Factors affecting the growth of *Microthrix parvicella*: Batch tests using bulking sludge as seed sludge

Niansi Fan^{a,b}, Rong Qi^{a,b}, Simona Rossetti^c, Valter Tandoi^c, Yingxin Gao^{a,b}, Min Yang^{a,b,*}

^a State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

^b University of Chinese Academy of Sciences, Beijing 100049, China

^c CNR, Water Research Institute, Via Reno 1, 00198 Roma, Italy

HIGHLIGHTS

- Population dynamics of *M. parvicella* and SVI variation identified the correlation between sludge settle ability and *M. parvicella* abundance.
- LCFA, anaerobic/aerobic alternation and low temperature are advantageous conditions for the competitive growth of *M. parvicella*.
- This study fosters physiological knowledge of *M. parvicella*, which lay a foundation for the subsequent pure culture of this microorganism.

GRAPHICAL ABSTRACT

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ABSTRACT

Sludge bulking caused by the overgrowth of filamentous bacteria, particularly *Microthrix parvicella*, is one of the challenges for the stable operation of municipal wastewater treatment plants (WWTPs). The driving forces for the development of sludge bulking, however, have not been well understood because of the extremely low growth rate of *M. parvicella*. In this study, batch experiments were performed using bulking sludge (sludge volume index (SVI), around 185 mL g⁻¹) from a full-scale WWTP as the seed sludge to investigate the influences of carbon source, anaerobic/aerobic alternation condition and temperature on the growth of *M. parvicella*. The qPCR results showed that the use of oleic acid as carbon source, anaerobic/aerobic alternation treatment and low temperature (13 °C) were favorable conditions for maintaining the dominance of *M. parvicella* in the tested activated sludge. Under these conditions, the SVI values remained at comparatively high values of 170.5 mL g⁻¹, 162.5 mL g⁻¹ and 129.5 mL g⁻¹ after operation for approximately two months, and the relative abundances of *M. parvicella* were 36.7%, 9.74% and 34.07%, respectively, in comparison with the initial values of 33.04%, 29.29% and 54.66%. However, the relative abundances of *M. parvicella* decreased to 0.86–4.44%, 0.7% and 4.94%, respectively, under the conditions of other carbon sources, aerobic-only treatment and a temperature of 20 °C. The FISH analysis gave a similar result. This study was performed with mixed sludge under controlled operating conditions, which provided a valuable information for the pure culture of *M. parvicella* and further investigations on its physiology and metabolism.

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* Corresponding author at: Shuangqing Road No. 18, Haidian District, Beijing 100085, China.
E-mail address: yangmin@rcees.ac.cn (M. Yang).

1. Introduction

Microthrix parvicella, with a hydrophobic cell surface, high intracellular storage capacity and low maintenance energy requirement, frequently causes sludge bulking in wastewater treatment plants (WWTPs) (Mamais et al., 1998; Miana et al., 2002; Slijkhuys, 1983a). Understanding the conditions favorable for the growth of *M. parvicella* have been one of the top hotspots for research on sludge bulking. Based on microautoradiography (MAR) analysis, Andreasen and Nielsen (2000) found that *M. parvicella* could take up long-chain fatty acids (LCFAs) under oxic, anoxic and anaerobic conditions. McIlroy et al. (2013) constructed a metabolic model for *M. parvicella* based on metagenomic analysis, proposing that under anaerobic condition this organism preferentially accumulated LCFAs. On the other hand, most of the sludge bulking events have been found to occur in enhanced nutrient removal processes including at least one anaerobic/aerobic alternation cycle (Eikelboom et al., 1998; Wagner et al., 1994), particularly at times when the temperature is low (normally below 15 °C) (Wang et al., 2014). Nielsen et al. (2012) revealed that *M. parvicella* was potentially active during the entire process, taking up substrates under anaerobic conditions (but no growth) and growing in the aerobic tanks by means of either stored materials or hydrolysis products. Kumari et al. (2009a) found that *M. parvicella* might prefer low temperature conditions for growth.

All of the previous studies on the utilization of LCFAs were mainly based on the observations of full-scale WWTPs or gene prediction, and the effects of LCFAs on the in situ growth of *M. parvicella* have not yet been elaborated, nor those of aerobic/anaerobic alternation and low temperature. Though pure cultures of *M. parvicella* 4B and RN1 strains were acquired through the micromanipulation method (Rossetti et al., 1997; Rossetti et al., 2002), studies on the behaviors of *M. parvicella* using pure cultures have gained little progress. One of the big challenges is the difficulty in getting a sufficient amount of biomass for the studies of *M. parvicella* due to its extremely low growth rate (Rossetti et al., 2002; Sheik et al., 2016). In this study, batch experiments were carried out to explore the favorable conditions for the growth of *M. parvicella* by using bulking sludge as seed sludge under different strategies. The impacts of carbon sources, anaerobic/aerobic conditions, and temperature on the growth of *M. parvicella* were evaluated over a period of approximately two months, respectively. Multiple approaches including microscopic observation, fluorescence in situ hybridization (FISH) and real-time polymerase chain reaction (qPCR) were employed to monitor the dynamics of *M. parvicella* under various conditions over the operating period. This is the first study to investigate the integrated effects of LCFAs, aerobic/anaerobic alternation and low temperature on the competitive growth *M. parvicella* using bulking sludge, which could not only provide useful information of selective conditions for subsequent pure culture, but also take one step forward in understanding the physiology and metabolism of *M. parvicella*.

2. Materials and methods

2.1. Source of seed sludge

Seed sludge was collected from the aeration tank of Xiaohongmen Wastewater Treatment Plant, Beijing, China, during the bulking period (SVI > 150 mL g⁻¹). This plant adopts the anaerobic-anoxic-oxic (A²/O) process (Fig. S1), designed for 193,000 population equivalents. The main operational parameters during the sampling time are summarized in Table S1. Sludge samples during moderate bulking events were collected on 26th Nov 2014 (SVI, 181.2 mL g⁻¹), 11th Mar 2015 (SVI, 189.5 mL g⁻¹), and 28th May 2015 (SVI, 183 mL g⁻¹), respectively, brought to the laboratory within 2 h, and immediately washed by phosphate buffer saline (PBS 1×) solution twice before the start of experiments.

2.2. Experimental set-up

Three sets of experiments were designed as shown in Table 1. Phases I, II and III were used to evaluate the effects of carbon source, anaerobic/aerobic alternation and temperature on the dynamics of *M. parvicella* separately. The experiments were performed in sequencing batch reactors (SBRs), and the running time of the three phases were 68, 67 and 61 d, respectively.

Seed sludge was inoculated into a 500 mL glass bottle containing 300 mL synthetic influents (Tables S2–S3), and 100 mL fresh artificial wastewater was added into the bottle every day after the same amount of treated wastewater was decanted following 30 min sedimentation. The sludge retention time (SRT) and hydraulic retention time (HRT) for all reactors were 60 d and 24 h, respectively. The long HRT was chosen to maintain sufficient COD and ammonium removal under different conditions. Besides, long HRT would result in a low sludge loading, which had been reported to be beneficial for the competitive growth of *M. parvicella* (Knoop and Kunst, 1998; Rossetti et al., 1994). Synthetic influents were freshly prepared every 3–4 d and stored at 4 °C. A stock solution of oleic acid was prepared in deionized water by adding Tween 80 (1:9, v/v), and heated at 60 °C for 30 min. Tween 80 is a water-soluble liquid containing 0.22 g oleic acid g⁻¹ Tween 80 (Noutsopoulos et al., 2012), which was added to promote the dissolution of oleic acid. The average chemical oxygen demand (COD), nitrogen (mainly ammonium salt, NH₄⁺-N) and phosphorus (mainly orthophosphate, PO₄³⁻-P) concentrations in the synthetic wastewater were adjusted to around 250 mg L⁻¹, 15 mg L⁻¹ and 5 mg L⁻¹, respectively.

The bottles were put in incubators under different conditions as shown in Table 1. For the aerobic treatment, bottles covered by sealing films with holes were shaken with a constant speed of 160 rpm. The anaerobic condition was achieved by bubbling with argon gas for at least 20 min and tightly sealing with a bottle cap. In the anaerobic/aerobic alternation experiment, a cycle of anaerobic condition for 8 h followed by aerobic condition for 16 h was adopted, and the last half hour of aerobic stage was taken for the sludge sedimentation. Nutrients feeding and argon inflation were carried out at 10:00 am each day.

This study was trying to verify three important assumptions for the preferential growth of filamentous bacteria: the presence of LCFAs, aerobic/anaerobic alternation and low temperature. In order to control the sampling numbers at a reasonable level, a progressive strategy was adopted to verify these three assumptions. Therefore, the selective conditions tested in each phase were chosen based on the results of the previous phase.

Table 1
The main conditions for the batch experiments.

Phase	Inoculation time ^a	SVI ^b (mL g ⁻¹)	Process type ^c	Carbon source ^d	Temperature (°C)
Phase I	26/11/2014	181.2	Aerobic (24 h)	Mixed NaAc LCFAs	20
Phase II	11/03/2015	189.5	Aerobic (24 h) Anaerobic (8 h)/Aerobic (16 h)	LCFAs	20
Phase III	28/05/2015	183.1	Anaerobic (8 h)/Aerobic (16 h)	LCFAs	13 20

Note:

^a The date indicates the sampling time (d/m/y) of the inoculated sludge for the three phases in the initial stage.

^b These values were SVI of the inoculum.

^c Duplicate experiments were performed for each condition and all results were analyzed based on average values.

^d Mixed carbon source included sodium carboxymethylcellulose (CMC-Na), starch, glucose and tryptone; NaAc: sodium acetate; LCFAs: long-chain fatty acids including oleic acid and Tween 80.

2.3. Chemical analysis and microscopic examination

The operating performance was monitored by taking wastewater samples twice a week for the determination of ammonium nitrogen ($\text{NH}_4^+\text{-N}$), nitrate nitrogen ($\text{NO}_3^-\text{-N}$), nitrite nitrogen ($\text{NO}_2^-\text{-N}$), ortho-phosphate phosphorus ($\text{PO}_4^{3-}\text{-P}$), sludge volume index (SVI), and mixed liquor suspended solids (MLSS) according to the Standard Methods (Eaton et al., 2005). Samples were collected and immediately filtered through 0.45 μm PES membranes (Millipore, USA). In addition, operating parameters including temperature, pH and dissolved oxygen (DO) were measured using a thermometer and Orion Star A portable DO/pH instrument (Thermo Scientific, USA). The DO value at the end of aerobic stage was 5–8 mg L^{-1} , and that of the anaerobic stage was below 0.1 mg L^{-1} . The reductive condition was also checked using a redox potential meter, and its value was around -180 mV at the end of anaerobic stage.

Samples used for quantification of filaments were collected once every week in duplicates from the mixed liquor. For DNA extraction, approximately 2 mL samples were centrifuged at 8000 g (Sigma Centrifuge, Germany) and the cell pellets were stored at -20 °C until DNA extraction. For FISH analysis, 2 mL samples were fixed in 9 mL 4% (w/v) paraformaldehyde for 2 h at 4 °C and stored in PBS/ethanol (1:1, v/v) at -20 °C until hybridization (Marneri et al., 2009). Samples were subjected to ultrasonic oscillation several times before cell fixation in order to destroy the floc structure and obtain a better view of the filaments during microscopic observation. In addition, a small amount of fresh sludge samples were taken for microscopic observation of sludge structure. The changes of sludge floc size and structure were observed under microscopy. Predominant microorganisms were identified based on morphological features by using Gram stain (Hucker and Conn, 1923) and Neisser stain (Westlund et al., 1998). The density of filamentous bacteria in activated sludge was roughly estimated in terms of the filament index (FI) according to Jenkins et al. (1993). Filamentous bacteria abundance could be evaluated using the FI on a scale of 0–6 representing none (0), few (1), some (2), common (3), very common (4), abundant (5) and excessive filaments (6), respectively. Activated sludge was generally considered to be in severe bulking state at $\text{FI} \geq 5$.

2.4. DNA extraction and quantitative PCR

To characterize the composition of the microbial community in the sludge samples, total genomic DNA was extracted using a Fast DNA Spin Kit for Soil from MP Biomedicals following the manufacturer's instructions. Extracted DNA was amplified in a PCR Cycler (Light Cycler 96, Roche) using 341f (5'-CTACGGGAGGCAGCAG-3') and 534r (5'-TTACCGCGCTGCTGGCAC-3') primer sets (Bru et al., 2008). The amplified products were visualized on 1% agarose gel stained with ethidium bromide (the final concentration of 10 mg mL^{-1}).

Standard plasmids carrying the target genes were obtained by utilizing TA clones and extracted using a TIANpure Mini Plasmid kit (Tiangen, China). The concentrations of the standard plasmids ($\text{ng } \mu\text{L}^{-1}$) were determined with a NanoDrop ND-1000 (NanoDrop, USA), and their copy concentrations ($\text{copies } \mu\text{L}^{-1}$) were subsequently calculated by using the following equation (Pei et al., 2006).

$$\frac{\text{DNA mass concentration } [\text{ng } \mu\text{L}^{-1}]}{\text{DNA molecular weight } [\text{g mol}^{-1}]} \times (6.02 \times 10^{23}) \times 10^{-9} \\ = \text{copy concentration } [\text{copies } \mu\text{L}^{-1}]$$

Triplicate quantitative real-time PCR assays were performed for the decimally diluted standard plasmids to obtain the standard curves, with no amplification signal from two negative controls (one without primers and the other without template DNA). Duplicate assays were performed for all samples on an ABI7300 apparatus (ABI, USA) using

SYBR Green chemistry. Quantification of *M. parvicella* was performed by qPCR using the 16S rRNA gene primer set S-S-M.par-0828-S-21/S-S-M.par-1018-A-17 (Kaetzke et al., 2005) and SYBR Green as the fluorescent dye with the program of 95 °C 10 min, 95 °C 15 s, 62 °C 30 s, 60 °C 30 s, and 39 cycles. The amplification was carried out in 25 μL reaction mixtures containing 2 μL of a 1:10 dilution of extracted DNA, 12.5 μL SYBR-Green (Applied Biosystems Power SYBRGreen), 0.5 μL fluorescent dye, 0.5 μL forward primer (10 mM), 0.5 μL reverse primer (10 mM), 0.5 μL BSA (20 mg mL^{-1} , Fermentas) and 8.5 μL RNA/DNA-free water (Fermentas). The reaction mixture was transferred to a thin 96-well PCR plate with a micropipette. The plate was covered with optical-quality PCR caps (ABI, USA) and centrifuged briefly to bring all reagents to the bottom of the wells. Melting curve (72–92 °C) analysis was conducted afterwards to determine the specificity of amplification. The average amplification efficiency of the specific qPCR primer set was 98% with an r^2 of 0.997 for the standard curve. The 16S rRNA gene copies of total bacteria were also quantified using 16S rRNA primer set S-D-Bact-0509-S-17/S-DBact-0784-A-22 (Table 2) according to the procedures described by Rupf et al. (1999). The relative abundance of *M. parvicella* could be calculated by dividing the 16S rRNA gene copy numbers of *M. parvicella* by that of total bacteria (Kaetzke et al., 2005).

2.5. Fluorescence in situ hybridization

It has to be taken into consideration that DNA studies deliver no direct information about microbe viability and that even inactive *M. parvicella* cells may promote foaming (de los Reyes et al., 2002). Thus, FISH was performed on ethanol-fixed activated sludge samples for Gram-positive bacteria. The fixed cells were sonicated for 60 s with an ultrasonic processor (Vibra-Cell, Sonics) at a pulse of 5 s and an output power of 4 W in order to break the sludge flocs and perform a better FISH quantification. The oligonucleotide probes EUB_{mix} (equimolar concentrations of EUB338, EUB338-II, and EUB338-III) labelled with fluorescein isothiocyanate (FITC) and MPA_{mix} (equimolar concentrations of MPA60, MPA223, and MPA645) labelled with the sulfoindocyanine dye Cy3 (Table 3), were used for FISH. In brief, each sample was diluted and homogenously spotted to the Teflon-coated 12-well slides. After hybridization, the slides were covered with ProLong® Gold Antifade Reagent (Thermo Fisher Scientific, USA). Detailed procedures were performed according to the protocols described by Nielsen et al. (2009) and stained images were captured with an Olympus BX51 epifluorescence microscope equipped with a cooled charged-coupled device (CCD) camera in true color using a suitable filter set and exposure times of a few milliseconds. At least 20 random fields at the magnification of 1000 were chosen to acquire images with a specific probe-defined population and the general EUB_{mix}. The area of *M. parvicella*, relative to the total biomass area, was determined by image analyzing software (ImageJ, <http://rsbweb.nih.gov/ij/>). The standard deviation for all values and probes was between 16% and 20% of the average value indicated and only averages are shown in the figures. The percentage of FISH detectable cells was determined as the fraction of bacterial cells responding to probe EUB_{mix} over the total number of cells stained by 4',6'-diamidino-2-phenylindole (DAPI) (Kapuscinski, 1995).

2.6. Statistical analysis

Statistical analysis to evaluate the effects of carbon source and environmental factors were performed using the SPSS16.0 software package (SPSS®, Chicago, IL, USA). The *p* values of 0.05 and 0.01 were regarded as the cutoff for statistical significance between two data sets.

3. Results and discussion

The key operating performance parameters are listed in Table 4. The average removal efficiencies for COD and ammonium nitrogen were

Table 2Primers used for the quantification of *M. parvicella* and total bacteria in this study.

Primer	Target	Sequence (5'–3')	Reference
S-D-Bact-0509-S-17	Total bacteria	ACTACGTGCCAGCAGCC	(Rupf et al., 1999) ^a
S-D-Bact-0784-A-22		GGACTACCAGGTATCTAATCC	
S-S-M.par-0828-S-21	<i>Microthrix parvicella</i>	GGTGTGGGAGAACTCAACTC	(Kaetzke et al., 2005) ^b
S-S-M.par-1018-A-17		GACCCGAAGGACACCG	

^a Rupf S, Merte K, Eschrich K (1999) Quantification of bacteria in oral samples by competitive polymerase chain reaction. J Dent Res 78(4):850–856.^b Kaetzke A, Jentsch D, Eschrich K (2005) Quantification of *Microthrix parvicella* in activated sludge bacterial communities by real-time PCR. Lett Appl Microbiol 40(3):207–211.

84.53% and 94.81%, respectively, showing that each system was functioning well under different strategies.

3.1. Influence of carbon source on *M. parvicella* growth

As shown in Fig. 1, the average values of SVI decreased from 185 mL g⁻¹ to 118 mL g⁻¹ and 131 mL g⁻¹, respectively, when mixed carbon source and acetate were fed in Phase I. However, the SVI was almost a constant value of 170 mL g⁻¹ over the whole period when LCFA was used as the sole carbon source. As shown in Fig. 2a, the relative abundance of *M. parvicella* revealed by qPCR decreased very rapidly for the mixed carbon source and acetate systems, from around 33% to <5% within 60 d. However, the relative abundance of *M. parvicella* for the LCFA system remained at a high level, ending at 36.7%. Based on the absolute quantification results (Fig. S2), *M. parvicella* gene copy numbers in the LCFA system varied between 8.04×10^{11} and 1.52×10^{12} gene copies g⁻¹ sludge over the whole period, in comparison with 1.96×10^{10} and 8.25×10^{10} gene copies g⁻¹ sludge in the end for the other two carbon source systems. The FISH analysis results exhibited a very similar trend, as shown in Fig. 2b. The relative abundance of *M. parvicella* decreased from 36.17% to 3.62% and 5.28% respectively, for the mixed carbon source and acetate systems, while that of the LCFA system was 37.3%. According to statistical analysis (Table 5), the LCFA system was significantly different from the other two systems in terms of *M. parvicella* abundance and SVI ($p < 0.01$). Microscopic examination results gave similar results (Fig. S3). The filamentous index (FI) of the LCFA system was an almost constant value of 5, while those of the mixed carbon source and acetate systems decreased to 4 and 3, respectively. Even for the LCFA system, however, changes of filaments were observed. The length of filaments was shorter and hollow cells appeared in comparison with the initial state.

The behaviors of *M. parvicella* have been studied mainly through the observation of full-scale WWTP due to the difficulty in getting sufficient amounts of pure culture biomass. In this study, three strategies, the preferences of *M. parvicella* for carbon sources, aerobic/anaerobic alternation and temperature, were evaluated by using activated sludge under bulking state as seed sludge. As shown in Figs. 1 and 2, the SVI values decreased quickly together with the relative abundance of

M. parvicella when the carbon sources did not contain oily compounds. However, both the SVI value and the abundance of *M. parvicella* were almost constant over a period of two months when LCFA was used as the carbon source. Thus, the presence of hydrophobic compounds in wastewater seems to be an important condition in keeping the growth of *M. parvicella* in activated sludge. Hydrophobic compounds including lipids and LCFA have been reported to constitute 15–40% of total COD in municipal wastewater (Dueholm et al., 2000; Henze et al., 1994; Quéméneur and Marty, 1994). The ability of *M. parvicella* to take up LCFA has been documented for both mixed culture (Andreasen and Nielsen, 1998; Mamais et al., 1998) and pure culture (Slijkhuys, 1983a; Slijkhuys and Deinema, 1988). By using the MAC-test Nielsen et al. (2002) found that compared to other unidentified filamentous bacteria, *M. parvicella* was more hydrophobic, allowing lipids and LCFA to be preferably adsorbed to its cells. At the same time, by using an enzyme-linked fluorescence method the presence of lipases was identified on the cell surface of *M. parvicella*, which also make it easier to take up the hydrophobic compounds (Nielsen et al., 2012).

3.2. Influence of anaerobic/aerobic alternation on *M. parvicella* growth

By employing LCFA as the sole carbon source, the aerobic strategy was compared with the anaerobic/aerobic alternation strategy in Phase II. As indicated in Table 4, ammonium nitrogen was almost completely transformed into nitrate in the aerobic stage. A part of nitrate (~30%) was decomposed into nitrogen gas in the anaerobic stage because some treated water was decanted after aerobic treatment. No significant changes of phosphate were observed.

In the aerobic system, the SVI value decreased from 182.5 mL g⁻¹ to 65.5 mL g⁻¹, while the SVI value only decreased slightly to 163 mL g⁻¹ in the anaerobic/aerobic alternation system (Fig. 1). Both FISH and qPCR results showed that the relative abundance of *M. parvicella* decreased in the two systems over the experimental period (Fig. 2). The relative abundance of *M. parvicella* revealed by qPCR decreased from around 26.52% to 0.7% and 9.74%, respectively for the aerobic and aerobic/anaerobic alternation systems over the experimental period. However, the relative abundance of *M. parvicella* remained almost constant from Day 40 on (Fig. 2a). According to absolute qPCR results (Fig. S2), the

Table 3The oligonucleotide probes for *M. parvicella* and total bacteria.

Probe	Target	Sequence (5'–3')	Target site (16S rRNA position)	Fluorochrome labelled	Reference
EUB _{mix}	Total bacteria	EUB338:GCTGCCTCCCGTAGGAGT	338–355	FITC	(Amann et al., 1990) ^a
		EUB338-II:GCAGCCACCCGTAGGTGT			
		EUB338-III:GCTGCCACCCGTAGGTGT			
MPA _{mix}	<i>Microthrix parvicella</i>	MPA60:GGA TGGCCGCTTCGACT	60–77	Cy3	(Erhart et al., 1997) ^b
		MPA223:GCCGCGAGACCCTCTAG	223–240		
		MPA645:CCGGACTCTAGTCAGAGC	645–661		

^a Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microb 56(6):1919–1925.^b Erhart R, Bradford D, Seviour RJ, Amann R, Blackall LL (1997) Development and use of fluorescent in situ hybridization probes for the detection and identification of “*Microthrix parvicella*” in activated sludge. System Appl Microbiol 20(2):310–318.

Table 4The overall performance of batch experiments under different operating conditions (mean $\pm$ standard deviation).

Experimental systems	MLSS	COD (mg L ⁻¹)		NH ₄ ⁺ -N (mg L ⁻¹)		NO ₃ ⁻ -N (mg L ⁻¹)		PO ₃ ³⁻ -P (mg L ⁻¹)	
	(g L ⁻¹)	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Phase I									
Mixed carbon source	5.1 ± 0.4	224 ± 29	41 ± 10	12.6 ± 0.9	0.8 ± 0.6	~0	14.8 ± 2.7	4.8 ± 1.5	5.6 ± 2.3
NaAc	5.0 ± 0.6	231 ± 24	32 ± 5	11.9 ± 1.3	0.4 ± 0.2	~0	11.6 ± 1.4	4.4 ± 1.8	6.1 ± 1.9
LCFA	5.0 ± 0.4	216 ± 19	32 ± 4	12.4 ± 1.1	1.0 ± 0.7	~0	16.5 ± 3.4	4.5 ± 1.0	5.5 ± 2.5
Phase II									
Aerobic	3.8 ± 0.7	226 ± 35	32 ± 10	16.1 ± 2.1	0.5 ± 0.3	~0	19.8 ± 3.5	4.6 ± 0.7	4.8 ± 0.7
Anaerobic/Aerobic	3.8 ± 0.6	247 ± 29	40 ± 15	16.2 ± 1.6	0.6 ± 0.2	~0	14.5 ± 2.2	3.9 ± 0.8	3.8 ± 1.2
Phase III									
13 °C	3.2 ± 0.9	232 ± 29	37 ± 15	14.5 ± 3.0	0.6 ± 0.1	~0	14.8 ± 3.2	3.8 ± 0.3	4.2 ± 1.5
20 °C	3.4 ± 0.6	253 ± 33	38 ± 16	12.9 ± 2.4	0.7 ± 0.8	~0	12.5 ± 2.7	4.4 ± 0.5	5.0 ± 1.7

M. parvicella gene copy numbers in the aerobic/anaerobic alternation system decreased from around 1.09×10^{12} gene copies g⁻¹ sludge to 9.70×10^{10} gene copies g⁻¹ sludge in the end of the experiment,

while that in the aerobic system decreased to a level of 4.47×10^9 gene copies g⁻¹ sludge. As shown in Fig. 2b, the relative abundances of *M. parvicella* revealed by FISH decreased from 43.64% to 2.2% and 16.4%, respectively, for the aerobic and aerobic/anaerobic alternation systems. According to statistical analysis (Table 5), the aerobic system was significantly different ($p < 0.01$) from the aerobic/anaerobic alternation one in terms of *M. parvicella* abundance and SVI. Microscopic examination results showed that the FI value dramatically decreased to 2.5 in the aerobic system, while the FI value was 4 in the aerobic/anaerobic alternation one (Fig. S4). The Gram staining images showed that some filaments became short and hollow inside in both systems.

In comparison with the aerobic system, the anaerobic/aerobic alternation system allowed a much higher presence of the filamentous bacteria, as shown in Fig. 2, suggesting the advantage of the anaerobic/aerobic alternation condition in keeping the dominant position of *M. parvicella* in activated sludge. This result may explain why *M. parvicella*-caused sludge bulking normally occurred in biological nutrient removal processes containing at least one anaerobic/aerobic alternation (Rossetti et al., 2005). An in situ investigation (Andreasen and Nielsen, 2000) indicated that *M. parvicella* had a considerable capacity to take up oleic acid under aerobic, anoxic or anaerobic conditions, with the most significant uptake occurring under the anaerobic condition. However, the uptake of LCFA for other filaments and floc formers was significantly diminished or even lost in the absence of aerobic conditions. According to a metabolic model of *M. parvicella* established by McIlroy et al. (2013), LCFAs were accumulated as neutral lipids (triacylglycerides, TAGs) under the anaerobic condition, and subsequently processed in the oxidative tricarboxylic acid (TCA) cycle, providing energy and intermediates required for *M. parvicella* growth under aerobic conditions. Thus, the presence of anaerobic condition may provide *M. parvicella* an advantage against other bacteria which do not possess storage capacity under such conditions.

It should be noted that the aerobic system in Phase II was almost the same with the LCFA system in Phase I in terms of the operational conditions and carbon source. However, the growth states of *M. parvicella* were significantly different. The only difference for the two systems was the inocula. In Phase II, seed sludge was collected on 11th March when sludge bulking had passed the peak, and the abundance of active *M. parvicella* was beginning to decrease, suggesting that *M. parvicella* might have entered its decline phase. Microscopic observation results (Fig. S5) showed that *M. parvicella* was in a healthy state with long and regular filaments in the seed sludge of Phase I, because sludge bulking was just beginning in the full-scale WWTP. Some empty or deformed cells of filamentous bacteria were observed in the seed sludge of Phase II although the SVI value was the highest in mid-March (Fig. S6). It seemed that the decay trend of *M. parvicella* could not easily be reversed. On the other hand, cultures of *M. parvicella* could grow over a wide range of oxygen partial pressures, but good growth was obtained under microaerophilic conditions (Rossetti et al., 2005). High DO concentration (>6 mg L⁻¹) might be toxic to this microorganism

Fig. 1. SVI changes in three phases.

Fig. 2. Quantification of *M. parvicella* in activated sludge under different conditions by qPCR and FISH analysis. (a) Relative abundance of *M. parvicella* in activated sludge quantified by qPCR. (b) Relative abundance of *M. parvicella* in activated sludge quantified by FISH analysis.

Table 5
The *t*-test results of batch experiment.

Experimental phases	Group ^a	p value ^b			
		qPCR	FISH	SVI	
Phase I	Mixed NaAc	0.0678	0.0564	0.0851	
	Mixed LCFA	0.0077	0.0002	0.0007	
	NaAc LCFA	0.0016	0.0001	0.0012	
Phase II	Aerobic Altern	0.0028	0.0001	0.0001	
Phase III	13 °C 20 °C	0.0255	0.0137	0.0087	

Note: $p > 0.05$, there is no significant difference between two groups; $0.01 < p < 0.05$, there is a significant difference between two groups; $p < 0.01$, there is a highly significant difference between two groups (Holm, 1979).

^a 'Mixed' is mixed carbon source and 'Altern' is anaerobic/aerobic alternative condition.

^b p value reflects the significance of the difference between two groups.

(Slijkhuys, 1983b). The DO values in aerobic condition of Phase I (5.8 mg L^{-1}) and Phase II (6.7 mg L^{-1}) were quite similar. The effects of DO on *M. parvicella* growth, however, could not be excluded since DO was not strictly controlled over the whole experimental period.

3.3. Influence of temperature on *M. parvicella* growth

By employing LCFA as the sole carbon source and adopting the anaerobic/aerobic alternation strategy, the influence of temperature on *M. parvicella* growth was investigated in Phase III. At a temperature of 20°C , the SVI value decreased quickly from 183.2 mL g^{-1} to 46.5 mL g^{-1} , while the SVI value was 129.5 mL g^{-1} in the end at 13°C . Similar to the trend of SVI, the relative abundances of *M. parvicella* were found to decrease from around 54.70% to 34.07% and 4.94%, and from around 56.41% to 37.38% and 7.88%, respectively, by using FISH

and qPCR methods for the 13 °C and 20 °C systems (Fig. 2). In addition, the *M. parvicella* gene copy numbers in activated sludge decreased from 8.21×10^{11} to 2.36×10^{11} in the 13 °C system, and from 1.29×10^{12} to 1.41×10^{10} gene copies g^{-1} sludge in the 20 °C system (Fig. S2). According to statistical analysis (Table 5), the 13 °C system was significantly different ($p < 0.05$) from the 20 °C one in terms of *M. parvicella* abundance and SVI. Microscopic examination results (Fig. S7) showed that the FI value dramatically decreased from 5 to 2 at 20 °C, while the FI value was kept at 4 at 13 °C. The Gram staining images showed the presence of short and hollow filaments after around two-month operation, and the sludge flocs were relatively compact with lower abundance of filaments extending outside.

Among various factors inducing filamentous sludge bulking, temperature seems to be correlated with the changes in both SVI and *M. parvicella* abundance in activated sludge (Wang et al., 2014). This study showed that a low temperature (13 °C) favored keeping a high relative abundance of *M. parvicella*. Though the optimal temperature for *M. parvicella* was determined to be 25 °C using pure cultures (Slijkhuis, 1983a), most previously sludge bulking events caused by *M. parvicella* mainly occurred at a low temperature (Miana et al., 2002; Wanner, 1994). The low temperature preference of *M. parvicella* has already been reported both in a full-scale plant and in a laboratory system. Knoop and Kunst (1998) found that *M. parvicella* could grow well in long and active filaments at low temperature around 12 °C and destruction of filament structure appeared in a lab-scale system with the increase of temperature from 12 °C to 20 °C. *M. parvicella* may produce enzymes that could destroy its cell wall in warmer water (>20 °C) (Franz and Matsche, 1994; Pitman, 1996). Cold climate in winter and spring was regarded as the main stimulator for the dominant growth of *M. parvicella* (Pitman, 1996). The growth rate of *M. parvicella* was still quite high even when the temperature was as low as 7 °C, while the bioactivity of most bacteria dramatically decreased at such a low temperature (Rossetti et al., 2002). Sheik et al. (2016) also performed temperature-dependent incubation experiments under aerobic conditions over wide range of temperatures (4–35 °C) and compared *M. parvicella* ^{13}C -oleic acid assimilation rates at the 5 h time point. The observed two temperature optima (4 °C and 25 °C) may be attributed to the differences in the bioavailability of ^{13}C -oleic acid (higher levels of bioavailability are expected at the lower temperatures) (Rossetti et al., 2005) and the altered activity of the acyl-CoA ligases for ^{13}C -oleic acid assimilation (higher assimilation rates might be expected at the higher temperature). Therefore, it is obvious that low temperature contributes to the competitive advantage of *M. parvicella* in activated sludge. However, it is still dubious to conclude whether low temperature stimulates the growth of *M. parvicella*, or just slows down its death rate by inhibiting metabolic rate; more knowledge is needed about the low-temperature response mechanism and ecophysiology of *M. parvicella*.

Correlations between SVI and *M. parvicella* abundance were calculated, as shown in Table 6. As a result, significant positive correlations ($p < 0.05$) were observed, which was in accordance with some previous studies (Kaetzke et al., 2005; Kumari et al., 2009b). Moreover, highly significant correlation ($r = 0.964$, $p < 0.01$) was also observed between

SVI and *M. parvicella* abundance for sludge from Xiaohongmen WWTP (seed sludge source) over the sampling period.

Although SVI is a convenient parameter to monitor the state of activated sludge in practice, it should be noted that it could not fully reflect the dynamic state of sludge bulking. Some studies have demonstrated poor correlation between SVI and filamentous bacteria (Valverde-Pérez et al., 2016). Recently, some other parameters, such as yield stress parameter and floc strength factor, have been used to better represent the bulking state of sludge (Burger et al., 2017; Wágner et al., 2015).

4. Conclusion

Though the preferences of *M. parvicella* for carbon sources, aerobic/anaerobic alternation and temperature were confirmed in this study by using bulking activated sludge as seed sludge, we could not find a condition to further enrich *M. parvicella*. At the same time, the three phases of the experiments were performed using seed sludge collected in different bulking stages, which makes it difficult to compare the results in different experimental phases. Besides, the HRT and SRT adopted in this study were much longer than real WWTPs, which might affect the bacterial population. In order to better understand the factors affecting the growth of *M. parvicella*, further studies are required to perform the experiments using the same seed sludge and rational technological parameters.

In conclusion, this study investigated the effects of carbon source, anaerobic/aerobic conditions and temperature on the growth of the filamentous bacterium *M. parvicella* in batch-scale systems by using seed sludge collected from a full-scale WWTP suffering sludge bulking. It was revealed for the first time that integrated conditions including the use of LCFA as carbon source, anaerobic/aerobic alternation treatment and low temperature favored the survival of *M. parvicella*, which laid a foundation for subsequent pure culture study. However, more investigations are required to rich the knowledge on physiology and metabolism of *M. parvicella*.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2017.07.261>.

Table 6
The correlation between SVI and *M. parvicella* abundance in three phases.

Correlation coefficient	Carbon source			Aerobic/Anaerobic		Temperature	
	Mixed	NaAc	LCFA	Aerobic	Aerobic/Anaerobic	13 °C	20 °C
r^a	0.883	0.867	0.473	0.993	0.699	0.879	0.976
p^b	<0.001**	<0.001**	0.121	<0.001**	0.011*	0.001**	<0.001**

Note: $p < 0.01$, highly significant correlation; $0.01 < p < 0.05$, significant correlation.

^a r is the correlation coefficient. $r > 0$, positive correlation; $r < 0$, negative correlation.

^b p is the significant coefficient.

References

- Amann, R.L., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56, 1919–1925.
- Andreasen, K., Nielsen, P.H., 1998. In situ characterization of substrate uptake by *Microthrix parvicella* using microautoradiography. *Water Sci. Technol.* 37, 19–26.
- Andreasen, K., Nielsen, P.H., 2000. Growth of *Microthrix parvicella* in nutrient removal activated sludge plants: studies of in situ physiology. *Water Res.* 34, 1559–1569.
- Bru, D., Martin-Laurent, F., Philippot, L., 2008. Quantification of the detrimental effect of a single primer-template mismatch by real-time PCR using the 16S rRNA gene as an example. *Appl. Environ. Microbiol.* 74, 1660–1663.
- Burger, W., Krysiak-Baltyn, K., Scales, P.J., Martin, G.J., Stickland, A.D., Gras, S.L., 2017. The influence of protruding filamentous bacteria on floc stability and solid-liquid separation in the activated sludge process. *Water Res.*
- Dueholm, T., Andreasen, K., Nielsen, P.H., 2000. Transformation of lipids in activated sludge. *Water Sci. Technol.* 43, 165–172.
- Eaton, A.D., Clesceri, L., Rice, E., Greenberg, A., Franson, M., Franson, M.A.H., Clesceri, L.S., Rice, E., Greenberg, A.S., 2005. Standard Methods for the Examination of Water and Wastewater. 21st Ed. Washington, DC, USA, American Public Health Association (APHA).
- Eikelboom, D.H., Andreadakis, A., Andreasen, K., 1998. Survey of filamentous populations in nutrient removal plants in four European countries. *Water Sci. Technol.* 37, 281–289.
- Erhart, R., Bradford, D., Seviour, R.J., Amann, R., Blackall, L.L., 1997. Development and use of fluorescent in situ hybridization probes for the detection and identification of "*Microthrix parvicella*" in activated sludge. *Syst. Appl. Microbiol.* 20, 310–318.
- Franz, A., Matsche, N., 1994. Investigation of a bacteria-enzyme additive to prevent foaming in activated sludge plants. *Water Sci. Technol.* 29, 281–284.
- Henze, M., Kristensen, G.H., Strube, R., 1994. Rate-capacity characterization of wastewater for nutrient removal processes. *Water Sci. Technol.* 29, 101–107.
- Holm, S., 1979. A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* 6, 65–70.
- Hucker, G.J., Conn, H.J., 1923. *Methods of Gram Staining*.
- Jenkins, D., Richard, M.G., Daigger, G.T., 1993. Manual on the causes and control of activated sludge bulking and foaming. 2. Manual on the Causes and Control of Activated Sludge Bulking and Foaming. 2. ed. Lewis.
- Kaetzke, A., Jentsch, D., Eschrich, K., 2005. Quantification of *Microthrix parvicella* in activated sludge bacterial communities by real-time PCR. *Lett. Appl. Microbiol.* 40, 207–211.
- Kapuscinski, J., 1995. DAPI: a DNA-specific fluorescent probe. *Biotech. Histochem.* 70, 220–233.
- Knoop, S., Kunst, S., 1998. Influence of temperature and sludge loading on activated sludge settling, especially on *Microthrix parvicella*. *Water Sci. Technol.* 37, 27–35.
- Kumari, S.K.S., Marrengane, Z., Bux, F., 2009a. Application of quantitative RT-PCR to determine the distribution of *Microthrix parvicella* in full-scale activated sludge treatment systems. *Appl. Microbiol. Biotechnol.* 83, 1135–1141.
- Kumari, S.K.S., Marrengane, Z., Bux, F., 2009b. Application of quantitative RT-PCR to determine the distribution of *Microthrix parvicella* in full-scale activated sludge treatment systems. *Appl. Microbiol. Biotechnol.* 83, 1135–1141.
- Mamais, D., Andreadakis, A., Noutsopoulos, C., Kalergis, C., 1998. Causes of, and control strategies for, *Microthrix parvicella* bulking and foaming in nutrient removal activated sludge systems. *Water Sci. Technol.* 37, 9–17.
- Marneri, M., Mamais, D., Koutsouki, E., 2009. *Microthrix parvicella* and *Gordonia amarae* in mesophilic and thermophilic anaerobic digestion systems. *Environ. Technol.* 30, 437–444.
- McIlroy, S.J., Kristiansen, R., Albertsen, M., Karst, S.M., Rossetti, S., Nielsen, J.L., Tandoi, V., Seviour, R.J., Nielsen, P.H., 2013. Metabolic model for the filamentous '*Candidatus Microthrix parvicella*' based on genomic and metagenomic analyses. *ISME J.* 7, 1161–1172.
- Miana, P., Grando, L., Caravello, G., Fabris, M., 2002. *Microthrix parvicella* foaming at the Fusina WWTP. *Water Sci. Technol.* 46, 499–502.
- Nielsen, P.H., Roslev, P., Dueholm, T.E., Nielsen, J.L., 2002. *Microthrix parvicella*, a specialized lipid consumer in anaerobic-aerobic activated sludge plants. *Water Sci. Technol.* 46, 73–80.
- Nielsen, P.H., Kragelund, C., Seviour, R.J., Nielsen, J.L., 2009. Identity and ecophysiology of filamentous bacteria in activated sludge. *FEMS Microbiol. Rev.* 33, 969–998.
- Nielsen, P.H., Saunders, A.M., Hansen, A.A., Larsen, P., Nielsen, J.L., 2012. Microbial communities involved in enhanced biological phosphorus removal from wastewater—a model system in environmental biotechnology. *Curr. Opin. Biotechnol.* 23, 452–459.
- Noutsopoulos, C., Mamais, D., Andreadakis, A., 2012. A hypothesis on *Microthrix parvicella* proliferation in biological nutrient removal activated sludge systems with selector tanks. *FEMS Microbiol. Ecol.* 80, 380–389.
- Pei, R., Kim, S.C., Carlson, K.H., Pruden, A., 2006. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* 40, 2427–2435.
- Pitman, A.R., 1996. Bulking and foaming in BNR plants in Johannesburg: problems and solutions. *Water Sci. Technol.* 34, 291–298.
- Quéméneur, M., Marty, Y., 1994. Fatty acids and sterols in domestic wastewaters. *Water Res.* 28, 1217–1226.
- de los Reyes, F.L., Rothauszky, D., Raskin, L., 2002. Microbial community structures in foaming and nonfoaming full-scale wastewater treatment plants. *Water Environ. Res.* 74, 437–449.
- Rossetti, S., Carucci, A., Rolle, E., 1994. Survey on the occurrence of filamentous organisms in municipal wastewater treatment plants related to their operating conditions. *Water Sci. Technol.* 29, 305–308.
- Rossetti, S., Christensson, C., Blackall, L.L., Tandoi, V., 1997. Phenotypic and phylogenetic description of an Italian isolate of "*Microthrix parvicella*". *J. Appl. Microbiol.* 82, 405–410.
- Rossetti, S., Tomei, M.C., Levantesi, C., Ramadori, R., Tandoi, V., 2002. *Microthrix parvicella*: a new approach for kinetic and physiological characterization. *Water Sci. Technol.* 46, 65–72.
- Rossetti, S., Tomei, M.C., Nielsen, P.H., Tandoi, V., 2005. "*Microthrix parvicella*", a filamentous bacterium causing bulking and foaming in activated sludge systems: a review of current knowledge. *FEMS Microbiol. Rev.* 29, 49–64.
- Rupf, S., Merte, K., Eschrich, K., 1999. Quantification of bacteria in oral samples by competitive polymerase chain reaction. *J. Dent. Res.* 78, 850–856.
- Sheik, A.R., Muller, E.E., Audinot, J.N., Lebrun, L.A., Grysan, P., Guignard, C., Wilmes, P., 2016. In situ phenotypic heterogeneity among single cells of the filamentous bacterium *Candidatus Microthrix parvicella*. *ISME J.* 10, 1274–1279.
- Slijkhuis, H., 1983a. *Microthrix parvicella*, a filamentous bacterium isolated from activated sludge: cultivation in a chemically defined medium. *Appl. Environ. Microbiol.* 46, 832–839.
- Slijkhuis, H., 1983b. The Physiology of the Filamentous Bacterium *Microthrix parvicella*. Landbouwhogeschool Press.
- Slijkhuis, H., Deinema, M.H., 1988. Effect of environmental conditions on the occurrence of *Microthrix parvicella* in activated sludge. *Water Res.* 22, 825–828.
- Valverde-Pérez, B., Wágner, D.S., Lóránt, B., Gülay, A., Smets, B.F., Plósz, B.G., 2016. Short-sludge age EBPR process—microbial and biochemical process characterisation during reactor start-up and operation. *Water Res.* 104, 320–329.
- Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., Schleifer, K.H., 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. *Appl. Environ. Microbiol.* 60, 792–800.
- Wágner, D.S., Ramin, E., Szabo, P., Dechesne, A., Plósz, B.G., 2015. *Microthrix parvicella* abundance associates with activated sludge settling velocity and rheology—quantifying and modelling filamentous bulking. *Water Res.* 78, 121–132.
- Wang, J., Li, Q., Qi, R., Tandoi, V., Yang, M., 2014. Sludge bulking impact on relevant bacterial populations in a full-scale municipal wastewater treatment plant. *Process Biochem.* 49, 2258–2265.
- Wanner, J., 1994. *Activated Sludge: Bulking and Foaming Control*. CRC Press.
- Westlund, A.D., Hagland, E., Rothman, M., 1998. Foaming in anaerobic digesters caused by *Microthrix parvicella*. *Water Sci. Technol.* 37, 51–55.