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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* (12.83%) 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; Świątczak 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; Zeng et al., 2007; Adav et al., 2009; Xu et al., 2010; Gao et al., 2012; Yuan et al., 2012; Wan et al., 2014a,b; 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

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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 diguanosine 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 reported 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. SV_{30}/SV_5 (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 (SOUR) and $SOUR_{Heterotrophic\ bacteria}/SOUR_{Nitrifying\ bacteria}$ ($SOUR_H/SOUR_N$) were 37.14 ± 4.36 mg O_2/(g MLSS h) and 5.59 ± 1.86, respectively, and the granulation rate & 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 reacstion (PCR). The polymerase chain reaction (PCR) primers were the V3–V4 universal primers 341F (CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG) and 805R (GACTGGAGTTCCTTGGCACCCGAG AATTCCAGACTACHVG GGTATCTAATCC). 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:

$$H_{shannon} = \sum_{i=1}^{Sobs} \frac{ni}{N} \ln \frac{ni}{N}.$$

$$D_{simpson} = \frac{\sum_{i=1}^{Sobs} n_i(n_i - 1)}{N(N-1)}.$$

Where

S_{obs}-actual number of operational taxonomic unit (OTU) observed;
n_i-the number of sequences contained in the i^{th} 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 SV_{30}/SV_5 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	OUT 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 *Elusimicrobia*, *Spirochaetes* and *Lentisphaerae*. 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*, *Flavobacteriia*, *Bacteroidia* and *Sphingobacteriia*. During the storage process, 5 classes, namely, *Cytophagia*, *Planctomycetia*, *Verrucomicrobiae*, *Spartobacteria* and *Acidobacteria_Gp3*, disappeared, but 3 new classes (*Endomicrobia*, *Spirochaetia* and *Lentisphaeria*) 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*, *Devosia*, *Succinispira*, *Sunxiuqinia* and *Portibacter*, 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*, *Aquimonas*, *Acinetobacter*, *Reyranella*, *Novosphingobium*, *Ferruginibacter*, *Pedobacter*, *Roseibacillus* and *Brevifollis* 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*, *Chryseoline*, *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, *Rhodoferax* (0.58%), *Desulfovibrio* (0.52%) and *Fluviicola* (0.41%), are capable of using a variety of electron acceptors, such as nitrate, nitrite, heavy metal, Fe(III), sulfate and so on (Dahal and Kim, 2018). *Propionivibrio* (0.32%), *Pseudorhodobacter* (0.37%), *Clostridium_III* (5.14%), *Ruminococcus* (0.29%), *Acetoanaerobium* (0.71%), *Anaerovorax* (0.42%), *Macellibacteroides* (12.83%), *Prevotella* (1%) and *Victivallis* (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.

3.2.2. Archaea communities

The coverage of sample sequences was 0.98 both before and after storage (Table 1), which can fully reflect the microbial community structures of the granules. The community richness indices (Chao1 and ACE) of the stored granules were much higher than those of the mature AGS, indicating that the bacterial richness of the former increased significantly. Compared with the mature AGS, the stored granules had a much lower Shannon index and a higher Simpson index, indicating that their diversity decreased during storage.

The archaea community compositions were distributed in 2 phyla, 6 classes and 19 genera (Table 3). The mature AGS included 2 phyla (*Euryarchaeo* and *Thaumarchaeota*), but *Thaumarchaeota* disappeared during storage. *Euryarchaeo* includes most species of archaea and all the methanogens. *Thaumarchaeota* relies on ammonia oxidization for energy, which can live independently from the outside ecosystem. At the class level, there were 6 classes in the mature AGS. However, only 3 classes (*Thermoplasmata, Methanomicrobia* and *Methanobacteria*, belonging to the phylum *Euryarchaeo*) survived in the stored granules, and all of them were methanogens.

At the genus level, the mature AGS contained 18 genera, while the stored granules had 11 genera. The results were in accord with the significant decrease in archaeal biodiversity during storage. Among the 10 identical genera, the relative abundances of *Methanomassiliicoccus* (24.51%–11.64%), *Methanoculleus* (22.88%–0.03%), *Methanoregula* (8.41%–0.01%), *Methanolinea* (7.47%–0.01%), *Methanothrix* (12.91%–0.01%), *Methanosarcina* (1.73%–1.09%), *Methanobacterium* (3.51%–1.15%) and *Methanobrevibacter* (2.18%–0.02%) all decreased significantly. However, the relative abundances of *Methanosphaerula* (0.07%–13.47%) and *Methanospirillum* (1.97%–72.01%) increased obviously, and *Methanospirillum* eventually became the dominant archaea with the greatest abundance. *Methanospirillum* produces methane by reducing carbon dioxide, formate or acetate and proliferates rapidly under high

Table 2. Bacteria community compositions of the mature AGS (A1) and the stored granules (A2).

Phylum	Class	Genus	Relative Abundance (%)			Function
			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 oxidization*
		Rhodoferax	0	0.58	New	Fe(III) reduction & Denitrification*
		Propionivibrio	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	0.32	0	Disappear	Refractory pollutants degradation & EPS secretion*
	Deltaproteobacteria	Bdellovibrio	1.31	2.07	+0.76	Bacterium predator*
		Desulfovibrio	0	0.52	New	Sulfate reduction & Denitrification*
	Alphaproteobacteria	Gemmobacter	0.87	0.53	-0.34	Denitrification (Sheu et al., 2013)
		Devosia	0.47	0.55	+0.08	EPS secretion and denitrification (Xia et al., 2018)
		Reyranella	0.30	0	Disappear	Organic compounds degradation (Lee et al., 2017)
		Sphingopyxis	0.21	0	Disappear	Refractory pollutants degradation (Kolvenbach and Corvini. 2012)
		Novosphingobium	0.19	0	Disappear	Refractory pollutants degradation (Chen et al., 2012)
		Pseudorhodobacter	0	0.37	New	Hydrolysis & Fermentation (Jung et al., 2017)
Firmicutes	Clostridia	Clostridium_sensu_stricto	11.17	0	Disappear	Fermentation*
		Proteocatella	0.28	0	Disappear	Fermentation (Pikuta et al., 2009)
		Clostridium_ III	0	5.14	New	Fermentation*
		Ruminococcus	0	0.29	New	Fermentation*
		Acetoanaerobium	0	0.71	New	Fermentation (Bes et al., 2015)
		Anaerovorax	0	0.42	New	Fermentation (Matthies et al., 2000)
	Negativicutes	Succinispira	0.93	1.45	+0.52	Fermentation (Janssen and O' Farrell, 1999)
		Phascolarctobacterium	0.24	0	Disappear	Fermentation (Watanabe et al., 2012)
Bacteroidetes	Flavobacteriia	Chryseobacterium	7.99	3.08	-4.91	EPS secretion & Denitrification*
		Flavobacterium	2.95	1.60	-1.35	EPS secretion & Polysaccharide decomposition*
		Fluviicola	0	0.41	New	Hydrolysis (Dahal and Kim, 2018)
	Bacteroidia	Paludibacter	0.85	5.24	+4.39	Fermentation (Qiu et al., 2014a)
		Sunxiuqinia	0.24	0.92	+0.68	Fermentation*
		Macellibacteroides	0	12.83	New	Fermentation (Jabari et al., 2012)
		Prevotella	0	1.0	New	Fermentation*
		Mangrovibacterium	0	0.58	New	Organic compounds degradation & Nitrogen fixation (Huang et al., 2014)
	Sphingobacteriia	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 (Szabó et al., 2016)
	Cytophagia	Chryseolinea	0.36	0	Disappear	Nitrogen fixation (Kim et al., 2013)
Planctomycetes	Planctomycetia	Planctopirus	1.42	0	Disappear	Organic compounds degradation*
		Aquisphaera	0.35	0	Disappear	Organic compounds degradation (Bondoso 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)
		Brevifollis	0.02	0	Disappear	Organic compounds degradation (Otsuka et al., 2013)
	Spartobacteria	Terrimicrobium	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_ Endomicrobium	0	0.44	New	——
Spirochaetes	Spirochaetia	Treponema	0	0.44	New	Carbohydrates degradation*
Lentisphaerae	Lentisphaeria	Victivallis	0	0.34	New	Fermentation (Zoetendal et al., 2003)
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).

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

Phylum	Class	Genus	Relative Abundance (%)			Function
			A1	A2	Profile	
Euryarchaeo	Thermoplasmata	Methanomassiliicoccus	24.51	11.64	-12.87	Methane production*
	Methanomicrobia	Methanoculleus	22.88	0.03	-22.85	Methane production*
		Methanoregula	8.41	0.01	-8.4	Methane production (Yamamoto et al., 2014)
		Methanolinea	7.47	0.01	-7.46	Methane production*
		Methanosphaerula	0.07	13.47	+13.4	Methane production*
		Methanospirillum	1.97	72.01	+70.04	Methane production*
		Methanocalculus	0.03	0	Disappear	Methane production*
		Methanothrix	12.91	0.01	-12.9	Methane production*
		Methanosarcina	1.73	1.09	-0.64	Methane production*
		Methanomethylovorans	0.1	0	Disappear	Methane production*
		Methanolobus	0.01	0	Disappear	Methane production*
		Methanocella	0	0.55	New	Methane production (Liu and Lu. 2018)
	Methanobacteria	Methanosphaera	7.05	0	Disappear	Methane production*
		Methanobacterium	3.51	1.15	-2.36	Methane production*
		Methanobrevibacter	2.18	0.02	-2.16	Methane production*
		Methanothermobacter	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	Nitrososphaera	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).

carbon dioxide concentrations. Most *Methanomassiliicoccus* species only utilize methanol to produce methane, but they are capable of utilizing many compounds, such as methanol, dimethylamine, trimethylamine, dimethyl sulfide and acetate. *Methanosphaerula* uses only H_2/CO_2 and formate as substrates to produce methane, but it can assimilate acetate as a carbon source. In other words, these methanogens predominated in the agar because of low requirements for carbon sources and other growth factors. By contrast, many other methanogens are picky, so their relative abundance decreased, or they were eliminated. For example, *Methanoregula* thrives in extremely low pH (Yamamoto et al., 2014), *Methanobacterium* is sensitive to salinity and prefers high temperature, and *Methanothermobacter* requires casamino acids, tryptone, yeast extract, or vitamins for growth. A new genus, *Methanocella*, appeared during storage, which produced methane by reducing carbon dioxide or acetate (Liu and Lu, 2018). Eight genera were eliminated during storage. Among them, *Thermococcus* and *Nitrososphaera* were obligate aerobes; other archaea were eliminated because of their specific growth requirements. For example, *Halomarina* is the most well-known halophilic archaea, which preferentially lives in mesophilic and neutrophilic environments (Zhou et al., 2017).

3.3. Microbial community compositions of the recovered AGS

3.3.1. Bacterial communities

The coverage of sample sequences (A3) was 0.96 (Table 4), and the results can truly reflect the bacterial community structures of the granules. The community richness indices (Chao1 and ACE) were similar to those of the mature AGS and higher than those of the stored granules, 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, while there was 1 identical genus between the recovered AGS and the stored granules. *Streptococcus* (43.64%), *Clostridium_sensu_stricto* (12.36%), *Lactococcus* (11.47%), *Weissella* (10.24%), *Alcaligenes* (5.02%), *Lactobacillus* (3.03%), *Leuconostoc* (2.47%), *Pseudomonas* (1.15%) and *Sporanaerobacter* (1.13%) were the main genera in the recovered AGS. It should be pointed out that most species of *Streptococcus*, *Lactococcus*, *Weissella* and *Leuconostoc* 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,

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	35895.71	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*
		Weissella	10.24	Organic compounds degradation *
		Leuconostoc	2.47	Fermentation*
		Lactobacillus	3.03	EPS secretion & Fermentation*
		Enterococcus	0.45	Fermentation*
		Trichococcus	0.09	Fermentation (Parshina et al., 2019)
	Clostridia	Clostridium_sensu_stricto	12.36	Fermentation*
		Sporanaerobacter	1.13	Fermentation & sulfur reduction (Hernandez-Eugenio et al., 2002)
		Anaerosalibacter	0.13	Organic compounds degradation (Rezgui et al., 2012)
		Lachnospiracea_incertae_sedis	0.15	——
		Clostridium_IV	0.09	Fermentation*
	Negativicutes	Phascolarctobacterium	0.55	Fermentation (Watanabe et al., 2012)
		Megasphaera	0.22	Organic compounds degradation (Srinivasan et al., 2018)
Bacteroidetes	Bacteroidia	Dysgonomonas	0.15	Organic compounds degradation (Duan 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)
		Lactivibrio	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
		Methanosphaerula	0.15	Methane production*
		Methanospirillum	7.34	Methane production*
		Methanocalculus	0.11	Methane production*
		Methanothrix	6.88	Methane production*
		Methanosarcina	0.64	Methane production*
		Methanolobus	0.02	Methane production*
	Methanobacteria	Methanobacterium	13.1	Methane production*
		Methanobrevibacter	0.07	Methane production*
		Methanothermobacter	0.3	Methane production*
	Methanococci	Methanothermococcus	0.01	Methane production*
	Thermococci	Thermococcus	0.06	Hydrogen sulfide production*
	Halobacteria	Halomarina	0.04	Hydrolysis (Zhou et al., 2017)
Thaumarchaeota	Thaumarchaeota_class	Nitrososphaera	0.32	Ammonia oxidation*
		Nitrosopumilus	0.08	Ammonia oxidation*
Woesearchaeota	Woesearchaeota_class	Woesearchaeota_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).

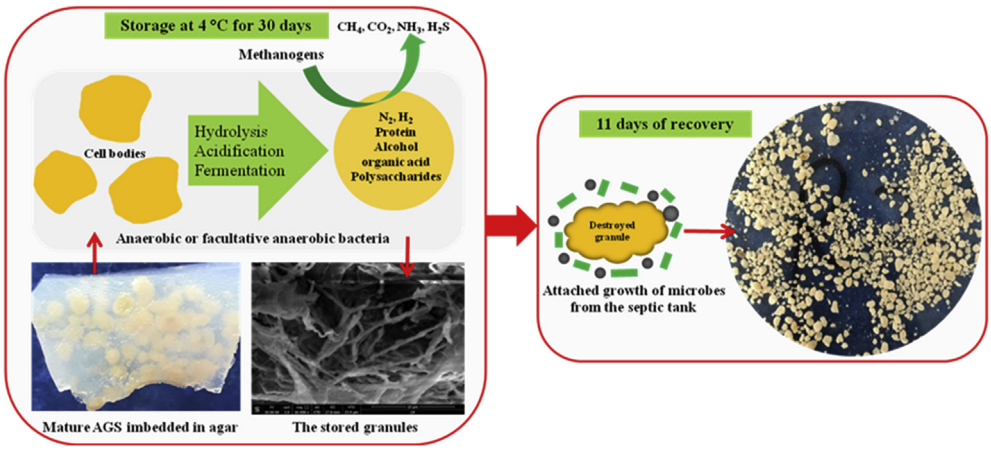

Figure 1. Mechanism of granular stability loss and recovery.

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 results 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%), *Methanothrix* (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 *Nitrososphaera* 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*, *Chryseobacterium* 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%), *Chryseobacterium* (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 (Adav 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 insides 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*, *Reyranella*, *Ferruginibacter*, *Roseibacillus* and *Brevifollis*, 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 mature AGS, stored granules and the recovered AGS, but methanogens were always the dominant archaeal species during the different periods. Metatrophic and anaerobic bacteria proliferated and dominated 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

Linan 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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