Research article

Evolution of microbial community during dry storage and recovery of aerobic granular sludge

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ABSTRACT

Aerobic granular sludge (AGS) was imbedded in agar and stored at 4 °C for 30 days, and then the stored granules were recovered in a sequencing batch reactor fed real wastewater within 11 days. Variations in microbial community compositions were investigated during dry storage and recovery of AGS, aiming to elucidate the mechanism of granular stability loss and recovery. The storage and recovery of AGS involved microbial community evolution. The dominant bacterial genera of the mature AGS were Zoogloea (relative abundance of 22.39%), Thauera (16.03%) and Clostridium sensu stricto (11.17%), and those of the stored granules were Acidovorax (26.79%), Macellibacteroides (12.3.6%) and Pseudoxanthomonas (5.69%), respectively. However, the dominant genera were Streptococcus (43.64%), Clostridium sensu stricto (12.3.6%) and Lactococcus (11.47%) in the recovered AGS. Methanogens were always the dominant archaeal species in mature AGS (93.01%), stored granules (99.99%) and the recovered AGS (94.84%). Facultative anaerobes and anaerobes proliferated and dominated in the stored granules, and their metabolic activities gradually led to granular structure destruction and property deterioration. However, the stored granules served as carriers for the microbes originated from the real septic tank wastewater during recovery. They proliferated rapidly and secreted a large number of extracellular polymeric substances which helped to recover the granular structure in 11 days.

1. Introduction

Aerobic granular sludge (AGS) is a biological aggregate formed by the self-aggregation of a large number of microorganisms, which has the advantages of a large settling velocity, tolerance to high toxicity, simultaneous nitrogen and phosphorus removal (Xia et al., 2018). To date, several aerobic granular projects with good pollutant removal performance have been built, and the operational results have indicated that the process could significantly reduce the construction and operational cost (Li et al., 2014; Pronk et al., 2015; Świetczak and Cydzik-Kwiatkowska, 2018). Therefore, AGS is considered to be a promising biological wastewater treatment technology for the 21st century. However, the cultivation of AGS is a time-consuming process (Xia et al., 2018), and AGS technology will inevitably have excess sludge and sludge treatment problems at the scale of reactor capacity. Thus, storage and reuse of AGS have attracted some scholars’ concerns. Currently, there are two main kinds of granular storage methods: dry storage and wet storage of AGS. Between them, wet storage of AGS is adopted by most scholars, as there are large quantities of aquatic microbes that inhabit AGS (Tay et al., 2002; Zhu and Wilderer, 2003; Zhang et al., 2005; Gao et al., 2012; Yuan et al., 2012; He et al., 2017). In contrast, limited studies of dry storage of AGS have thus far been reported (Hu et al., 2016; Cheng et al., 2018; Lv et al., 2018), and the dilemma is restricted mainly by complex sludge dewatering processes.

Although existing preservation methods of AGS differ greatly, they cannot inhibit the granular stability loss regardless of how complicated the method adopted is. It was found that granular structure destruction or activity decrease was usually detected and even accompanied by substantial migration and transformation between different phases (Gao et al., 2012; He et al., 2017; Zeng et al., 2007; Xu et al., 2010). Therefore, methods that can be employed to effectively maintain the stability of AGS are in high demand. Compared with the reactor operating environment that maintains intense substance and energy conversion (Ali et al., 2019), the storage environment of AGS is relatively stable, in which in-situ information inside granules during storage can be detected. Zhu and
Wilderer (2003) reported that sulfides released by microbial endogenous respiration darkened the surface of the AGS during storage. Wan et al. (2014a,b) found that the harsh storage environment stimulated cell secretion of cyclic di-guanosine monophosphate and pentaphosphate. The former can promote the transition of cells from a motile state to an aggregate state (Wan et al., 2013), while the latter inhibits ribose nucleic acid (RNA) synthesis and deoxyribonucleic acid (DNA) replication, resulting in microbial cells entering viable but non-culturable status for self-protection. In addition, most studies revealed that the instability of AGS was ascribed to EPS degradation during storage (Adav et al., 2009; Xu et al., 2010; Gao et al., 2012; He et al., 2017; Cheng et al., 2018). To elucidate the mechanism of AGS stability loss during storage, scholars tend to analyse the microbial community changes to infer the metabolic pathways involved. However, most studies were based on wet storage of AGS (Adav et al., 2009; Lv et al., 2013; Wan et al., 2014a,b; He et al., 2017), and little relevant information could be found during dry storage of AGS (Lv et al., 2018). Interestingly, the stored AGS could be recovered to normal within several weeks after re-aeration as inoculated sludge (Zhang et al., 2005; Zeng et al., 2007; Gao et al., 2012; Yuan et al., 2012; Lv et al., 2018; Hu et al., 2016; He et al., 2017), while it usually takes months from floc to AGS. The results indicate that the stored AGS is still a useful biological resource and has a positive significance for shortening the start-up time of the reactor.

To achieve dry storage and reuse of AGS, the storage of embedded AGS and its recovery were investigated in our previous work (Cheng et al., 2018). The results showed that the simple agar-embedding method was beneficial for granular morphology observation. Although granular activity decreased and the microstructure was destroyed, the AGS recovered within 11 days after re-aeration. The granular mass loss was 1.6393 g after 30 days of dry storage, which confirmed that substantial migration and transformation occurred between the gas and solid phases. AGS is composed of different types of functional bacteria (Xia et al., 2018), and the microbial community will vary with environmental change, which is the source of property changes of AGS. Therefore, the evolution of the microbial community was analysed to explore the microbial metabolic pathways during dry storage and reactivation of embedded AGS, which aims to reveal the mechanism of AGS stability loss and lay a theoretical basis for efficient application of AGS in wastewater treatment.

2. Materials and methods

2.1. Storage of AGS

The sludge-liquid mixture from a laboratory-scale sequencing batch reactor (SBR) fed organic simulated wastewater was screened through a 0.3 mm standard sieve, and the obtained AGS was collected and washed three times with tap water. Then, the granules were embedded in an open container (inner diameter of 14 cm, height of 20 cm) with a 3% agar solution, and placed in a refrigerator at 4 °C after solidification. SV30/SV5 (SV: sludge volume) & sludge volume index (SVI) of the AGS were 0.91 ± 0.02 and 45.87 ± 7.53 mL/g, respectively, mixed liquor volatile suspended solid/mixed liquor suspended solid (MLVSS/MLSS) was 0.56 ± 0.05, respectively, extracellular polymeric substances (EPS) & polysaccharides/proteins ratio (PN/PS) were 129.54 ± 16.47 mg/g MLSS and 0.55 ± 0.16, respectively, specific oxygen utilization rate (SUR) and SOURHeterotrophic bacteria/SOURNitrifying bacteria (SOURR/SOURN) were 37.14 ± 4.36 mg O2/g (MLSS h) and 5.59 ± 1.86, respectively, and the granule radius & average particle size were 92.79% ± 2.66% and 1.87 ± 0.11 mm, respectively.

2.2. Analytical methods

SV, SVI, MLSS and MLVSS were determined according to standard methods (APHA, 2005). Sludge with a particle size larger than 0.3 mm was defined as AGS. The size distribution was measured by the wet sieving separation method, the average particle size was calculated from the mass distribution curve, a heat extraction method was adopted to extract EPS from AGS and other detection methods (such as SOUR & scanning electron microscope), were applied as suggested by Long et al. (2019).

2.3. Microbial communities

Samples of mature AGS (A1) to be stored, granules stored after 30 days (A2) and recovered AGS (A3) were washed three times with distilled water. The sample used for high-throughput sequencing was a mixture of three parallel sludge samples obtained under the same condition. DNA was extracted by using an E.Z.N.A.TM Soil DNA Kit (Omega, Bio-Tek, Norcross, GA, USA) according to the manufacturer’s instructions. Then, a Qubit 2.0 DNA detection kit was used to exactly quantify the amount of DNA for polymerase chain reaction (PCR). The polymerase chain reaction (PCR) primers were the V3–V4 universal primers 341F (CCCTACGAGCCGTCTTCCGATCTG) and 805R (GACTGGAGTTCCTTGGCACCCGAG AATTCCAGACTACHVGGGTATCTAACTC). The detailed first and second amplification processes were applied as described by Chen et al. (2017). Finally, the extracted DNA was subjected to sequencing analysis of the V3–V4 region of the 16S rDNA gene with the MiSeq sequencing platform (Illumina, Inc., San Diego, CA, USA) in Sangon Biotech Co., Ltd., Shanghai, China. Shannon and Simpson indices are often used to estimate the microbial diversity of the samples. Greater Shannon index means higher community diversity, while the higher Simpson index indicates lower community diversity. The two indices are calculated as follows:

\[
H_{\text{shannon}} = \sum_{i=1}^{n_{\text{obs}}} \frac{n_i}{N} \ln \frac{n_i}{N},
\]

\[
D_{\text{simpson}} = \frac{\sum_{i=1}^{n_{\text{obs}}} n_i (n_i - 1)}{N(N - 1)}.
\]

Where

\(S_{\text{obs}}\)-actual number of operational taxonomic unit (OTU) observed;
\(n_i\)-the number of sequences contained in the ith OUT;
N- the total number of sequences.

3. Results and discussion

3.1. Variations in the properties of AGS

Most granules maintained their colour and appearances after 30 days of storage. Only a small number of granules with black cores were observed. However, it was found that a large number of holes were formed on the surface of the stored granules, as observed by SEM, and the mass of the AGS decreased by 45.17%. Comparing the granular properties before and after storage, it was found that SVI and SV30/SV5 had no large changes, but MLVSS/MLSS decreased by 62.5%, EPS decreased by 86.0%, SOUR decreased by 72.4%, the granulation rate decreased by 10.2%, and the average particle size decreased by 9.1%. The results indicated that granular stability decreased significantly during storage. The stored granules were then inoculated into a SBR fed real septic tank wastewater for reactivation, and most properties recovered to the levels before storage in 11 days. The storage and reactivation process were discussed in detail by Cheng et al. (2018).

3.2. Variations in microbial community compositions during storage

3.2.1. Bacterial communities

High-throughput sequencing results showed that the coverage of the sample (A1 and A2) sequences was both 0.95 (Table 1), which can truly
Table 1. OTUs, richness and diversity of bacteria and archaea during storage.

| Sample          | Sequencing Number | OTU Number | Richness index | Diversity index | Coverage |
|-----------------|-------------------|------------|----------------|-----------------|----------|
|                 |                   |            | ACE            | Chao1           | Shannon  | Simpson |
| Bacteria (A1)   | 33514             | 2173       | 37404.63       | 14616.88        | 3.98     | 0.07    | 0.95   |
| Bacteria (A2)   | 34245             | 2440       | 29746.90       | 14331.97        | 4.19     | 0.07    | 0.95   |
| Archaea (A1)    | 49344             | 1426       | 33914.12       | 12379.09        | 3.21     | 0.09    | 0.98   |
| Archaea (A2)    | 59029             | 1225       | 61351.97       | 23927.69        | 1.66     | 0.28    | 0.98   |

reflect the microbial community structures of the granules. Community richness indices (Chao1 and ACE) of the stored granules were lower than those of the mature AGS, indicating that the bacterial richness of the former decreased. Compared with that of the mature AGS, the Simpson index of the stored granules remained unchanged, but the Shannon index increased, indicating that the diversity of the stored granules increased.

Bacterial community compositions of the mature AGS and the stored granules were distributed among 9 phyla, 17 classes and 55 genera (Table 2). At the phylum level, mature AGS (A1) and the stored granules (A2) both consisted of 6 phyla. Among them, the three identical phyla were Proteobacteria, Firmicutes and Bacteroidetes. Proteobacteria is the largest phylum of bacteria. All Proteobacteria are gram-negative bacteria, including varieties of nitrogen-fixing bacteria, nitrifying bacteria and denitrifying bacteria. Most species of Firmicutes are gram-positive bacteria, including varieties of anaerobes and facultative anaerobes that are able to resist dehydration and survive in extreme environments. Most species of Bacteroides are gram-negative, anaerobic bacteria. They participate in many important metabolic activities, including fermentation of carbohydrates and utilization of nitrogenous substances. During the storage process, Verrucomicrobia, Planctomycetes and Acidobacteria disappeared, but 3 new phyla appeared, which were Eusikismicrobia, Spicrochaetes and Lentisphaeraceae. At the class level, there were 14 and 12 classes in mature AGS and stored granules. Among them, the 8 identical classes were Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Alphaproteobacteria, Negativicutes, Flavobacteria, Bacteroidia and Sphingobacteria. During the storage process, 5 classes, namely, Cytophaga, Planctomycetes, Verrucomicrobiae, Spartobacteria and Acidobacteria Gp3, disappeared, but 3 new classes (Endomicrobia, Spirochaeta and Lentisphaeraceae) appeared.

At the genus level, the mature AGS and stored granules contained 39 and 30 genera respectively, of which 14 identical genera were retained during storage. The relative abundance of Acidovorax (9.62%–26.79%) and Paludibacter (0.85%–5.24%) increased significantly, and Acidovorax eventually became the dominant bacteria with the maximum abundance. Acidovorax is not only a common plant pathogen in the world but also a wastewater treatment functional bacterium with strong degradation ability (Fan et al., 2008). Most species of Paludibacter are facultative anaerobes or anaerobes that can utilize many kinds of carbon sources (Qiu et al., 2014a). The relative abundance of Zoogloea (22.39%–0.46%), Thauera (16.03%–2.7%), Chryseobacterium (7.99%–3.08%) and Flavobacterium (2.95%–1.6%) all decreased obviously. Zoogloea is one of the most important aerobic bacterial genera in wastewater treatment (Xia et al., 2018), and it was not surprising that a low-oxygen and substrate-deficient environment led to the death of most of the species. Thauera (16.03%–2.7%) and Chryseobacterium (7.99%–3.08%) are capable of denitrification (Xia et al., 2018), but their growth was inhibited due to the lack of nitrite or nitrate. It was found that Flavobacterium (2.95%–1.6%) could only use a few polysaccharides. Pseudoxanthomonas, Aeromonas, Bdellovibrio, Gemmobacter, Devesia, Succinispira, Sunxuqiang and Pottibacter, in contrast, showed little change (abundances less than 0.82%), indicating that they were very adaptable.

Twenty-five genera with a total abundance of 17.68% were eliminated during storage. Among them, Nitrosomonas, Arenimonas, Aquimona, Acinetobacter, Reyrunella, Novosphingobium, Ferruginibacter, Pedobacter, Roseibacillus and Brevibacillus were mainly strictly aerobic bacteria, while Clostridium sensu stricto, Proteocatella and Phascolarctobacterium were strictly anaerobic bacteria, which were apt to grow under high pH. Sphingopyxis, Proteocatella, Phascolarctobacterium, Sediminibacterium, Chryseolinea, Prosthecobacter and Terrimicrobium and Gp3 originally had low relative abundances (each of them did not exceed 0.36%), so it was speculated that they were eliminated due to a lack of appropriate temperature, pH or carbon source. Sixteen new genera with a total relative abundance of 24.4% appeared during storage, which satisfactorily explained the bacterial diversity increase in the stored granules. Most of them were facultative anaerobes or anaerobes. For example, Rhodofexar (0.58%), Desulfovibrio (0.52%) and Flavivolina (0.41%), are capable of using a variety of electron acceptors, such as nitrate, nitrite, heavy metal, Fe(III), sulfate and so on (Dahlak and Kim, 2018). Propionivibrio (0.32%), Pseudorhodothrobacter (0.37%), Clostridium III (5.14%), Rumimococcus (0.29%), Acetanaeroaerobium (0.71%), Anaerovarax (0.42%), Macellibacteroides (12.83%), Prevotella (1%) and Viciuillus (0.34%), are all organics-fermenting anaerobes. In addition, Macellibacteroides can use many carbon sources in a wide pH range (Jabari et al., 2012), while Clostridium III has a strong toxicity resistance. The results showed that a large number of aerobic bacteria disappeared under the low-oxygen and substrate-deficient environment, and anaerobic bacteria were enriched by decomposing their bodies.
| Phylum                  | Class           | Genus               | Relative Abundance (%) | Function                                      |
|------------------------|-----------------|---------------------|------------------------|-----------------------------------------------|
| Bacteria               |                 |                     | A1 | A2 | Profile                  |
| *Proteobacteria*       | Betaproteobacteria | Zoogloea            | 22.39 | 0.46 | -21.93 | EPS secretion & denitrification (Xia et al., 2018) |
|                        |                 | Thauera             | 16.03 | 2.7 | -13.33 | EPS secretion & denitrification (Xia et al., 2018) |
|                        |                 | Acidovorax          | 9.62  | 26.79 | +17.17 | Arsenite oxidation (Fan et al., 2008), organic compounds degradation & EPS secretion (Xia et al., 2018) |
|                        |                 | Nitrosomonas        | 0.67  | 0  | Disappear | Aerobic ammonia oxidation* |
|                        |                 | Rhodobactera        | 0  | 0.58 | New | Fe(III) reduction & Denitrification* |
|                        |                 | Propionibacera      | 0  | 0.32 | New | Fermentation & polyphosphate accumulation (Albertsen et al., 2016) |
| *Gammaproteobacteria*  |                 | Pseudoxanthomonas   | 4.87  | 5.69 | +0.82 | Denitrification (Xia et al., 2018) |
|                        |                 | Arenimonas          | 0.38  | 0  | Disappear | Organic compounds degradation (Zhu et al., 2017) |
|                        |                 | Aquimonas           | 0.29  | 0  | Disappear | Organic compounds degradation (Saha et al., 2005) |
|                        |                 | Aeromonas           | 1.72  | 1.06 | -0.66 | EPS secretion, sulfate reduction & fermentation* |
|                        |                 | Acinetobacter       | 1.31  | 2.07 | +0.76 | Bacterium predator* |
|                        |                 | Desulfovibacera     | 0  | 0.52 | New | Sulfate reduction & Denitrification* |
| *Alphaproteobacteria*  |                 | Deivosia            | 0.47  | 0.55 | +0.08 | EPS secretion and denitrification (Xia et al., 2018) |
|                        |                 | Erynanella          | 0.30  | 0  | Disappear | Organic compounds degradation (Lee et al., 2017) |
|                        |                 | Sphingopyxis        | 0.21  | 0  | Disappear | Refractory pollutants degradation & EPS secretion (Xia et al., 2018) |
|                        |                 | Novosphingobactera  | 0.19  | 0  | Disappear | Refractory pollutants degradation (Chen et al., 2012) |
|                        |                 | Pseudomonadobactera | 0  | 0.37 | New | Hydrolysis & Fermentation (Jung et al., 2017) |
| *Firmicutes*           | Clostridiales    | Clostridium_sensu_stricto | 11.17 | 0  | Disappear | Fermentation* |
|                        |                 | Proteocatella       | 0.28  | 0  | Disappear | Fermentation (Pikuts et al., 2009) |
|                        |                 | Clostridiaceae      | 0  | 5.14 | New | Fermentation* |
|                        |                 | Rumincoccus         | 0  | 0.29 | New | Fermentation* |
|                        |                 | Acetoanaerobicum    | 0  | 0.71 | New | Fermentation (Bies et al., 2015) |
|                        |                 | Anaerovorax         | 0  | 0.42 | New | Fermentation (Matthies et al., 2000) |
| *Bacteroidetes*        | Flavobacteriales | Chyrosebacera       | 7.99  | 3.08 | -4.91 | EPS secretion & Denitrification* |
|                        |                 | Flavobacterium      | 2.95  | 1.60 | -1.35 | EPS secretion & Polyaccharide decomposition* |
|                        |                 | Fluvicola           | 0  | 0.41 | New | Hydrolysis (Oshali and Kim, 2018) |
| *Bacteroidia*          |                 | Paludibacter        | 0.85  | 5.24 | +4.39 | Fermentation (Qiu et al., 2014a) |
|                        |                 | Sunxiiquisna        | 0.24  | 0.92 | +0.68 | Fermentation* |
|                        |                 | Macellibacteroides  | 0  | 12.83 | New | Fermentation (Jabarri et al., 2012) |
|                        |                 | Prevotella          | 0  | 1.0 | New | Fermentation* |
| *Sphingobacteria*      |                 | Mangrovibacterium   | 0  | 0.58 | New | Organic compounds degradation & Nitrogen fixation (Huang et al., 2014) |
|                        |                 | Portibacter         | 0.39  | 1.11 | +0.72 | Organic compounds degradation (Jaewoo et al., 2012) |
|                        |                 | Ferruginibacter     | 0.33  | 0  | Disappear | Hydrolysis (Jin et al., 2014) |
|                        |                 | Sediminibacterium   | 0.25  | 0  | Disappear | Organic compounds degradation (Song et al., 2017) |
|                        |                 | Pedobacter          | 0.20  | 0  | Disappear | Hydrolysis (Zhang et al., 2019) |
|                        |                 | Taibaiella          | 0  | 0.42 | New | Hydrolysis (Zsabo et al., 2016) |
| *Clophyagia*           |                 | Chrysoelina         | 0.36  | 0  | Disappear | Nitrogen fixation (Kim et al., 2013) |
| *Planctomycetes*       | Planctomycetia   | Planctopiruris      | 1.42  | 0  | Disappear | Organic compounds degradation* |
|                        |                 | Aquisphaera         | 0.35  | 0  | Disappear | Organic compounds degradation (Bondos et al., 2011) |
|                        |                 | Schlesneria         | 0.03  | 0  | Disappear | Organic compounds degradation (Kulichevskaya et al., 2007) |
|                        |                 | Thermogutta         | 0.01  | 0  | Disappear | Organic compounds degradation (Slobodkina et al., 2015) |
|                        |                 | Pirellula           | 0.01  | 0  | Disappear | Organic compounds degradation* |
| *Verrucomicrobia*      | Verrucomicrobiae | Haloferula          | 0.05  | 0  | Disappear | Organic compounds degradation (Yoon et al., 2008a) |
|                        |                 | Prosthecobacter     | 0.21  | 0  | Disappear | Organic compounds degradation* |
|                        |                 | Roseibacillus       | 0.02  | 0  | Disappear | Organic compounds degradation (Yoon et al., 2008b) |
|                        |                 | Brevifillaceae      | 0.02  | 0  | Disappear | Organic compounds degradation (Otuka et al., 2013) |
| *Spartobacteria*       | Terrimicrobiae   | Terrimicrobia      | 0.16  | 0  | Disappear | Fermentation (Qiu et al., 2017) |
| *Acidobacteria*        | Acidobacteria_Gp3 | Gp3                 | 0.21  | 0  | Disappear | Organic carbon decomposition (Fan et al., 2019) |
| *Elusimicrobia*        | Endomicrobia     | Candidatus_          | 0  | 0.44 | New | —— |
|                        |                 | Endomicrobium      | 0  | 0  | New | —— |
| *Spirochaetes*         | Spirochaetia     | Treponema           | 0  | 0.44 | New | Carbohydrates degradation* |
| *Lentisphaeraceae*     | Lentisphaeraceae | Victivallis         | 0  | 0.34 | New | Fermentation (Zoetendal et al., 2005) |
| *Unclassified*         |                  |                     | —— | 9.6 | 15.16 | +5.56 | —— |
| Total                  |                  |                     | —— | 97.91 | 93.22 | -4.69 | —— |

*Information of the microbes is summarized from MicrobeWiki (https://microbewiki.kenyon.edu/index.php/MicrobeWiki).
carbon dioxide concentrations. Most Methanomasilllicoccus species only utilize methanol to produce methane, but they are capable of utilizing many compounds, such as methanol, dimethylamine, trimethylamine, dimethyl sulfide and acetate. Methanosaeta uses only H2/CO2 and formate as substrates to produce methane, but it can assimilate acetate as a carbon source. In other words, these methanogens predominated in the absence of acetate, which means they are highly competitive in substrate utilization. In addition, Streptococcus and Weissella are facultative anaerobes, and their metabolic pathway can switch to aerobic respiration, fermentation or anaerobic respiration according to the environment, which means they are highly competitive in substrate utilization. In addition, Streptococcus and Lactococcus are capable of secreting EPS. Clostridium sensu stricto species are strictly anaerobes, and they mainly resided in the anaerobic cores of the granules because it was found that anaerobic bacterium and dead microbial cells usually resided at a depth of 800–1000 μm in AGS (Zheng et al., 2006). According to our previous work, the mass percentages of 2–3 mm and 3–4 mm granules were 51.29% and 7.26% after 11 days of reactivation, indicating that the bacterial richness almost recovered after 11 days of reactivation. Compared with the mature AGS and stored granules, the recovered AGS had a lower Shannon index and a higher Simpson index, indicating that its diversity was lower than that of the former two. The reason is probably ascribed to the fact that many species had not yet proliferated during the short reactivation period.

The bacterial community of the recovered AGS included 6 phyla, 10 classes and 28 genera (Table 5). The number of genera in the recovered AGS was smaller than that in the mature AGS (30) and the stored granules (30), and the results were consistent with the lower diversity of the former. The recovered AGS had only 3 identical genera to that of the mature AGS and stored granules. The bacterial richness almost recovered after 11 days of reactivation. Compared with the mature AGS and stored granules, the recovered AGS had a lower Shannon index and a higher Simpson index, indicating that its diversity was lower than that of the former two. The reason is probably ascribed to the fact that many species had not yet proliferated during the short reactivation period.

Table 3. Archaea community compositions of the mature AGS (A1) and the stored granules (A2).

| Phylum         | Class            | Genus                  | Relative Abundance (%) | Function                      |
|----------------|------------------|------------------------|------------------------|-------------------------------|
| Euryarchaeo    | Thermoplasma     | Methanomasilllicoccus  | 24.51                  | Methane production*           |
|                | Methanomicrobia  | Methanoculleus         | 22.88                  | Methane production*           |
|                | Methanoregula    | 8.41                   | -8.4                   |                               |
|                | Methanolinea     | 7.47                   | -7.46                  | Methane production*           |
|                | Methanosphaerula | 0.07                   | +13.4                  | Methane production*           |
|                | Methanospirillum | 1.97                   | +70.04                 | Methane production*           |
|                | Methanocalculus  | 0.03                   | 0                      | Methane production*           |
|                | Methaneoarcha    | 12.91                  | -12.9                  | Methane production*           |
|                | Methanosarcina   | 1.73                   | -0.64                  | Methane production*           |
|                | Methanomethylovorans | 0.1              | 0                      | Disappear Methane production*|
|                | Methanobus       | 0.01                   | 0                      | Disappear Methane production*|
|                | Methanocella     | 0                     | 0.55                   | New Methane production (Liu and Lu, 2018) |
| Methanosphaergera | Methanospafera | 7.05                   | 0                      | Disappear Methane production*|
|                | Methanobacterium | 3.51                   | 1.15                   | -2.36 Methane production*     |
|                | Methanobrevibacter | 2.18               | 0.02                   | -2.16 Methane production*     |
|                | Methanotrichacter | 0.18                  | 0                      | Disappear Methane production*|
| Thermococci    | Thermococcus     | 0.03                   | 0                      | Disappear Hydrogen sulfide production* |
| Halobacteria   | Halomarina       | 0.01                   | 0                      | Disappear Hydrolysis (Zhou et al., 2017) |
| Thaumarchaeota | Thaumarchaeota_class | 0.19              | 0                      | Disappear Ammonia oxidation*  |
| Unclassified   | —                | 4.55                   | 0.01                   | -4.54 —                      |
| Total          | —                | 97.79                  | 100                    | -2.21 —                      |

*Function of the genus is summarized from MicrobeWiki (https://microbewiki.kenyon.edu/index.php/MicrobeWiki).

Table 4. OTUs, richness and diversity of bacteria and archaea in recovered AGS.

| Sample          | Sequencing Number | OUT Number | Richness index   | Diversity index   | Coverage |
|-----------------|-------------------|------------|------------------|-------------------|----------|
|                 |                   |            | ACE | Chao1 | Shannon | Simpson |         |
| Bacteria (A3)   | 39471             | 1888       | 25895.72        | 14770.42          | 3.09     | 0.16    | 0.96     |
| Archaea (A3)    | 53436             | 1552       | 47523.10        | 15737.59          | 3.04     | 0.13    | 0.97     |
### Table 5. Bacteria community composition of the recovered AGS (A3).

| Phylum            | Class          | Genus           | Relative Abundance (%) | Function                                                                 |
|-------------------|----------------|-----------------|------------------------|--------------------------------------------------------------------------|
| Proteobacteria    | Betaproteobacteria | Alcaligenes    | 5.02                   | Arsenite oxidation & denitrification*                                      |
|                   | Gammaproteobacteria | Pseudomonas    | 1.15                   | Organic compounds degradation, denitrification & phosphorous accumulation* |
|                   |                 | Enterobacter    | 0.65                   | EPS secretion, Fermentation, denitrification & phosphorous accumulation*  |
|                   |                 | Proteus         | 0.42                   | Organic compounds degradation*                                           |
|                   |                 | Providencia     | 0.24                   | EPS secretion, hydrolysis & Fermentation*                                |
|                   |                 | Stenotrophomonas| 0.24                   | Organic compounds degradation*                                           |
|                   | Deltaproteobacteria | Syntrophobacter| 0.31                   | Organic compounds degradation*                                           |
|                   |                 | Desulfovibrio   | 0.16                   | Sulfate reducing & Denitrification*                                      |
| Firmicutes        | Bacilli         | Streptococcus   | 43.64                  | EPS secretion & Organic compounds degradation*                           |
|                   |                 | Lactococcus     | 11.47                  | EPS secretion, Fermentation*                                             |
|                   |                 | Weisella        | 10.24                  | Organic compounds degradation*                                           |
|                   |                 | Leuconostoc     | 2.47                   | Fermentation*                                                           |
|                   |                 | Lactobacillus   | 3.03                   | EPS secretion & Fermentation*                                           |
|                   |                 | Enterococcus    | 0.45                   | Fermentation*                                                           |
|                   | Clostridia      | Clostridium_sensu_stricto | 12.36         | Fermentation*                                                           |
|                   |                 | Sporanoferrobuter | 1.13              | Fermentation & sulfur reduction (Hernandez-Eugenio et al., 2002)          |
|                   |                 | Anaerobutyricum | 0.13                   | Organic compounds degradation (Jorgui et al., 2012)                       |
|                   |                 | Lachnospiraceae_incertae_sedis | 0.15            | ——                                                                       |
|                   | Clostridium_IV  | 0.09            | Fermentation*                                                        |
|                   | Negativicutes   | Phascolactobacterium | 0.55              | Fermentation (Watanabe et al., 2012)                                     |
|                   |                 | Megalhaer        | 0.22                   | Organic compounds degradation (Srinivasan, et al., 2019)                  |
| Bacteroidetes     | Bacteroidia     | Dygonoformica   | 0.15                   | Organic compounds degradation (Pan et al., 2016)                          |
| Planctomycetes    | Planctomycetia  | Thermogutta     | 0.09                   | Organic compounds degradation (Slobodkina et al., 2015)                   |
| Synergistetes     | Synergistia     | Aminobacterium  | 0.4                    | Amino acid degradation (Hamdi et al., 2015)                               |
|                   |                 | Lactinivibrio   | 0.14                   | Fermentation (Qiu et al., 2014b)                                         |
| Actinobacteria    | Actinobacteria_class | Corynebacterium | 0.12                   | Organic compounds degradation*                                           |
|                   |                 | Actinomyces     | 0.1                    | Fermentation*                                                           |
| Unclassified      |                 | ——              | 2.36                   | ——                                                                        |
| Total             |                 | ——              | 97.57                  | ——                                                                        |

*Function of the genus is summarized from MicrobeWiki (https://microbewiki.kenyon.edu/index.php/MicrobeWiki).

### Table 6. Archaea community composition of the recovered AGS (A3).

| Phylum            | Class          | Genus           | Relative Abundance (%) | Function                                |
|-------------------|----------------|-----------------|------------------------|-----------------------------------------|
| Euryarchaeo       | Thermoplasmata | Methanomassiliicoccus | 8.24                   | Methane production*                     |
|                   | Methanomicrobia | Methanoculleus  | 38.37                  | Methane production                      |
|                   |                | Methanoregula   | 6.65                   | Methane production (Yamamoto et al., 2014) |
|                   |                | Methanolinea    | 12.96                  | Methane production                      |
|                   |                | Methanospaera   | 0.15                   | Methane production*                     |
|                   |                | Methanospirillum| 7.34                   | Methane production*                     |
|                   |                | Methanocalculus | 0.11                   | Methane production*                     |
|                   |                | Methanobrix     | 6.88                   | Methane production*                     |
|                   |                | Methanosarcina  | 0.64                   | Methane production*                     |
|                   |                | Methanobacterium| 13.1                   | Methane production*                     |
|                   |                | Methanobrevibacter | 0.07             | Methane production*                     |
|                   |                | Methanobacterium| 0.3                    | Methane production*                     |
|                   |                | Methanococcus   | 0.01                   | Methane production*                     |
|                   |                | Thermococcus    | 0.06                   | Hydrogen sulfide production*            |
|                   |                | Halobacteria    | 0.04                   | Hydrolysis (Zhou et al., 2017)          |
| Thaumarchaeota    | Thaumarchaeota_class | Nitrososphaera | 0.32                   | Ammonium oxidation*                     |
|                   |                | Nitrosopumilus  | 0.08                   | Ammonium oxidation*                     |
| Woearearchaeota   | Woearearchaeota_class | Woearearchaeota_Incertae_Sedis_AR16 | 0.07 | —— |
| Unclassified      |                 | ——              | 2.62                   | ——                                      |
| Total             |                 | ——              | 98.03                  | ——                                      |

*Function of the genus is summarized from MicrobeWiki (https://microbewiki.kenyon.edu/index.php/MicrobeWiki).
which provided a large number of habitats for the growth of anaerobes or facultative anaerobes.

### 3.3.2. Archaea communities

The coverage of sample sequences was 0.97 (Table 4), meaning the communities can truly reflect the archaeal community structures of the granules. The community richness indices (Chao1 and ACE) were between those of the mature AGS and the stored granules (A1 < A3 < A2). The results indicated that the proportion of archaea was higher in the recovered AGS than in the mature AGS. The reason might be that the real wastewater quality and large numbers of large granules provided a suitable growth environment for the archaea. Compared with the mature AGS, the recovered AGS had a lower Shannon index and a higher Simpson index, indicating that its diversity was lower. However, the diversity of the recovered AGS was much higher than that of the stored granules, according to their diversity indices.

The archaeal community of the recovered AGS included 3 phyla, 8 classes and 19 genera, which were similar to those of the mature AGS (Table 6). *Methanoculleus* (38.37%), *Methanobacterium* (13.1%), *Methanolinea* (12.96%), *Methanomassiliicoccus* (8.24%), *Methanospirillum* (7.34%), *Methanolithix* (6.88%) and *Methanoregula* (6.65%) were the dominant archaea. All of them could be found in the mature AGS and the stored granules, and many of them were also the dominant species. A new AOB (*Nitrosopumilus*) appeared in the recovered AGS, and the relative abundance of *Nitrosospira* increased, which was biological selection of high ammonia nitrogen in the real septic tank wastewater.

### 3.4. Mechanism of granular stability loss and recovery

Although the instability mechanism of AGS is still not fully understood, it is generally believed that the maintenance of granular stability needs a high selection pressure (Franca et al., 2018), such as large hydraulic shear force, feast-famine operation, appropriate pollutant load and short settling time. Under the high selection pressure, microorganisms with fast settling velocity and strong cohesive ability can be retained in the reactor and become the dominant functional species. Owing to the unique stratified structure (Xia et al., 2018), it was found that a large number of functional bacteria (such as Zoogloea, Thauera, Christesbacterium and Flavobacterium) inhabited the stable AGS. However, dry storage environment is difficult to create sufficient selection pressure for these functional bacteria. It was found that the abundance of Zoogloea (22.39%–0.46%), Thauera (16.03%–2.7%), Christesbacterium (7.99%–3.08%) and Flavobacterium (2.95%–1.6%) all decreased significantly owing to the lack of oxygen and nutrients. The loss of functional bacteria also led to the decrease of EPS secretion and a weakened mutual cohesion between cells (Li et al., 2019). Research has shown that EPS secreted by Zoogloea and Thauera played an adhesive role in the formation of granules (Xia et al., 2018). Therefore, the loss of Zoogloea and Thauera resulted in a decrease in EPS secretion. On the other hand, EPS was consumed as a carbon source by other microbes (Aday et al., 2009; Xu et al., 2010; Gao et al., 2012; He et al., 2017; Cheng et al., 2018), which also led to a decrease in EPS. The breakage of a large number of granules after re-aeration at the early stage of recovery also proved the damage of the granular structure during the storage (Cheng et al., 2018). In addition, Zoogloea is composed of mainly aerobic bacteria, and the decrease in its abundance also leads to the decline in SOUR. Therefore, the evolution of the microbial community is the source of granular stability loss during storage, and the structure destruction and property deterioration are the external expression of the microbial community change and microbial metabolism.

With the increase of particle size of AGS, anaerobic cores are often formed inside the granules due to oxygen transfer resistance, and the activity of anaerobes in the anaerobic cores is considered to be one of the main causes of granular instability during operation (Verawaty et al., 2013; Long et al., 2015, 2019; Zhang et al., 2015). This effect also occurred in the stored granules, and it was even more intense in the agar block as oxygen transfer restriction. Almost half of the archaeal genera of the mature AGS disappeared during storage. However, the abundance of archaea increased during storage, and methanogenic archaea were still the dominant genera after storage. The results indicated that anaerobic microorganisms proliferated during storage, such as *Clostridium III* (0–5.14%), *Paludibacter* (0.85%–5.24%), *Macellibacteroides* (0–12.83%), *Methanosphaerula* (0.07–13.47%) and *Methanospirillum* (1.97%–72.01%). In addition, the metabolic activity of facultative and anaerobic microbes increased, and these microbes obtained nutrients from the dead cells to proliferate, which not only destroyed the granular structure, but also caused a significant decrease in AGS mass due to the transformation of a large number of cellular substances into carbon dioxide, methane and odour (Gao et al., 2012; He et al., 2017; Cheng et al., 2018). It was speculated that the storage environment created a new food chain between the bacterial community and archaea community during storage (Figure 1). Dead aerobic bacteria, such as Zoogloea, *Nitrosomonas*, *Arenimonas*, *Aquimonas*, *Reynella*, *Ferruginibacter*, *Rosebacillus* and *Brevifilum*, were degraded as nutrients by metatrophic and anaerobic bacteria (such as *Macellibacteroides*, *Paludibacter* and *Clostridium III*). Then, their fermentation products, such as organic acids, alcohols and amines were further utilized by methanogenic archaea as carbon sources. Finally, the metabolites were converted into methane and other gases released into the air, which eventually led to the destruction of the granular structure from the inside out.

The genera of the mature AGS and the stored granules were quite disparate from those of the recovered AGS, and most of the genera were not found in the former two. Therefore, it was reasonable to conclude that the new species probably originated from the real septic tank.
wastewater. These microbes were very adaptable to the real wastewater quality. They proliferated quickly and replaced the original species in the SBR with a large aeration rate. Although the structure of the mature AGS was destroyed during storage, it served as a carrier for the new species. EPS increased rapidly during the recovery period, increasing from 18.46 mg/g MLSS to 49.56 mg/g MLSS during the recovery period (Cheng et al., 2018). Thus, EPS secreted by the new species (such as Streptococcus and Lactococcus) played an important role in the reconstruction of the destroyed granular structure.

4. Conclusion

Dry storage and recovery of AGS involved obvious microbial community evolution. The dominant bacterial genera were quite disparate in the stored granules, and their metabolic activity gradually led to granular structure destruction and property deterioration. However, the stored granules served as carriers for the microbes originating from the real septic tank wastewater during recovery. These new species proliferated rapidly and secreted a large amount of EPS that recovered the granular structure in 11 days.

Declarations

Author contribution statement

Lian Zhang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Bei Long: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Junfeng Wu, Yuanyuan Cheng: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Binchao Zhang, Yu Zeng, Sinong Huang, Mingjing Zeng: Performed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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