic digestion process of PHA cells? Does PHA affect the anaerobic digestion of non-PHA cells? If it does, how does it influence these cells?

On the basis of these questions, this study aims to reveal the underlying mechanism of how PHA affects methane production from anaerobic WAS digestion. To gain a comprehensive understanding, the effect of sludges containing different PHA levels on methane production was first compared. Then the facts of what happens in the PHA-involved anaerobic digestion systems were explored. Finally, the hypothesis that further enhancement of methane production can be achieved by combining this PHA enriched sludge approach with other existing digester control strategies, as mentioned above, was confirmed. To our knowledge, this is the first study revealing details of how PHA-rich sludge enhances methane production. The findings presented here can guide engineers to develop some new strategies for improved operation of WWTPs.

MATERIALS AND METHODS

Source and Characteristics of Sludges with Different PHA Levels. The following five bioreactors were operated to produce sludges with different PHA levels since they are not available in real WWTPs. Seed sludge was collected from a municipal WWTP in Shanghai, China, and was simultaneously inoculated into five replicate sequencing batch reactors with working volumes of 40 L. Four cycles were performed daily in all reactors. Each cycle was comprised of 240 min aerobic and 55 min settling periods, followed by a 5 min decanting period and a 60 min idle period. To produce sludges with different PHA levels, these five bioreactors were fed synthetic wastewater containing 200, 400, 600, 800, or 1000 mg/L chemical oxygen demand (COD) with acetate as the sole organic carbon source. Hereinafter sludges wasted from these five reactors are defined as sludge I, sludge II, sludge III, sludge IV, and sludge V, respectively. Other components in these synthetic media were the same for each reactor, and the details are as follows (per liter): 0.1 g of NH_4Cl, 0.04 g of KH_2PO_4, 0.005 g of $CaCl_2$, 0.01 g of $MgSO_4{\cdot}7H_2O$, and 0.5 mL of a trace element solution. The components of the trace element solution are detailed in the literature.[28] All reactors were aerated at a flow rate of 20 L/min during the aerobic phase. Every cycle, 30 L of supernatant was discharged from each reactor after the settling phase and was replaced with 30 L of the respective wastewater during the first 6 min of the subsequent aerobic phase. At 1.5 h of the aerobic phase, approximately 5.7 L of the sludge mixture was withdrawn daily from each reactor. The sludge retention time was maintained at about 7 d in all reactors. After about 60 d of operation, the levels of protein, carbohydrate, and PHA in WAS wasted from the five reactors did not vary with the operational time significantly; these sludges were then employed in the following anaerobic digestion experiments. Before use, these wasted sludges were concentrated at 4 °C for 12 h.

Table S1 summarizes the main characteristics of sludges wasted from the five reactors after concentration. These sludges have approximately the same total COD levels. Protein, carbohydrate, and PHA are the major components in these sludges. With an increase of the sludge PHA, both the protein and carbohydrate levels decreased. Moreover, poly(hydroxybutyrate) (PHB) is the main PHA in all sludges, and the fractions of PHB, poly(hydroxyvalerate) (PHV), and poly(3-hydroxy-2- methylvalerate) (PH2MV) among these sludges are very similar.

Methane Production from Sludges with Different PHA Levels. This batch experiment was carried out in five serum bottles, each with a working volume of 1 L. Five serum bottles were first fed with 540 mL of sludge I, sludge II, sludge III, sludge IV, or sludge V. Then 300 mL of inoculum, which was collected from an anaerobic sludge digester in our laboratory, was equally divided and added to these five bottles. The volume ratio of sludge to inoculum was 9:1. This anaerobic sludge digester was fed with WAS wasted from a municipal WWTP in Shanghai, China. The total suspended solids (TSS), VSS, and total COD concentrations of inoculum were respectively 7.2 ± 0.2, 6.3 ± 0.2, and 7.8 ± 0.3 g/L, which resulted in feed/inoculum ratios of approximately 17.5 in TSS, 18.0 in VSS, and 17.3 in COD. One blank reactor was also conducted to test the methane productivity from the inoculum alone. This blank reactor contained 60 mL of inoculum and 540 mL of Milli-Q water without WAS. The pH in all mixtures was adjusted to 7.0 ± 0.1 by adding 4 M hydrochloric acid or 4 M

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262

sodium hydroxide, because it is generally accepted to be the suitable pH for methanogenic Archaea.[10,12] All bottles were flushed with nitrogen gas for 30 s to remove oxygen. Afterward, all serum bottles were capped with rubber stoppers, sealed, and placed in an air-bath shaker (120 rpm) at 37 ± 1 °C. The pH value in all reactors was maintained at 7.0 ± 0.1 during the whole digestion period by 4 M hydrochloric acid or 4 M sodium hydroxide with an automatic titrator. All tests were performed in triplicate. In the following batch tests, all experiments, unless otherwise described, were carried out in triplicate with a blank reactor containing 60 mL of inoculum and 540 mL of Milli-Q water. Moreover, all data reported below are net values, the values determined in the blank reactors having already been subtracted. During the digestion, the production of gas was periodically measured by releasing the pressure in the serum bottle using a 300 mL glass syringe to equilibrate with the room pressure according to the method reported previously.[29] The cumulative volume of methane was calculated by the following equation:

$$V_{M,i} = V_{M,i-1} + C_{M,i}V_{G,i} - C_{M,i-1}V_{G,i-1} \quad (1)$$

where $V_{M,i}$ and $V_{M,i-1}$ are respectively the cumulative volumes of methane in the current (i) and previous ($i - 1$) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are respectively the total gas volumes in the current and previous time intervals, and $C_{M,i}$ and $C_{M,i-1}$ are the respective percentages of methane gas measured by gas chromatography in the current and previous time intervals.

Operation of the Long-Term Semicontinuous Reactors for Monitoring Archaea Abundances and for Detection of Key Enzyme Activities. Two semicontinuous reactors were operated for this microbial study. The two reactors were fed with 540 mL of either sludge I or sludge V. All other operational conditions were the same as described for the above batch experiments, except the semicontinuous operation described here. According to the results of the above batch test, the maximal methane yield occurred at 25 d in the sludge I reactor and 19 d in the sludge V reactor; thus, the sludge retention time in the sludge I and sludge V reactors should be controlled at 25 and 19 d, respectively. On each day, 24 and 31.6 mL of digestion mixtures were manually taken from the sludge I and sludge V reactors, respectively. Then the respective same volumes of new sludge I and sludge V were added to the two reactors. To remove oxygen, both reactors were sparged with nitrogen gas for 30 s before they were recapped and resealed. The VSS loading rate was 0.49 kg/(m^3·d) in the sludge I reactor and 0.66 kg/(m^3·d) in the sludge V reactor. It took about 3 months for methane production from these two reactors to become relatively stable, and then the determinations of Archaea abundances and enzyme activities were made.

Comparison of Biochemical Methane Potential among Carbohydrate, Protein, and PHA. In this batch test, three identical serum bottles with a working volume of 1 L each were operated. The three reactors first received 0.52 g of dextran (average molecular weight 23800, a model polysaccharide compound), 0.52 g of bovine serum albumin (BSA; average molecular weight 67000, a model protein compound), or 0.52 g of PHA (88% PHB and 12% PHV, purchased from Sigma-Aldrich Chemical Co.). It should be noted that PHB and PHV are the major components of PHA in activated sludge involved in WWTPs. It is indicated that the amount of PHB and PHV usually accounts for more than 90% of the total PHA.[7] In the sludges of this study, PH2MV accounted for less than 5% of the total PHA (Table S1). Thus, only PHB and PHV were selected and tested in this study. After the addition of dextran, BSA, and PHA, 60 mL of identical inoculum from the same anaerobic sludge digester, as mentioned above, was added into each reactor. Then each reactor was diluted with tap water to 600 mL. The pH in all reactors was adjusted to 7.0 ± 0.1. After removal of oxygen with nitrogen gas, all serum bottles were capped, sealed, and placed in an air-bath shaker (120 rpm) at 37 ± 1 °C. All other digestion conditions of these three reactors were the same as those described for the above batch test.

Comparison of Anaerobic Digestion of Non-PHA Sludge with and without the Addition of PHA. Another batch experiment was conducted to assess the potential impact of PHA on the anaerobic digestion performance of sludge containing no detectable levels of PHA (this sludge hereafter is called non-PHA sludge). Two identical reactors with working volumes of 1 L were operated in this batch test. One received 60 mL of inoculum and 540 mL of non-PHA sludge, while the other received 60 mL of inoculum, 394 mL of non-PHA sludge, and 1.24 g of exogenous PHA (88% PHB and 12% PHV). The inoculum used here was collected from the same anaerobic sludge digester, as described above. The non-PHA sludge was withdrawn from the 1000 mg/L influent COD fed reactor at 6 h of aeration as it was measured that PHA was nondetectable at this time. After 12 h of settling at 4 °C, it was detected that this non-PHA sludge contained 12210 ± 370 mg/L VSS, 14460 ± 410 mg/L total COD, 582 ± 35 mg/g of VSS of protein, and 243 ± 20 mg/g of VSS of carbohydrate. The two digestion systems had approximately the same total COD (7.8 g of COD except for the inoculum). The pH in each reactor was also controlled at 7.0 ± 0.1. Both reactors were capped, sealed, and placed in an air-bath shaker (120 rpm) at 37 ± 1 °C after removal of oxygen with nitrogen gas. All other digestion conditions were also the same as those described for the other batch tests above. It took about 25 d for the two reactors to achieve maximal methane production.

Methane Production from WAS Anaerobic Digestion When the PHA-Based Method Was Combined with Other Pretreatment Strategies. Six reactors were used in this batch experiment. Three of the reactors (group I) were operated for the digestion of non-PHA sludge, while the other three (group II) were operated for the digestion of sludge V, taken directly from the parent reactor, which was enriched with PHA (Table S1). The sludges in reactor 1 of both groups were not pretreated and were set as the controls, whereas the sludges in the other two reactors of each group were pretreated either by incubation at 70 °C for 9 h or by incubation with FNA at 2.13 mg of FNA/L for 24 h according to the methods previously reported.[13,15] After pretreatment, the pH value in these four reactors was adjusted to 7.0 ± 0.1, and then 60 mL of inoculum was added to each reactor. All other operational conditions of the six reactors were the same as those described for the batch experiments depicted above.

Analytical Methods. The methane fraction in the gas samples was determined by use of a gastight syringe to inject 0.2 mL of the samples into a gas chromatograph (GC112A, China) equipped with a thermal conductivity detector and a 4 mm × 32 m stainless column with nitrogen as the carrier gas (flow rate 30 mL/min). The temperatures of the injection port, column, and detector were controlled at 40, 40, and 80 °C, respectively. The measurements of COD, VSS, and TSS were performed in accordance with standard methods.[30] The sludge

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262

levels of PHA, protein, carbohydrate, lipid, and volatile fatty acids were determined according to the methods reported in previous publications.[31,32] The molecular weight (M_w) distribution of the digestion liquid was determined by a gel-filtration chromatography analyzer (Shimadzu Co., Japan) as previously described.[6,7] The measurements of microbial EPSs including loosely bound EPSs and tightly bound EPSs of the sludge were the same as described in the literature.[33] The carbon, hydrogen, and nitrogen elemental compositions of the sludge were analyzed by an elemental analyzer (Elemental Analyzer NA 2500). Fluorescence in situ hybridization (FISH) was used to quantify the abundance of methanogenic Archaea in the sludges of the two long-term reactors as described in the Supporting Information. The oligonucleotide probe, ARC915, was employed to target Archaea, while the fluorescent stain 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the total cells. In addition, the detailed analytical procedures of protease, acetate kinase, and coenzyme F420 activities are also provided in the Supporting Information.

Statistical Analysis. All measurements were performed in triplicate. An analysis of variance was used to evaluate the significance of the results, and $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of the Sludge PHA Levels on Methane Production. Figure 1 shows the time curve of cumulative methane production from the anaerobic digestion reactors fed with sludges containing different PHA contents. The methane yield in the lowest PHA sludge (i.e., sludge I) reactor increased gradually with the digestion time from day 1 to day 25, and no significant increase occurred between 25 and 27 d ($p < 0.05$). The optimal digestion time for sludge I (21 ± 4 mg of PHA/g of VSS, Table S1) was 25 d, and at this time the maximal methane yield of 168.0 ± 4.2 mL/g of VSS was detected. It is seen that, with an increase of the sludge PHA, the methane production levels increased (Figure 1). For example, when sludge V (184 ± 16 mg of PHA/g of VSS, Table S1) was digested, the maximal methane yield of 246.1 ± 4.1 mL/g of VSS was determined at 19 d, which was 1.46 times that from sludge I digestion. However, the time required for maximum methane production for the high-PHA sludge was shorter than that required for the low-PHA sludges (Figure 1). A similar observation was made in the other high-PHA sludge reactors. Further investigation determined that the maximal methane yield correlated well with the starting PHA levels in the sludges ($R^2 = 0.9834$, Figure S1). All these results showed that the PHA levels in the sludge could affect methane production, which is consistent with the results obtained previously.[27] In the following experiments, the mechanisms of how PHA affects the methane production will be explored.

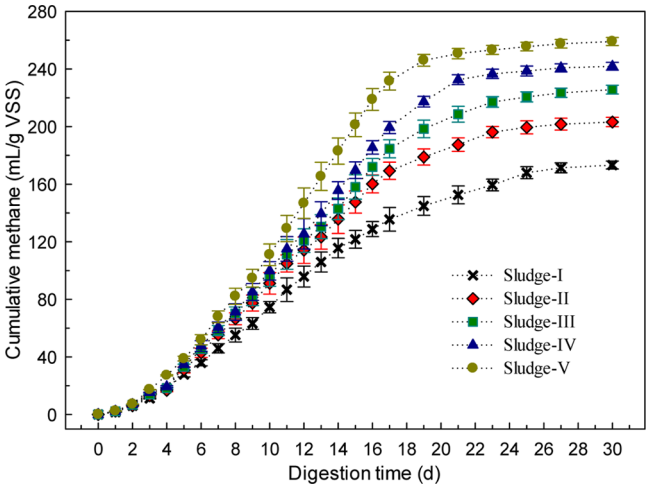


Figure 1. Cumulative methane production from anaerobically digested WAS containing different levels of PHA. Error bars represent the standard deviations of triplicate tests.

Does PHA Have a Higher Biochemical Methane Potential on a Unit VSS Basis Than the Other Main Cell Components? It is reported that PHA can be completely degraded and used as a substrate to produce methane under anaerobic conditions.[27,34,35] By determining the variation of the sludge PHA with the digestion time, it was also found that more than 96% of the sludge PHA was degraded already within the initial 84 h of the anaerobic digestion for all of the PHA-enriched sludges investigated in this study (Figure S2). However, it is unknown whether PHA has a higher methane production potential on a unit VSS basis in comparison to the other main constituents of the sludge. Protein and carbohydrate are generally considered to be the major components of a sludge cell. Apart from PHA, they are also the top two organic compounds, with their contents above 60% in all tested sludges in the current study (Table S1). Thus, the biochemical methane potentials among PHA, protein, and carbohydrate were first compared.

Table 1 shows the methane production from dextran (a model polysaccharide compound), BSA (a model protein compound), and PHA digestion. It can be seen that all three of these organic matters were progressively degraded in the anaerobic digestion over time. After 25 d of digestion, 97.5 ± 2.2% of BSA was degraded, while dextran and PHA were completely decomposed. On a unit VSS basis, the methane yield from PHA digestion was 260.5 ± 15.1 mL, whereas the

Table 1. Comparison of the Degradation Percentage and Methane Yield during Anaerobic Digestion of Dextran, BSA, and PHA[a]

digestion time (d)		dextran	BSA	PHA[b]
1	degradation percentage (%)	48.6 ± 4.2	29.1 ± 2.3	28.5 ± 3.6
2	degradation percentage (%)	72.9 ± 5.4	53.8 ± 5.6	53.4 ± 4.7
3	degradation percentage (%)	89.4 ± 4.3	69.6 ± 3.9	67.8 ± 4.5
4	degradation percentage (%)	92.6 ± 3.7	82.3 ± 5.1	83.9 ± 6.7
25	degradation percentage (%)	100 ± 0	97.5 ± 2.2	100 ± 0
	methane yield (mL)	214.7 ± 13.5	233.9 ± 15.7	260.5 ± 15.1

[a]Results are the averages and their standard deviations of triplicate tests. [b]PHA used here contained 88% PHB and 12% PHV.

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262

Table 2. Comparison of Soluble Substrates and VSS Reduction among the Reactors Fed with Different PHA Sludges after 1 d of Digestion[a]

parameter	sludge I	sludge II	sludge III	sludge IV	sludge V
soluble carbohydrate/total carbohydrate (%)	5.4 ± 0.3	6.2 ± 0.3	6.4 ± 0.6	6.8 ± 0.7	7.2 ± 0.8
soluble protein/total protein (%)	6.3 ± 0.4	7.1 ± 0.5	7.4 ± 0.4	7.9 ± 0.8	8.6 ± 0.9
soluble COD (mg/L)	161 ± 12	184 ± 11	193 ± 15	201 ± 18	209 ± 21
reduction of total VSS (%)	8.7 ± 0.8	13.1 ± 1.2	15.3 ± 1.5	17.8 ± 2.1	18.8 ± 1.9
VSS reduction of non-PHA composition[b] (%)	8.1 ± 0.6	9.5 ± 0.9	10.3 ± 1.1	10.7 ± 1.6	11.4 ± 1.7

[a]Results are the averages and their standard deviations of triplicate tests. [b]These values were calculated according to the following equation: total VSS (g) × measured VSS reduction ratio (%) = PHA reduction (g) + non-PHA cell composition (g) × VSS reduction ratio of non-PHA composition (%).

corresponding yields from dextran and BSA digestion were 214.7 ± 13.5 and 233.9 ± 15.7 mL, respectively. Consequently, per gram of substrate digested, PHA will generate more methane than dextran and BSA.

Anaerobic sludge digestion involves internal redox reactions that convert organic substrates to CH_4 and CO_2; the amounts of these gases produced are related to the composition of the digested sludge. For the digestion of protein and carbohydrate, the ideal stoichiometry can theoretically be written as eqs 2 and 3, respectively, such that for the digestion of 1 g of protein or dextran, the respective theoretical methane yields are 0.318 and 0.296 g (or 445.5 and 414.4 mL). In general, the biomass composition without PHA accumulation in wastewater and WAS treatment can be expressed as $C_5H_7O_2N$,[36] and its theoretical stoichiometry can be further written as eq 4. On the basis of this equation, it can be calculated that 0.249 g (or 349 mL) of methane will be produced per gram of sludge that is completely degraded.

$$C_{10}H_{20}O_6N_2\ (\text{protein}) + 3.5H_2O \rightarrow 5.25CH_4 + 4.75CO_2 + 2NH_3 \quad (2)$$

$$C_6H_{10}O_5\ (\text{dextran}) + H_2O \rightarrow 3CH_4 + 3CO_2 \quad (3)$$

$$C_5H_7O_2N\ (\text{cell}) + 3H_2O \rightarrow 2.5CH_4 + 2.5CO_2 + NH_3 \quad (4)$$

According to the theoretical stoichiometry of PHB and PHV degradation presented in eqs 5 and 6, it can be calculated that the theoretical methane production from PHB digestion is 0.419 g/g degraded (or 586.0 mL/g degraded), while 0.48 g/g degraded (or 672 mL/g degraded) of methane can be produced from PHV degradation. This value will reach 0.526 g/g degraded (or 736.8 mL/g degraded) if 1 g of PH2MV is completely degraded according to eq 7. It is seen that all components of PHA show higher biochemical methane potential than protein, dextran, and sludge not enriched with PHA, which is one reason for PHA promoting methane production.

$$C_4H_6O_2\ (\text{PHB}) + 1.5H_2O \rightarrow 2.25CH_4 + 1.75CO_2 \quad (5)$$

$$C_5H_8O_2\ (\text{PHV}) + 2H_2O \rightarrow 3CH_4 + 2CO_2 \quad (6)$$

$$C_6H_{10}O_2\ (\text{PH2MV}) + 2.5H_2O \rightarrow 3.75CH_4 + 2.25CO_2 \quad (7)$$

How Does PHA Affect the Process of Anaerobic Digestion? The following five steps are often included in sludge anaerobic digestion: solubilization, hydrolysis, acidification, acetogenesis, and methanogenesis. Methane is generated in the last step. If the four former steps are affected, the methane yield will inevitably be influenced. Thus, how PHA affects these processes of anaerobic digestion was investigated second.

Microbial PHA is typically accumulated intracellularly, and this suggests that the microbial cells need to be disrupted prior to the degradation of PHA to methane. Additionally, in general, cell disruption is a major limiting step in anaerobic digestion. Therefore, one hypothesis to explain the increased methane production is that intracellular PHA makes the cells more amenable to disruption. To investigate this possibility, we compared the ratio of soluble protein (carbohydrate) to total protein (carbohydrate) and the VSS reduction among the reactors fed with different PHA sludges after 1 d of digestion, and the results are summarized in Table 2. As the sludges tested in this study contained almost the same levels of protein and carbohydrate in the EPSs (Table S1), the change of the ratio soluble protein (carbohydrate) to total protein (carbohydrate) was used to indicate cell breakage. It was found that, with an increase of the sludge PHA, the ratio of soluble protein (carbohydrate) to total protein (carbohydrate) increased, which indicated that the increased PHA was beneficial to cell disruption. Another possible explanation is that PHA associates with protein in its biogenesis and degradation, and the degradation of PHA will increase the solubilization of protein. Further verification was provided by the VSS reduction data. Also, as seen in Table 2, both the reduction of total VSS and the reduction of VSS of non-PHA composition increased as the PHA content of the sludge increased. It has been observed previously that microbial cells are more fragile with increasing levels of intracellular PHA.[35,37,38] As a result, more soluble productions were released from these sludges containing higher PHA, which could thereby provide more substrates for the subsequent hydrolysis, acidification, and methanogenesis processes. It should be emphasized that the sludges used here were taken from different bioreactors. These sludges might have different microbial compositions and disruption thresholds, which might also affect cell disruption. However, a previous study showed that a PHA-rich sludge had a higher soluble protein rate and VSS reduction even using the sludge wasted from the same bioreactor.[7]

To further evaluate the potential impact of released PHA on the disruption of non-PHA cells, a batch test was performed on non-PHA sludge in the presence and absence of added PHA. It can be seen from Table 3 that although the total VSS reduction at 1 d of digestion in the non-PHA sludge with added PHA was greater than that in the non-PHA sludge alone, differences in both the reduction of non-PHA cell composition and the soluble carbohydrate to total carbohydrate ratio between the two reactors were insignificant ($p > 0.05$). These results

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262

Table 3. Comparison of Anaerobic Digestion from Non-PHA Sludge with and without Addition of Exogenous PHA[a]

digestion time (d)		non-PHA sludge	non-PHA sludge + exogenous PHA[b]
1	total VSS reduction (%)	8.5 ± 0.7	12.9 ± 1.1
	reduction of non-PHA cell composition[c] (%)	8.5 ± 0.7	8.6 ± 0.9
	soluble carbohydrate/total carbohydrate (%)	5.3 ± 0.3	5.1 ± 0.6
25	total VSS reduction (%)	39.3 ± 3.1	51.7 ± 3.4
	reduction of non-PHA cell composition[c] (%)	39.3 ± 3.1	39.1 ± 2.7
	soluble protein (mg of COD/L)	663 ± 43	375 ± 15
	protein consumption ratio (%)	32.6 ± 1.4	45.4 ± 3.6
	soluble carbohydrate (mg of COD/L)	15.0 ± 1.2	13.4 ± 1.0
	carbohydrate consumption ratio (%)	38.2 ± 1.4	38.4 ± 2.2
	methane produced (mL/g of VSS)	172 ± 6	197 ± 10

[a]Results are the averages and their standard deviations of triplicate tests. The measured C/N ratio was 7.06 ± 0.25 in non-PHA sludge and 9.32 ± 0.37 in exogenous-PHA-added non-PHA sludge. [b]The total VSS included both the non-PHA sludge and exogenous PHA. The exogenous PHA used in this study contained 88% PHB and 12% PHV. [c]These values were calculated on the basis of the following equation: total VSS (g) × total VSS reduction (%) = PHA reduction (g) + non-PHA cell composition (g) × reduction of non-PHA cell composition (%).

indicated that the lysis of non-PHA cells was unaffected by the released PHA.

After cell lysis, the released substrates undergo hydrolysis, acidification, and acetogenesis processes before they are finally bioconverted to methane. It may be that the hydrolysis rate of released PHA may be different from that of other cell components, which would affect the digestion time. Additionally, the released PHA may bring some positive or negative impacts on the digestion of other substrates, which would affect the methane yield.

It is seen that more than 96% of PHA was degraded within the first 84 h of digestion, irrespective of the initial PHA level in the sludge (Figure S2). However, only 92.6 ± 3.7% of dextran (model polysaccharide compound) and 82.3 ± 5.1% of BSA (model protein compound) were degraded after 96 h of digestion (Table 1). A similar observation has been made in other digestion studies. For example, Zhao et al. found that only 54.5% of BSA and 84% of dextran were degraded after 84 h during an alkaline anaerobic fermentation,[39] although Wang et al. observed that more than 94% of sludge PHA was decomposed within the initial 72 h of the same alkaline fermentation conditions.[7] It seems that intracellular PHA is hydrolyzed faster than the other main cell components of protein and carbohydrate. The degradation rate of PHA presented in Table 1, however, showed an inconsistent result. It was observed that the degradation rate of PHA was similar to that of BSA but lower than that of dextran. The reason for these inconsistent observations might be the different types of PHA tested. PHA used in the former experiments was intracellular PHA accumulated in activated sludge, whereas exogenous PHA was utilized in the latter test. It is reported that exogenous PHA tends to be crystallized, which may be more resistant to enzymatic attack in comparison to the intracellular PHA.[7]

Figure 2a shows a COD mass balance of the five digestion reactors at the time of maximal methane production. There is no significant variation between input and output COD ($p > 0.05$). With an increase of the sludge PHA, the COD output of

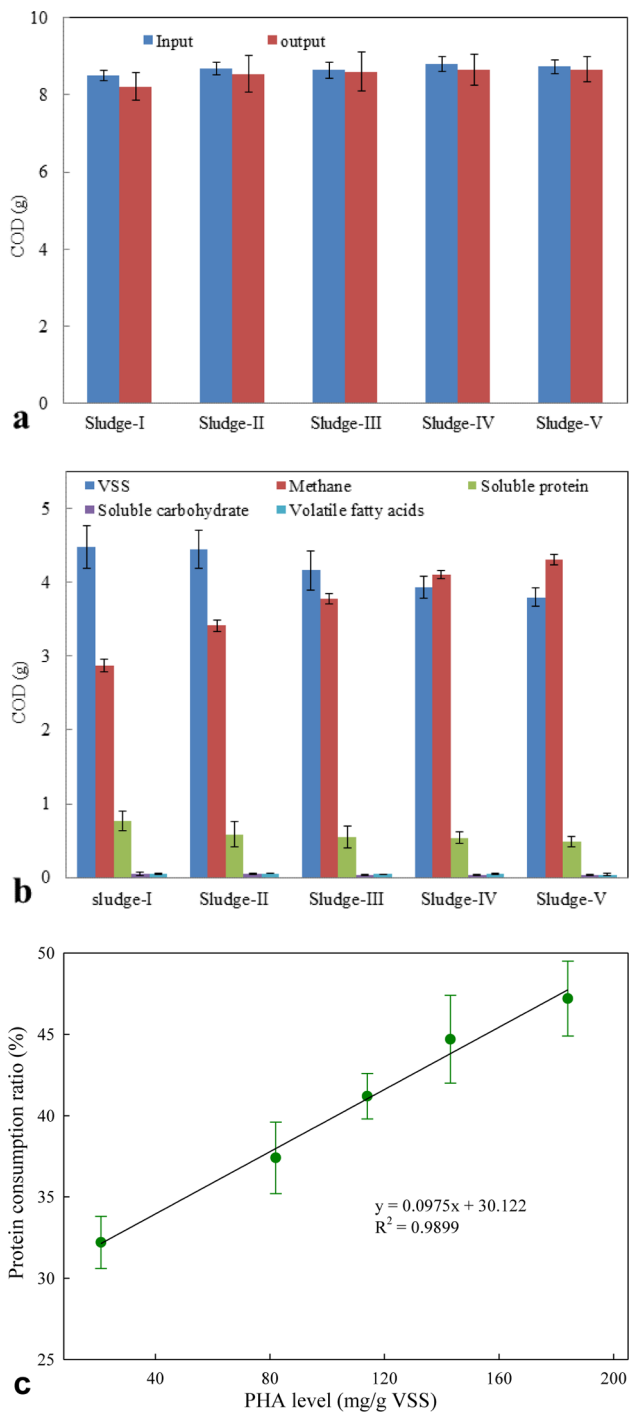


Figure 2. COD mass balance analysis (a), composition of output COD (b), and correlation between the PHA content and protein consumption ratio (c) in different digestion reactors at the time of maximal methane production. The input COD includes inoculum and WAS. The COD conversion coefficients are 1.42 g of COD/g of VSS, 4 g of COD/g of CH_4, 1.5 g of COD/g of protein, 1.06 g of COD/g of carbohydrate, 1.07 g of COD/g of acetic acid, 1.51 g of COD/g of propionic acid, 1.82 g of COD/g of butyric acid, and 2.04 g of COD/g of valeric acid.

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262

methane production increased, accompanied by a decreased of the remaining VSS ratio (Figure 2b). The amounts of volatile fatty acids and soluble carbohydrate were almost the same and at very low levels (<1%). The soluble protein showed a decreasing trend with an increase of the PHA level.

There are two possibilities that can cause this decrease. As the protein level in the higher PHA sludge is lower than that in the lower one, one possibility is that the released protein in the higher PHA sludge digestion reactor is lower, as compared with that in the lower one. The alternative explanation is that the higher PHA reactor consumed more soluble protein. Further analysis revealed that protein consumption had a linear positive correlation with the PHA levels (R^2 = 0.9899, Figure 2c). Similar results were also observed in the batch test of the non-PHA sludge in the presence and absence of added PHA (Table 3). This result supported the latter but not the former and indicated that the increased PHA level in the sludge benefited the conversion of released protein and methane production. It was seen that the C/N ratio in the sludge increased with increasing sludge PHA (Table S1), which may be the reason for the increased protein conversion. Previous publications have reported that an increase of the C/N ratio benefits the conversion of protein.[7,40] Additionally, it is seen that, as the sludge C/N ratio was increased or decreased, an improved or declined methane yield was consequently obtained during its anaerobic digestion.[12]

Verification of the above analysis regarding the hydrolysis process could be further provided by the M_w distribution of solubilized substrates. As shown in Figure 3a, solubilized substrates with low M_w in the sludge V (i.e., the highest PHA sludge) reactor were at higher levels than those in the sludge I (i.e., the lowest PHA sludge) reactor. Further study showed that the fraction of small soluble substrates with M_w < 1000 at 2 d of digestion correlated well with the sludge PHA (R^2 = 0.9673, Figure 3b). It is clear that the increased PHA level was beneficial to the hydrolysis process of solubilized substrates, which was another reason for the higher PHA sludge generating more methane.

Figure 3c presents the production of total volatile fatty acids in different PHA sludge reactors at the time of maximal methane production rate, and the concentration of individual volatile fatty acids among these reactors is detailed in Table S2. It can be seen that the levels of total volatile fatty acids and acetate were greater in the high-PHA sludge reactors than those in the lower ones. For example, 215.0 ± 12.6 mg of COD/g of VSS of total volatile fatty acids and 121.9 ± 7.9 mg of COD/g of VSS of acetate were measured in the sludge I reactor, while the corresponding levels were respectively 291.2 ± 15.9 and 165.7 ± 11.3 mg of COD/g of VSS in the sludge V reactor. It can be concluded that an increase of the PHA level also benefited sludge acidification and acetogenesis processes.

According to our investigations above, it can be found that the increased PHA in WAS not only enhanced cell disruption of PHA cells but also benefited the conversion of soluble protein of both PHA- and non-PHA cells, which thereby improved the sludge hydrolysis process. In addition, the reactor fed with the higher PHA sludge system showed greater sludge acidification and acetogenesis than that fed with the lower ones. Consequently, this provides an understanding to explain why the reactor fed with the higher PHA sludge produced more methane.

Comparison of Archaea Abundances and Key Enzyme Activities between the Two Long-Term Reactors. The abundance of methanogenic Archaea and the activities of key enzymes are directly relevant to the methane yield. Thus, we finally compared them in the two long-term reactors fed with different PHA sludges. It was detected that Archaea accounted for 34.5 ± 4.2% of the total cells in the lowest PHA sludge (i.e., sludge I) reactor (Figure 4a). When the reactor was fed with sludge V (the highest PHA sludge in this study), the abundance of Archaea increased to 52.6 ± 5.7% (Figure 4b). Figure 4c further exhibits the relative activities of key enzymes involved in hydrolysis, acidification, and methanogenesis processes. Protease, acetate kinase, and coenzyme F420, which are key enzymes for protein hydrolysis, acetate generation, and methane production, respectively, were selected to be assayed here. It can be seen that the activities of protease, acetate kinase, and coenzyme F420 in the sludge V reactor were 20.5%, 31.7%, and 48.7% higher than those in the sludge I reactor, respectively. All these observations are in accord with the above measured methane yield.

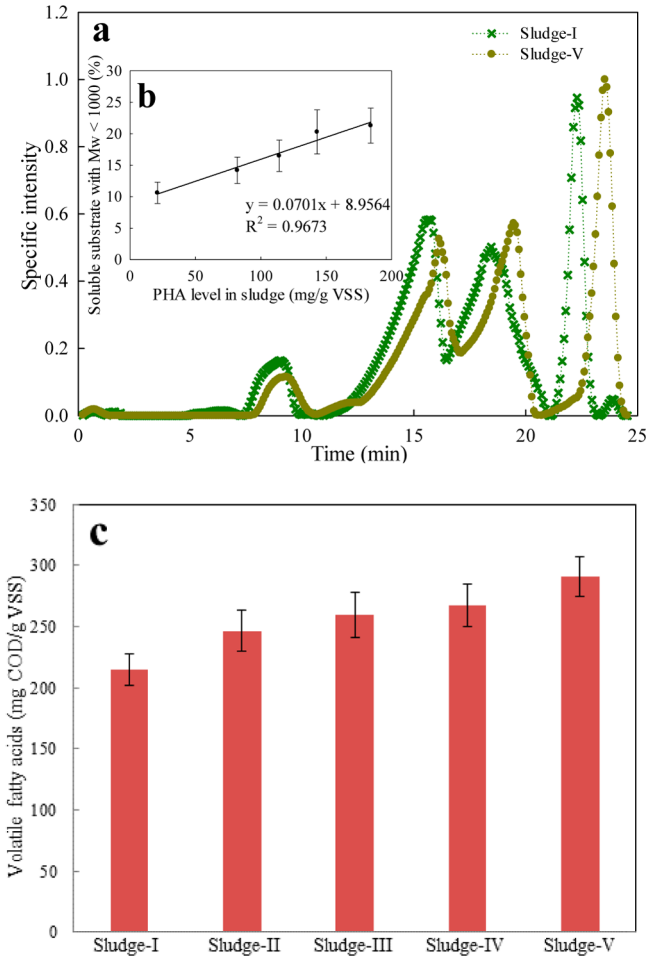


Figure 3. M_w distribution of soluble substrate in the sludge I and sludge V reactors (a), correlation between the sludge PHA content and percentage of soluble substrate with M_w < 1000 at 2 d of digestion time (b), and production of total volatile fatty acids in the different PHA sludge reactors at the time of maximal methane production rate, i.e., 12 d of digestion time (c). The data are the averages and their standard deviations of triplicate tests.

Improved Methane Production When Other Pretreatment Strategies Were Implemented. As demonstrated above, increased levels of intracellular PHA enhanced the methane production by modifying the characteristics of the

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262

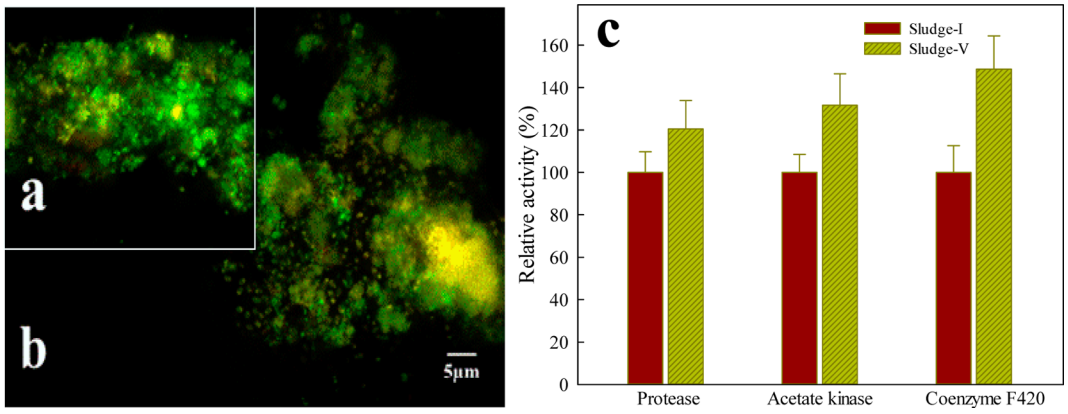


Figure 4. Fluorescence in situ hybridization image of microbial communities from the long-term reactors fed with sludge I (a) and sludge V (b) and comparison of the relative activities of protease, acetate kinase, and coenzyme F420 in the long-term-operated reactors (the unit for these enzymes is U/mg of VSS) after steady-state operation. The samples for FISH analysis were taken on day 120, while they were respectively taken on days 113, 120, and 127 for enzyme analysis. The oligonucleotide probe specific for Archaea was Arch915 (red), while fluorescent dye DAPI was used to target the total cells (green), and cells that were positive for both appeared yellow. Error bars represent standard deviations of triplicate measurements.

sludge. Thus, there is the possibility to further improve the methane production by application of other operational strategies of digestion. To investigate this hypothesis, two pretreatment methods, FNA and thermal, were tested, and the results are summarized in Table S3. When FNA and thermal pretreatments were applied, the sludge reduction and methane yields in the sludge V (high-PHA sludge) reactor increased from 56.4 ± 2.1% and 243.2 ± 4.5 mL/g of VSS to 65.3 ± 2.1% and 273.4 ± 4.8 mL/g of VSS and to 67.3 ± 2.5% and 291.7 ± 3.9 mL/g of VSS, respectively. It is clear that combining the PHA-enriched sludge approach with other digester strategies to improve methane production is feasible.

Implications for WWTPs. The phenomenon that PHA affects digestion, as explored in this work, provides a new approach to promote methane production from anaerobic sludge digestion. This is a recently found phenomenon; until now there has been little understanding of the microbial process. This study, for the first time, reveals details of how the intracellular PHA contributes to the increased methane production.

It is likely that this PHA effect has already had an unintentional impact on wastewater and WAS treatments. For example, a process configuration with two stages, called the A–B process, has been used for wastewater treatment for some time.[41] In such a process, most of the organic matter is adsorbed and stored by biomass at the A stage, and then the adsorbed/stored carbon is sent to the anaerobic digester for bioenergy recovery. Since volatile fatty acids are the main carbon sources in wastewaters, PHA is inevitably the major constituent of biomass storage. It is reported that the VSS reduction during the anaerobic digestion of an A-stage sludge was at ~70%, whereas the corresponding value during digestion of the general WAS was only around 40%.[13,42] This difference could be partly due to the effect of PHA storage on the digestion. Therefore, this work reveals an explanation for a previously unrecognized feature of sludge digestion, which may be of importance for future operation of WWTPs.

With the growing worldwide energy crisis, WWTPs are increasingly recognized as places for energy recovery rather than waste removal.[43] As a result, there is an ongoing paradigm shift in the operation of WWTPs from pollutant removal to energy recovery. The findings obtained in this work can enlighten and further guide engineers to develop improved strategies for WWTP operation to enhance energy recovery, which provide strong support to this ongoing paradigm shift. Consequently, we suggest a scheme for "wastewater treatment–WAS digestion" that includes the PHA-enriched sludge approach in an integrated environmental and economic perspective (Figure S3). In bioreactors of the proposed WWTP, organic compounds in wastewater are designed to be mainly removed by PHA accumulation instead of by CO_2 formation and cell growth. Following that, the sludge containing a high level of PHA is sent to the anaerobic digester for methane production. In such a WWTP operation, aeration costs are reduced, the amount of sludge for dewatering and final disposal is decreased, and the methane yield is enhanced.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03112.

Additional analytical methods, Tables S1–S3, and Figures S1–S3 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Phone: +86-731-8-8823701; fax: +86-731-8-8823701; e-mail: zgming@hnu.edu.cn.

*Phone: +86-21-65981263; fax: +86-21-65986313; e-mail: yg2chen@yahoo.com.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was financially supported by the National Natural Science Foundation of China (NSFC) (Grants 51508178, 51521006, and 51425802) and China Postdoctoral Science Foundation (Grant 2014M550249).

■ REFERENCES

(1) Hao, X.; Wang, Q.; Cao, Y.; van Loosdrecht, M. C. M. Evaluating sludge minimization caused by predation and viral infection based on the extended activated sludge model No. 2d. *Water Res.* **2011**, *45*, 5130–5140.

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253–12262

(2) Zhao, J.; Wang, D.; Li, X.; Yang, Q.; Chen, H.; Zhong, Y.; An, H.; Zeng, G. An efficient process for wastewater treatment to mitigate free nitrous acid generation and its inhibition on biological phosphorus removal. *Sci. Rep.* **2015**, *5*, 8602.
(3) Ni, B.; Yu, H. Growth and storage processes in aerobic granules grown on soybean wastewater. *Biotechnol. Bioeng.* **2008**, *100*, 664−672.
(4) Wang, Q.; Ye, L.; Jiang, G.; Yuan, Z. A free nitrous acid (FNA)-based technology for reducing sludge production. *Water Res.* **2013**, *47*, 3663−3672.
(5) Gao, Y.; Peng, Y.; Zhang, J.; Wang, S.; Guo, J.; Ye, L. Biological sludge reduction and enhanced nutrient removal in a pilot-scale system with 2-step sludge alkaline fermentation and A2O process. *Bioresour. Technol.* **2011**, *102*, 4091−4097.
(6) Wang, D.; Chen, Y.; Zheng, X.; Li, X.; Feng, L. Short-chain fatty acid production from different biological phosphorus removal sludges: The influences of PHA and gram-staining bacteria. *Environ. Sci. Technol.* **2013**, *47*, 2688−2695.
(7) Wang, D.; Zeng, G.; Chen, Y.; Li, X. Effect of polyhydroxyalkanoates on dark fermentative hydrogen production from waste activated sludge. *Water Res.* **2015**, *73*, 311−322.
(8) Chen, Y.; Li, X.; Zheng, X.; Wang, D. Enhancement of propionic acid fraction in volatile fatty acids produced from sludge fermentation by the use of food waste and *Propionibacterium Acidipropionici*. *Water Res.* **2013**, *47*, 615−622.
(9) Chen, H.; Chen, Y.; Zheng, X.; Li, X.; Luo, J. How does the entering of copper nanoparticles into biological wastewater treatment system affect sludge treatment for VFA production. *Water Res.* **2014**, *63*, 125−134.
(10) Appels, L.; Baeyens, J.; Degrève, J.; Dewil, R. Principles and potential of the anaerobic digestion of waste-activated sludge. *Prog. Energy Combust. Sci.* **2008**, *34*, 755−781.
(11) Carrère, H.; Dumas, C.; Battimelli, A.; Batstone, D. J.; Delgenès, J. P.; Steyer, J. P.; Ferrer, I. Pretreatment methods to improve sludge anaerobic degradability: A review. *J. Hazard. Mater.* **2010**, *183*, 1−15.
(12) Zhang, D.; Chen, Y.; Zhao, Y.; Zhu, X. New sludge pretreatment method to improve methane production in waste activated sludge digestion. *Environ. Sci. Technol.* **2010**, *44*, 4802−4808.
(13) Wang, Q.; Ye, L.; Jiang, G.; Jensen, P. D.; Batstone, D. J.; Yuan, Z. Free nitrous acid (FNA)-based pretreatment enhances methane production from waste activated sludge. *Environ. Sci. Technol.* **2013**, *47*, 11897−11904.
(14) Abelleira-Pereira, J. M.; Pérez-Elvira, S. I.; Sánchez-Oneto, J.; de la Cruz, R.; Portela, J. R.; Nebot, E. Enhancement of methane production in mesophilic anaerobic digestion of secondary sewage sludge by advanced thermal hydrolysis pretreatment. *Water Res.* **2015**, *71*, 330−340.
(15) Zhang, D.; Chen, Y.; Zhao, Y.; Ye, Z. A new process for efficiently producing methane from waste activated sludge: Alkaline pretreatment of sludge followed by treatment of fermentation liquid in an EGSB reactor. *Environ. Sci. Technol.* **2011**, *45*, 803−808.
(16) Kallistova, A. Y.; Goel, G.; Nozhevnikova, A. N. Microbial diversity of methanogenic communities in the systems for anaerobic treatment of organic waste. *Microbiology* **2014**, *83*, 462−483.
(17) Chen, Y.; Wang, D.; Zheng, X.; Li, X.; Feng, L.; Chen, H. Biological nutrient removal with low nitrous oxide generation by cancelling the anaerobic phase and extending the idle phase in a sequencing batch reactor. *Chemosphere* **2014**, *109*, 56−63.
(18) Wang, D.; Li, X.; Yang, Q.; Zheng, W.; Wu, Y.; Zeng, T.; Zeng, G. Improved biological phosphorus removal performance driven by the aerobic/extended-idle regime with propionate as the sole carbon source. *Water Res.* **2012**, *46*, 3868−3878.
(19) Wang, D.; Zheng, W.; Liao, D.; Li, X.; Yang, Q.; Zeng, G. Effect of initial pH control on biological phosphorus removal induced by the aerobic/extended-idle regime. *Chemosphere* **2013**, *90*, 2279−2287.
(20) Ni, B.; Yu, H. Storage and growth of denitrifiers in aerobic granules: Part I. Model development. *Biotechnol. Bioeng.* **2008**, *99*, 314−323.
(21) Takabatake, H.; Satoh, H.; Mino, T.; Matsuo, T. PHA (polyhydroxyalkanoate) production potential of activated sludge treating wastewater. *Water Sci. Technol.* **2002**, *45*, 119−126.
(22) Coats, E. R.; Loge, F. J.; Wolcott, M. P.; Englund, K.; McDonald, A. G. Synthesis of polyhydroxyalkanoates in municipal wastewater treatment. *Water Environ. Res.* **2007**, *79*, 2396−2403.
(23) Moralejo-Gárate, H.; Kleerebezem, R.; Mosquera-Corral, A.; van Loosdrecht, M. C. M. Impact of oxygen limitation on glycerol-based biopolymer production by bacterial enrichments. *Water Res.* **2013**, *47*, 1209−1217.
(24) Kasemsap, C.; Wantawin, C. Batch production of polyhydroxyalkanoate by low-polyphosphate-content activated sludge at varying pH. *Bioresour. Technol.* **2007**, *98*, 1020−1027.
(25) Verlinden, R. A.; Hill, D. J.; Kenward, M. A.; Williams, C. D.; Radecka, I. Bacterial synthesis of biodegradable polyhydroxyalkanoates. *J. Appl. Microbiol.* **2007**, *102*, 1437−1449.
(26) Jiang, Y.; Marang, L.; Kleerebezem, R.; Muyzer, G.; van Loosdrecht, M. C. M. Polyhydroxybutyrate production from lactate using a mixed microbial culture. *Biotechnol. Bioeng.* **2011**, *108*, 2022−2035.
(27) Huda, S. M. S.; Satoh, H.; Mino, T. Anaerobic digestion of polyhydroxybutyrate accumulated in excess activated sludge. *J. Water Environ. Technol.* **2013**, *11*, 429−438.
(28) Wang, D.; Li, X.; Yang, Q.; Zeng, G.; Liao, D.; Zhang, J. Biological phosphorus removal in sequencing batch reactor with single-stage oxic process. *Bioresour. Technol.* **2008**, *99*, 5466−5473.
(29) Owen, W. F.; Stuckey, D. C.; Healy, J. B., Jr.; Young, L. Y.; McCarty, P. L. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Res.* **1979**, *13*, 485−492.
(30) *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; American Public Health Association (APHA), American Water Works Association, and Water Environment Federation: Washington, DC, 1998.
(31) Wang, D.; Li, X.; Yang, Q.; Zheng, W.; Liu, Z.; Liu, Y.; Cao, J.; Yue, X.; Shen, T.; Zeng, G.; Deng, J. The probable metabolic relation between phosphate uptake and energy storages formations under single-stage oxic condition. *Bioresour. Technol.* **2009**, *100*, 4005−4011.
(32) Yuan, H.; Chen, Y.; Zhang, H.; Jiang, S.; Zhou, Q.; Gu, G. Improved bioproduction of short-chain fatty acids (SCFAs) from excess sludge under alkaline conditions. *Environ. Sci. Technol.* **2006**, *40*, 2025−2029.
(33) Mu, H.; Zheng, X.; Chen, Y.; Chen, H.; Liu, K. Response of anaerobic granular sludge to a shock load of zinc oxide nanoparticles during biological wastewater treatment. *Environ. Sci. Technol.* **2012**, *46*, 5997−6003.
(34) Reischwitz, A.; Stoppok, E.; Buchholz, K. Anaerobic degradation of poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-3-hydroxyvalerate. *Biodegradation* **1997**, *8*, 313−319.
(35) Budwill, K.; Fedorak, P. M.; Page, W. J. Methanogenic Degradation of Poly(3-Hydroxyalkanoates). *Appl. Environ. Microb.* **1992**, *58*, 1398−1401.
(36) Chen, Y.; Wang, D.; Zhu, X.; Zheng, X.; Feng, L. Long-term effects of copper nanoparticles on wastewater biological nutrient removal and N_2O generation in the activated sludge process. *Environ. Sci. Technol.* **2012**, *46*, 12452−12458.
(37) Page, W. J.; Cornish, A. Growth of Azotobacter vinelandii UWD in fish peptone medium and simplified extraction of polybhydroxybutyrate. *Appl. Environ. Microb.* **1993**, *59*, 4236−4244.
(38) Lee, S. Y. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.* **1996**, *49*, 1−14.
(39) Zhao, Y.; Chen, Y.; Zhang, D.; Zhu, X. Waste activated sludge fermentation for hydrogen production enhanced by anaerobic process improvement and acetobacteria inhibition: the role of fermentation pH. *Environ. Sci. Technol.* **2010**, *44*, 3317−3323.
(40) Feng, L.; Chen, Y.; Zheng, X. Enhancement of waste activated sludge protein conversion and volatile fatty acids accumulation during waste activated sludge anaerobic fermentation by carbohydrate substrate addition: the effect of pH. *Environ. Sci. Technol.* **2009**, *43*, 4373−4380.

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262

(41) Versprille, A. I.; Zuurveen, B.; Stein, T. The AeB process: a novel two stage wastewater treatment system. *Water Sci. Technol.* **1984**, *17*, 235−246.
(42) Ge, H.; Batstone, D. J.; Keller, J. Operating aerobic wastewater treatment at very short sludge ages enables treatment and energy recovery through anaerobic sludge digestion. *Water Res.* **2013**, *47*, 6546−6557.
(43) Li, W.; Yu, H.; He, Z. Towards sustainable wastewater treatment by using microbial fuel cells-centered technologies. *Energy Environ. Sci.* **2014**, *7*, 911−924.

DOI: 10.1021/acs.est.5b03112
Environ. Sci. Technol. 2015, 49, 12253−12262