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Effect of organic carbon on microbial characteristics in partial nitrification system

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ABSTRACT

Two same lab-scale sequencing batch reactors were developed to investigate the effect of organic carbon on microbial characteristics in partial nitrification system. Partial nitrification is effectively achieved through aeration time and dissolved oxygen control. Results indicated that the addition of organic carbon inhibited the nitrification reaction, thus increasing the total nitrification time. However, as organic matter was consumed, it did not inhibit the achievement of partial nitrification. Scanning electron microscope pictures showed that the morphology of biomass was affected by organic carbon, which made spherical, small rod-shaped, and filamentous cells both observed and distributed all over. 16SrDNA cloning results showed that organic carbon had little effect on competitive growth of phyla α-*Proteobacteria*, δ-*Proteobacteria*, and *Bacteroidetes*; was beneficial to that of phyla γ-*Proteobacteria* and *Planctomycetes*; went against that of phyla *Acidobacteria*, *Nitrospira*, *Spirochaetes*, and *Chloroflexi*. Bacterial species comprised by phylum β-*Proteobacteria* could be easily influenced by organic carbon. In addition, organic carbon affected the species related to nitrogen removal, especially that are not good for competitive growth of ammonia-oxidizing bacteria. Interestingly, the major function of *Denitratisoma oestradiolicum* may be affected by organic carbon. In addition, species of ammonia-oxidizing bacteria were affected by organic carbon, which was positive for *Nitrosomonas europaea*, but *Nitrosomonas* sp. was also the dominant one.

Keywords: Partial nitrification; Organic carbon; Microbial characteristics; Scanning electron microscope; Clone-sequencing

1. Introduction

The traditional full nitrification oxidizes ammonia nitrogen to nitrate nitrogen under the combined action of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Partial nitrification represses the growth of NOB, controls the production of ammonia nitrogen in nitrite nitrogen under the action of AOB, and saves about 25% of aeration [1]. In recent years, partial nitrification has been widely concerned as a type of pre-treatment process [2,3].

The main means of achieving and maintaining partial nitrification are high temperature, high pH, high free ammonia, high free nitrous acid, suitable sludge retention time (SRT), and low dissolved oxygen (DO) [4,5]. The use of DO

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control has been shown to be an effective method to accomplish partial nitrification [2,6]. In recent years, partial nitrification has been widely used in the treatment of sidestream wastewater such as landfill leachate and digestate [7]. Partial nitrification of low ammonia wastewater is relatively difficult to achieve.

For wastewater biological treatment process, pollutants removal is performed by two kinds of bacteria, heterotrophic bacteria and autotrophic bacteria. Carbons needed by heterotrophic and autotrophic bacteria are organic and inorganic, respectively. In principle, nitrification can be achieved without carbon source. However, actual organic carbon source in wastewater is one of the major pollutants; the previous research about effect of organic carbon is more focused on denitrification [8,9]. It is of practical significance to explore the impact of carbon source on partial nitrification. C/N (CH_3COONa-C/NH_4Cl-N) of municipal wastewater is fluctuant, and then the organic carbon enters nitrification system is fluctuant. The presence of organic matter affects the operation of the partial nitrification in the SHARON process, when the C/N ratio was higher than 0.3, for the feeding concentrations, the ammonia oxidization drastically decreased to levels around 10% [10]. Jia et al. [11] evaluated the effects of organic carbon on nitrogen conversion and microbial communities in a CANON system and found that at a C/N ratio of 1.2 both nitrogen and carbon were removed simultaneously; AOB, de-nitrifiers, and anammox bacteria coexist and removed N cooperatively. Liang et al. [12] pointed out that the presence of chemical oxygen demand (COD) performs activity inhibition on AOB and enables the survival of denitrifiers by adding COD into a CANON system [12]. In recent years, it is common to use C/N = 2 as a research in an experiment. In this experiment, we did not focus on the specific carbon concentrations to research the partial nitrification so we chose C/N = 2 to do the experiment.

Although some researches have demonstrated the effect of organic carbon on partial nitrification, the effect of organics on nitrification reaction is not clear. Several differences are existent in this study: (1) temperature is ambient temperature (about 19°C–22°C); (2) microbial community of total bacteria is investigated seriously, not limit to nitrogen removal functional bacteria; (3) no complete nitrogen removal process is set (only nitrification) for investigating the effect of organic carbon on startup of partial nitrification. Therefore, the main goals of this study were to (1) achieve partial nitrification according to the room temperature which is practical significance in laboratory-scale sequencing batch reactors (SBRs) using synthetic wastewater with and without organic carbon, respectively, (2) investigate the effects of organic carbon on partial nitrification by the microbiology of SBRs, using scanning electron microscope (SEM) and clone-sequencing techniques, and (3) explore that whether adopting the method of no complete nitrogen removal process (only nitrification) could distinguish the effects of organic carbon's existence on startup of partial nitrification.

2. Materials and methods

2.1. Experimental setup

Two same sequencing batch reactors with working volume of about 22 L (diameter: 200 mm, height: 800 mm, and loading height: 700 mm) were operated in the lobby of the laboratory. The reactors were made of polymethyl methacrylate. System mainly contained stirrer, DO probe, pH probe, water quality analyzer (WTW Multi 3420i meter, WTW company, Germany), computer, microporous aeration diffusors, rotor flow meter, and air pump. The synthetic wastewater used in Reactor 1 (R1) contained NH_4Cl and $NaHCO_3$ as main substrates, together with a small amount of KH_2PO_4. Their corresponding concentrations were 70 mg·L^{-1} (NH_4^+), 500 mg·L^{-1} ($CaCO_3$), and 3 mg·L^{-1} (phosphorus). CH_3COONa was additionally added to Reactor 2 (R2); its concentration was 140 mg·L^{-1} (COD). Ingredients of trace elements were the same as the research of Yin et al. [13]. Inoculated sludge was taken from aeration tank of a wastewater treatment plant, which had a good nitrification performance, and its f (MLVSS/MLSS) and sludge volume index (SVI) were 0.75 and 90 (no unit), respectively. During the experimental process, MLSS of the wastewater was kept at about 3,500 mg·L^{-1}. Through measurement, SRT of R1 and R2 was 70–80 and 50–60 h, respectively. 2–4 cycles were run each daytime, and reactors were idle for about 8 h in the night. Each cycle contained instantaneous influent, aeration, 30-min settling, 5-min drawing out, and washing stage, which was twice of instantaneous influent with only tap water, 30-min settling and 5 min-drawing out. Due to the dilution of washing stage, remaining un-decanted substrate could be neglected. Meanwhile, the inflow was concentrated solution not raw water, so the volume exchange rate could be considered as 100%.

2.2. Startup of partial nitrification through DO control

The startup of partial nitrification in two reactors was achieved through aeration time control and DO control; DO was controlled at 0.5–1 mg·L^{-1} through rotor flow meter, aeration time was controlled by timer, temperature was about 19°C–22°C, and the varying range of pH in each cycle was about 7.9–7.2. Nitrite accumulation ratio (ϱ) and nitrification time (t) were measured at every 10 cycles, which were known from variation of ammonium, nitrite, and nitrate in one cycle. Nitrification time was the time when the removal ratio of ammonia was about 100% in each cycle. Nitrite accumulation ratio was calculated as $\varrho = NO_2^-/(NO_2^- + NO_3^-) \times 100\%$ (where NO_2^- and NO_3^- all refers the produced and corresponding to nitrification time). Aeration times of every 10 cycles were different. They were the latest obtained nitrification time.

2.3. Analytical methods

All samples were analyzed after filtration with 0.45 μm filter paper. NH_4^+, NO_2^-, NO_3^-, COD, MLSS, MLVSS, and SVI were measured according to Standard Methods [14]. The temperature and pH were detected on line using WTW level 2 pH meters (WTW company, Germany). DO concentration was continuously monitored by WTW, pH/oxi340i meter, with DO probes (WTW Company, Germany).

2.4. SEM observation

The partial nitrification sludge was observed using a SEM. The specimens were prepared as follows. Samples were

fixed with 2.5% glutaraldehyde solution for 1.5 h with 4°C, after which they were washed thrice with phosphate buffer. Next, the samples were subjected to sequential ethanol dehydration (including 50%, 70%, 80%, 90%, and 100% ethanol). Following were twice metathesis, first with ethanol and isoamyl acetate of 1:1, second with only isoamyl acetate. Then the samples were dried for 24 h at freezer dryer (FD-1A-50). The specimens were finally sputter-coated with gold and then examined under a SEM (Jeol, Japan).

2.5. DNA extraction, polymerase chain reaction amplification and cloning-sequencing of the 16SrRNA gene

DNA was extracted from the sample using the Ultra Clean™ Soil DNA kit (MoBio Laboratories, Solana Beach, CA), which includes bead beating and a spin-column purification steps [15], and then DNA was stored at –20°C until processed further.

The amplification of the 16SrRNA gene of total bacteria was performed using the universal primer set27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′). The mixture solutions (50 μL) for polymerase chain reaction (PCR) amplification of total bacteria consisted of 10 × PCR buffer 5 μL, 1 μL dNTP (2.5 mmol·L^{-1}), 1 μL 27f (20 μmol·L^{-1}), 1 μL 1492r (20 μmol·L^{-1}), 0.5 μL Taq DNA polymerase, 0.5 μL template DNA, and 41 μL PCR-grade sterile water. The PCR program for the total bacterial 16SrRNA gene measurement was as follows: 1.5 min at 95°C; 5 cycles of 0.5 min at 95°C, 0.5 min at 60°C, 2 min at 72°C; 5 cycles of 0.5 min at 95°C, 0.5 min at 55°C, 2 min at 72°C; 15 cycles of 0.5 min at 95°C, 0.5 min at 50°C, 2 min at 72°C; 10 min at 60°C.

The amplification of the 16SrRNA gene of AOB was performed using the universal primer set amoA-1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′). The mixture solutions (50 μL) for PCR amplification of AOB consisted of 5 μL 10 × PCR buffer, 2 μL dNTP (2.5 mmol·L^{-1}), 1 μL amoA-1F (20 μmol·L^{-1}), 1 μL amoA-2R (20 μmol·L^{-1}), 0.5 μL Taq DNA polymerase, 2 μL template DNA, and 38.5 μL PCR-grade sterile water. The PCR program for the AOB 16SrRNA gene measurement was 5 min at 94°C; 35 cycles of 0.5 min at 94°C, 1 min at 55°C, 1 min at 72°C; 10 min at 72°C.

The PCR products were purified with the purification kit (Sangon, China) and cloned using pMD18-T plasmid vector system (TaKaRa, Japan). White colonies including the insert were randomly selected for sequencing. All sequences obtained were compared with the reference microorganisms available in Genbank by BLAST tool. The Genbank accession numbers of total bacteria in this study are KP411846-KP411869 and KP663383-KP663412.

3. Results and discussions

3.1. Reactors performance

The variations of nitrite accumulation ratio and nitrification time during 150 run cycles in two reactors are shown as Figs. 1(a) and (b), respectively. And, the variations of ammonia, nitrite, and nitrate concentration during 150 run cycles in two reactors are shown in Figs. 3(a) and (b), respectively.

As shown Figs. 1(a) and (b), variations of nitrite accumulation ratio and nitrification time between R1 and R2 were the same: nitrite accumulation ratio increased gradually and then remains stable; and nitrification time decreased gradually and then remained stable. Nitrite accumulation ratio of 90% was used as a sign to evaluate the success of partial nitrification startup, stable operation. Cycles needed in two reactors were about 96 and 99, which were similar. Dissimilarly, although the variation of nitrification time between R1 and R2 was the same, the values of nitrification time were significantly different. The reason was for synthetic wastewater with organic carbon, nitrification time contained the time needed by organic carbon consumption. Nitrification time of R2 was about 1.5 times that of R1 in this study. It was clear that for SBR process, the addition of organic carbon inhibited the nitrification reaction, thus increasing the total nitrification time. But as organic matter was consumed, it does not inhibit the achievement partial nitrification. As shown in Figs. 2(a) and (b), the concentrations of ammonia, nitrite, and nitrate were the same in the two reactors, and by controlling the nitrification time, the removal ratio of ammonia was about 100% in each cycle. Meanwhile, the accumulation of nitrite gradually achieved over 90%. In the achievement process of partial nitrification, nitrite showed the same variation trend in R1 and R2, rising and then remaining stable; the variation trend of nitrate and total nitrogen removal ratio was similar, declining, and then remaining stable; but the values in R1

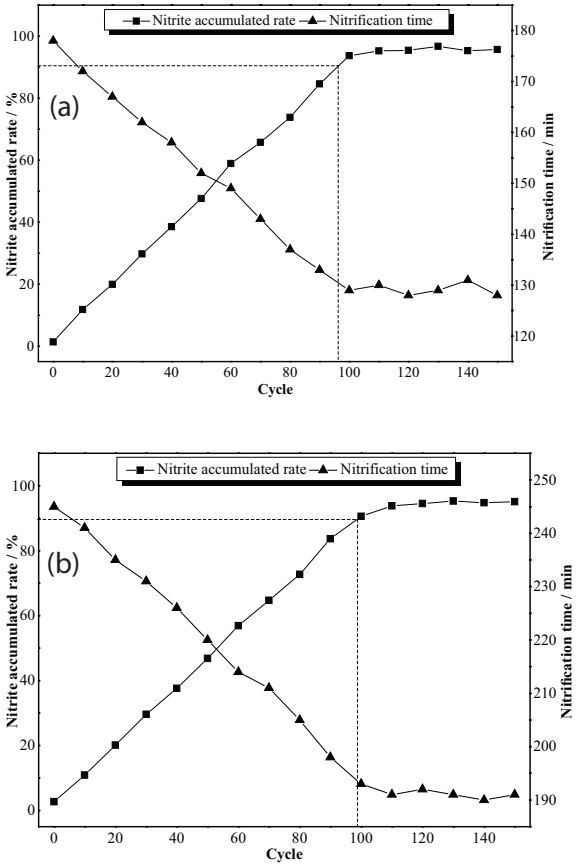

Fig. 1. Variations of nitrite accumulated rate and nitrification time during 150 run cycles in (a) R1 and (b) R2.

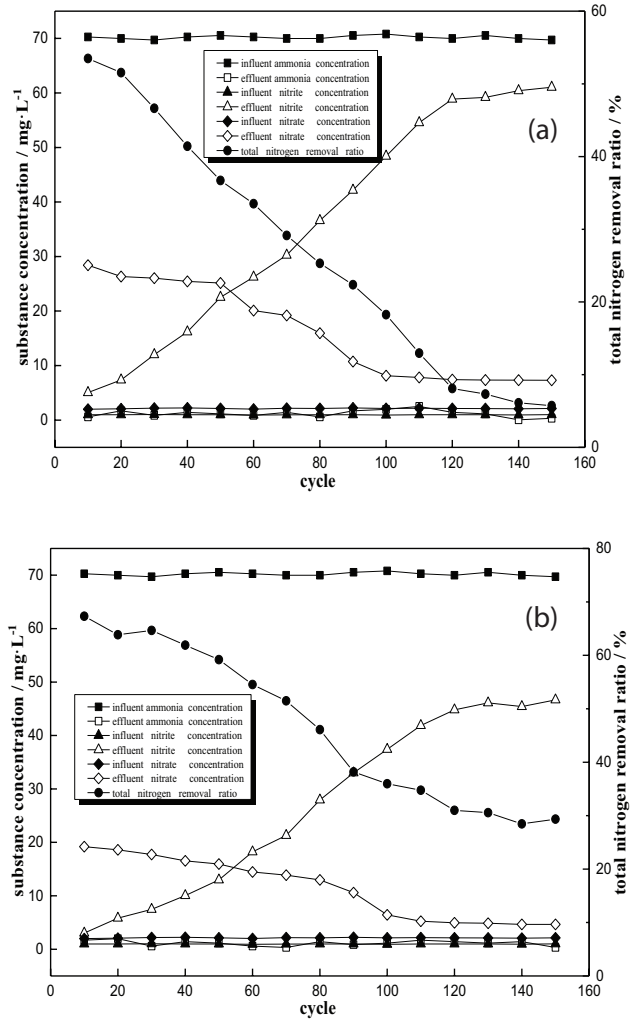

Fig. 2. Variation of ammonia, nitrite, nitrate concentration and total nitrogen removal ratio during 150 run cycles in (a) R1 and (b) R2.

and R2 were different. When partial nitrification was stable (cycles 120–150), nitrite and nitrate concentrations of R1 and R2 were about 58–62 and 45–48 mg·L^{-1} and 7 and 5 mg·L^{-1}, respectively. Total nitrogen (ammonia, nitrite, and nitrate concentration) removal ratio of R1 and R2 was about 5%–8% and 27%–32%, respectively. It might be that the organic carbon in R2 produced weak denitrification under such DO, so the nitrite and nitrate concentrations in R2 were lower than R1, and the total nitrogen removal ratio was higher than R1.

The variations of NH_4^+ and COD concentrations and nitrite and nitrate concentrations in one cycle of steady R1 and R2 are described in Figs. 3(a) and (b), respectively. As shown in Figs. 3(a), NH_4^+ concentration decreased evenly in R1, but in R2 decreased slowly in early stage. Oppositely, in R2, COD concentration decreased rapidly in early stage. Oxygen saturation constant of heterotrophic bacteria was less than autotrophic bacteria [16]. In R2, DO concentration was controlled at 0.5–1 mg·L^{-1}, initial period in one cycle, due to high COD concentration, DO was prior consumed by heterotrophic bacteria, resulting in the activity of autotrophic bacteria decrease, and NH_4^+ concentration decreased slowly.

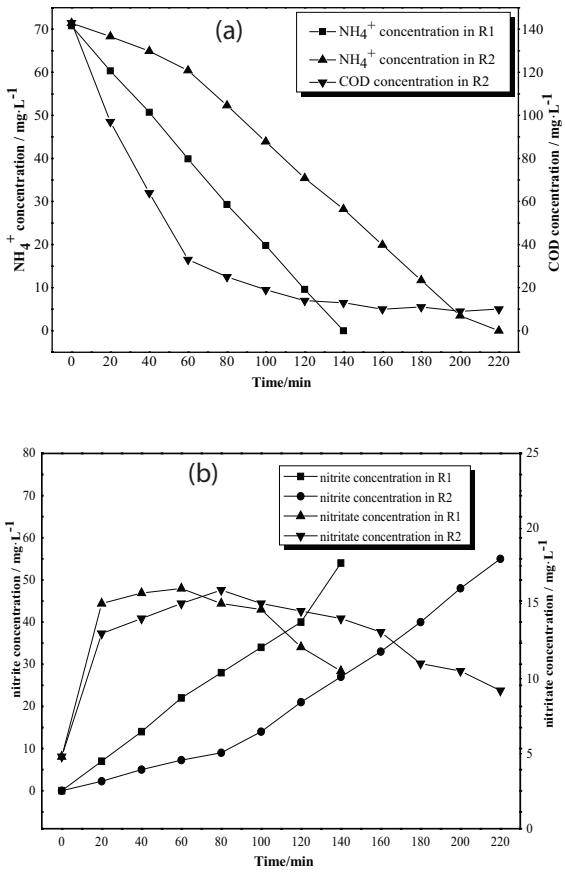

Fig. 3. Variations of (a) NH_4^+ and COD and (b) nitrite and nitrate concentrations in one cycle of steady R1 and R2.

As Fig. 3(b) shows, nitrite concentration increased evenly in R1, and in R2 increased slowly in early stage. Nitrate concentration increased quickly in two reactors in early stage because inoculated sludge was a good full nitrification performance. Following the next cycles, nitrate concentration increased steady first and then decreased gradually. The nitrite and nitrate concentrations increased with the gradual consumption of NH_4^+. At the later stage of the reaction, when the partial nitrification was gradually achieved, and nitrate slowly decreased.

3.2. SEM analysis

The morphology of the sludge was observed in more detail using SEM. Sludges were taken from Cycle 140 in R1 and R2 for SEM examination, respectively. Images taken from R1 (Fig. 4(a)) show that thick clusters of spherical cells were the dominant population structure than other forms. Images taken from R2 (Fig. 4(b)) show that spherical, small rod-shaped, and filamentous cells were observed and distributed all over. Many reports described that AOB and NOB were spherical or small rod-shaped, most of denitrifying bacteria were small rod-shaped [17–19]. With variation of nitrite accumulated ratio and total nitrogen removal in R1 and R2, it was speculated that spherical cells in Fig. 4(a) might be AOB and spherical, and small rod-shaped cells in Fig. 4(b) might be AOB and denitrifying bacteria. The results of SEM analysis

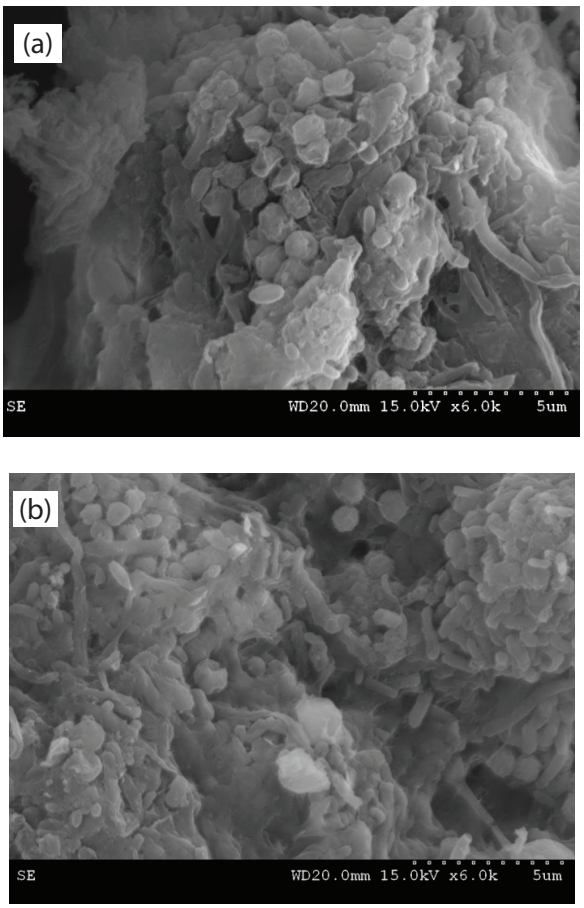

Fig. 4. SEM pictures taken from the steady operation of (a) R1 and (b) R2.

showed that the species of bacteria in R2 was more than that of R1. The denitrifying bacteria observed proved that R2 did undergo denitrification in the actual operation process. The next experiment wanted to focus on the impact of the presence of organic carbon on nitrifying bacteria, so the 16SrDNA cloning method was adopted to study the differences of bacteria.

3.3. Microbial community of total bacteria

Two sludge samples were taken from Cycle 140 in R1 and R2, respectively. 16SrDNA cloning results of total bacteria were described in supplementary material. Phyla comprised by R1 and R2 are described in Table 1. With Fig. 1, up to Cycle 140, the total aeration time (reaction time) of R1 and R2 was about 345 and 490 h, respectively. SRT of R1 and R2 was 70–80 h and 50–60 h, respectively. The total aeration time of R1 was about 4.6 times as large as its SRT, and R2's was about 8.9 times. $0.5^{4.6}\approx0.04$ and $0.5^{8.9}\approx0.002$. The remaining inoculum sludges in R1 and R2 were both less than 5%, which had little effect on microbial data obtained from Cycle 140.

As shown in Table 1, six phyla of *α-Proteobacteria*, *β-Proteobacteria*, *δ-Proteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, and uncultured bacterium were both comprised by R1 and R2. The proportions of *α-Proteobacteria*, *δ-Proteobacteria*, and *Bacteroidetes* between R1 and R2 are similar, which indicated

Table 1
Proportion of phylum comprised by R1 and R2

Phylum	Proportion in R1 (%)	Proportion in R2 (%)
α-Proteobacteria	1.25	2
β-Proteobacteria	32.5	18
γ-Proteobacteria	Not detected	7
δ-Proteobacteria	1.25	3
Bacteroidetes	17.5	17
Acidobacteria	7.5	Not detected
Nitrospira	3.75	Not detected
Spirochaetes	3.75	Not detected
Planctomycetes	Not detected	2
Verrucomicrobia	2.5	8
Chloroflexi	1.25	Not detected
Uncultured bacterium	28.75	43

that organic carbon had little effect on *α-Proteobacteria*, *δ-Proteobacteria*, and *Bacteroidetes*. Wagner and Loy [20] found that *β-Proteobacteria* and *Bacteroidetes* were always the dominant population in wastewater treatment system after summarizing a large number of literature reports. Similarly, apart from uncultured bacterium, both *β-Proteobacteria* and *Bacteroidetes* were the dominant population in R1 and R2. But the proportion of *β-Proteobacteria* between R1 and R2 are different, and the proportion in R1 was obvious greater than that in R2. As shown in the 16SrDNA cloning results of total bacteria (supplementary material), autotrophic bacteria (22.5%) became dominant in *β-Proteobacteria* which might be the explanation for significant difference of *β-Proteobacteria* between R1 and R2. Phylum *Verrucomicrobia* comprised gram-negative microorganisms, mostly chemoorganotrophic and found in several environments, including soils, leachates, etc. [21]. So understandably, the proportion of *Verrucomicrobia* in R2 was greater than that in R1. Both of R1 and R2 had high proportion of uncultured bacterium. This revealed the shortage of traditional microbiology analysis determination technique and the superiority of molecular biological technique. Molecular biological technique uncultured bacterium could be detected and the original microbial information in the sample would be obtained effectively and quickly.

Four phyla of *Acidobacteria*, *Nitrospira*, *Spirochaetes*, and *Chloroflexi* were only comprised by R1. Ramirez-Villanueva et al. [22] investigated bacterial community structure in maize residue amended soil with contrasting management practices and found that application of organic material generally decreased relative abundance of *Acidobacteria*. Tank and Bryant [23] pointed out that oligotrophic behavior was common among *Acidobacteria*. Phylum *Nitrospira*, which comprised autotrophic aerobic bacteria responsible for oxidation of nitrite to nitrate, was also found in R1. Detection of *Nitrospira* did not tell the bad effect of partial nitrification because the proportion of bacteria responsible for ammonia oxidation was much greater than bacteria responsible for nitrite oxidation (Table 2). At the same time, undetection of *Nitrospira* in R2 did not indicate no *Nitrospira* was existent, and only indicated that proportion of *Nitrospira* was less than 1% (100 clones were chosen for sequencing and the target

Table 2
Bacteria related to nitrogen removal in R1 and R2

Closest relative	Identity (%)	Access no.	Proportion to total bacteria (%)	Phylum
R1				
Nitrosomonas sp.	99	AJ224941	15	β-*Proteobacteria*
Denitratisoma oestradiolicum	98	KF810114	2.5	β-*Proteobacteria*
D. oestradiolicum	99	KF810117	7.5	β-*Proteobacteria*
Nitrosococcus mobilis Nc2	99	AF287297	5	β-*Proteobacteria*
Nitrosomonas sp.	99	AB079053	2.5	β-*Proteobacteria*
Candidatus Nitrospira defluvii	99	NR_074700	3.75	*Nitrospira*
R2				
D. oestradiolicum	99	KF810118	4	β-*Proteobacteria*

clone was not chosen). Phylum *Spirochaetes* was gram-negative bacteria with a distinctive spiral shape and was able to ferment carbohydrates and amino acids into mainly acetate, H_2 and CO_2 in anaerobic digesters [24]. In this study, reactors were idle at night and anaerobic state could be formed, which might be the reason for detection of *Spirochaetes* in R1. However, R2 did not comprise that *Spirochaetes* might be due to the existent of CH_3COONa which inhibited the growth of *Spirochaetes*. As we all know, generation time of heterotrophic bacteria was shorter than autotrophic bacteria. MLSS in this study was controlled at fixed value, and SRT of R1 was longer than that of R2. So, it was speculated that the long SRT involved in R1 might has induced the growth of phylum *Chloroflexi* [25].

Two phyla of γ-*Proteobacteria* and *Planctomycetes* were only comprised by R2. Some studies had shown that a shift of soil bacterial community structure toward a higher abundance of γ-*Proteobacteria* results from an input of organic carbon sources or irrigation with treated wastewater [26,27]. Phylum γ-*Proteobacteria* was related to reduction of NO_3^- to NO_2^- [28]. Considering the high total nitrogen loss (27%–32%) in R2, SND or SPND might occur. Addition of organic carbon promotes the growth of γ-*Proteobacteria*. Phylum *Planctomycetes* was facultative aerobic bacteria, chemoorganotrophic, except for the microorganisms responsible for the anaerobic ammonium oxidation, mostly gram-negative that grow slowly [29], so un-detection of *Planctomycetes* in R1 might be largely affected by organic carbon.

The detected bacteria related to nitrogen removal in R1 and R2 are described in Table 2. As shown in Table 2, R1 comprised four different species, and R2 comprised only one species.

Four different species related to nitrogen removal in R1 were *Nitrosomonas* sp., *Nitrosococcus mobilis* Nc2, *Candidatus* Nitrospira defluvii, and *Denitratisoma oestradiolicum*. *Nitrosomonas* sp. was well-known AOB. Through analyzing, Campbell et al. [30] proposed the following validation of '*N. mobilis*' as an additional species of the genus *Nitrosomonas* and Nc2 as its type strain. So, *N. mobilis* Nc2 belonged to AOB. *Ca.* N. defluvii was one of the dominant populations to converting nitrite to nitrate [31,32], and belonged to typical NOB. In general, the *D. oestradiolicum* was denitrifying bacteria, which was responsible for the reduction of nitrate to nitrogen [33]. In this study, total nitrogen removal of R1 was about 5%–8%, which indicates that little denitrification was involved. What more is the contribution of the organic carbon by decaying cells could not be ignored. As an evidence, the residual COD of about 5 $mg \cdot L^{-1}$ in Fig. S2 was probably from non-degradable soluble microbial products originated from cell decay, because acetate was very degradable. Even so, the total nitrogen removal of R1 was only about 5%–8%, but the proportion of *D. oestradiolicum* was up to 10%, so it could be speculated that the major function of *D. oestradiolicum* in R1 was not denitrifying. Meincke et al. [34] reported that *D. oestradiolicum* was able to oxidize ammonia to nitrate. The major function of *D. oestradiolicum* in R1 was may be ammonia oxidation, and the further research was needed. Taking no account of *D. oestradiolicum*, in R1, the proportion of AOB was 22.5%, and the proportion of NOB was 3.75%. Superiority of AOB was obvious.

Only *D. oestradiolicum* related to nitrogen removal was detected in R2. Because R2 was fed with 140 mg COD, the nitrogen consumption by synthesis could be significant (near 7 $mgN \cdot L^{-1}$), which comprised over about 10% of the fed ammonium. And now, considering the 27%–32% of total nitrogen removal and addition of organic carbon, the major function of *D. oestradiolicum* in R2 should be denitrifying. For R2, much ammonia and organic carbon were removed through microbial processes. Complete non-existence of AOB was not possible. Reliable explanation might be that the proportion of AOB was less than 1% (100 clones were chosen for sequencing and the target clone was not chosen), and another possibility was that the activated AOB in R2 was comprised by uncultured bacterium. Also, from Table 2, we could find that nearly all species related to nitrogen removal belonged to phylum β-*Proteobacteria*, which revealed the leading role acted by β-*Proteobacteria* in wastewater treatment.

3.4. Phylogenetic analysis of AOB

16SrDNA cloning results of AOB for R1 and R2 are described in Table 3.

As Table 3 shows, *Nitrosomonas* sp. was the only AOB detected in R1, and R2 comprised *Nitrosomonas* sp. and *Nitrosomonas europaea*. When organic carbon was existent, the proportion of *N. europaea* was 18.75%, which is much less than that of *Nitrosomonas* sp. (81.25%). Both in R1 and R2, *Nitrosomonas* sp. was the dominant AOB. *Nitrosospira* sp. was previously reported as dominant AOB when ammonia

Table 3
16SrDNA cloning results of AOB for R1 and R2

Closest relative	Identity (%)	Access no.	Proportion to total bacteria (%)	Phylum
R1				
Nitrosomonas sp.	99	JN367456	96.20	β-*Proteobacteria*
Nitrosomonas sp.	99	JN367453	1.27	β-*Proteobacteria*
Nitrosomonas sp.	99	AF272407	1.27	β-*Proteobacteria*
Nitrosomonas sp.	89	DQ228469	1.27	β-*Proteobacteria*
R2				
Nitrosomonas sp.	99	JN367456	48.44	β-*Proteobacteria*
Nitrosomonas sp.	99	JN367453	28.12	β-*Proteobacteria*
Nitrosomonas europaea	97	JN099309	18.75	β-*Proteobacteria*
Nitrosomonas sp.	96	AY958703	3.13	β-*Proteobacteria*
Nitrosomonas sp.	93	AB079055	1.56	β-*Proteobacteria*

was low while *N. europaea* predominated in the traditional nitrification and denitrification system fed with high ammonia [35]. However, ammonia concentrations between R1 and R2 were similar, so ammonia concentration should not be the reason for *N. europaea* only detected in R2. Jiang et al. [36] pointed out that *N. europaea* could potentially facilitate HCO_3^- transport under limiting inorganic carbon supply. As Fig. S2 shows, in R2, most of organic carbon was removed early in one cycle. In early stage of one cycle, NH_4^+ concentration in R2 decreased slower than R1. So, for R2, the alkalinity ($NaHCO_3$) in early stage of one cycle was excess, at the same time, no additional ionic inorganic carbon was supplied to R2, *N. europaea* would facilitate HCO_3^- transport for inorganic carbon demand. From Table 3, we could also find that all detected AOB belonged to phylum β-*Proteobacteria*, which was in accord with previous reports [37]. Access numbers of *Nitrosomonas* sp. were different, but for both R1 and R2, the proportion of access no. JN367456 was the maximum, which might be analyzed in the future.

4. Conclusions

During the nitrification of SBR process, the addition of organic carbon inhibited the nitrification reaction, thus increasing the total nitrification time. But, as organic matter was consumed, it does not inhibit the achievement partial nitrification. When C/N = 2, the nitrification time increased by about 50% compared with no organic carbon was added.

For steady operation of partial nitrification in SBR, the morphology of biomass was affected by organic carbon. When only NH_4Cl and $NaHCO_3$ were main substrates, spherical cells were the dominant population structure than other forms. However, addition of organic carbon made spherical, small rod-shaped and filamentous cells were both observed and distributed all over.

16SrDNA cloning results indicated that organic carbon had little effect on competitive growth of phyla α-*Proteobacteria*, δ-*Proteobacteria*, and *Bacteroidetes*; was beneficial to that of phyla γ-*Proteobacteria* and *Planctomycetes*; went against that of phyla *Acidobacteria*, *Nitrospira*, *Spirochaetes*, and *Chloroflexi*. Bacterial species comprised by phylum β-*Proteobacteria* were easily influenced by organic carbon, in which autotrophic bacteria became dominant when no organic carbon was added. Also, organic carbon had effect on species related to nitrogen removal, especially not good for competitive growth of AOB. Interestingly, organic carbon might have affected the major functions of *D. oestradiolicum*, which were ammonia oxidation in R1 and denitrifying in R2, and further research was needed. In addition, species of AOB were affected by organic carbon, which might be positive for *N. europaea*, but *Nitrosomonas* sp. was also the dominant one of AOB.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Supplementary material

Table S1
16SrDNA cloning results of total bacteria for R1 (without organic carbon)

Clone	Clone numbers of OTU	Proportion of OTU (%)	Sequence length (bp)	Accession number	The most similar bacteria (NCBI)	Similarity (%)	Phylum	Proportion of phylum (%)
T-57	1	1.25	1,440	KP411857	*Micavibrio* sp. EPC2 (DQ186614)	88	α-*Proteobacteria*	1.25
T-14	12	15	1,490	KP411848	*Nitrosomonas* sp. (AJ224941)	99	β-*Proteobacteria*	32.5
T-38	2	2.5	1,479	KP411853	*Denitratisoma oestradiolicum* (KF810114)	98		
T-66	6	7.5	1,493	KP411859	*D. oestradiolicum* (KF810117)	99		
T-84	4	5	1,484	KP411864	*Nitrosococcus mobilis* Nc2 (AF287297)	99		
T-85	2	2.5	1,361	KP411865	*Nitrosomonas* sp. (AB079053)	99		
T-16	1	1.25	1,479	KP411849	*Melittangium boletus* (AJ233908)	93	δ-*Proteobacteria*	1.25
T-62	5	6.25	1,465	KP411858	Uncultured *Bacteroidetes* bacterium (GQ274116)	86	*Bacteroidetes*	17.5
T-7	2	2.5	1,423	KP411847	*Bacteroidetes* bacterium (AB539999)	95		
T-75	5	6.25	1,477	KP411861	*Chryseolinea serpens* strain RYG (NR_108511)	92		
T-79	2	2.5	1,426	KP411862	*Chitinophagaceae* bacterium (FJ263933)	95		
T-6	3	3.75	1,470	KP411846	Bacterium *Ellin*6075 (AY234727)	92	*Acidobacteria*	7.5
T-25	1	1.25	1,497	KP411850	*Acidobacteria* bacterium (GU187027)	94		
T-104	2	2.5	1,466	KP411866	*Acidobacteria* bacterium (GU187039)	97		
T-109	3	3.75	1,480	KP411867	*Candidatus* Nitrospira defluvii (NR_074700)	99	*Nitrospira*	3.75
T-33	3	3.75	1,449	KP411852	*Leptonema illini* strain (NR_119299)	99	*Spirochaetes*	3.75
T-80	2	2.5	1,505	KP411863	*Spartobacteria* bacterium (GU129926)	88	*Verrucomicrobia*	2.5
T-43	1	1.25	1,456	KP411854	*Ornatilinea apprima* strain (NR_109544)	90	*Chloroflexi*	1.25
T-31	8	10	1,474	KP411851	Uncultured bacterium (JX040363)	99	*uncultured bacterium*	28.75
T-51	1	1.25	1,440	KP411855	Uncultured bacterium (AB286378)	99		
T-56	3	3.75	1,469	KP411856	Uncultured bacterium (GU454914)	94		
T-73	4	5	1,443	KP411860	Bacterium enrichment culture clone (KC539798)	99		
T-116	4	5	1,481	KP411868	Uncultured bacterium (HQ158632)	99		
T-120	3	3.75	1,452	KP411869	Uncultured bacterium (KC253303)	98		

OTU, optical transform unit.

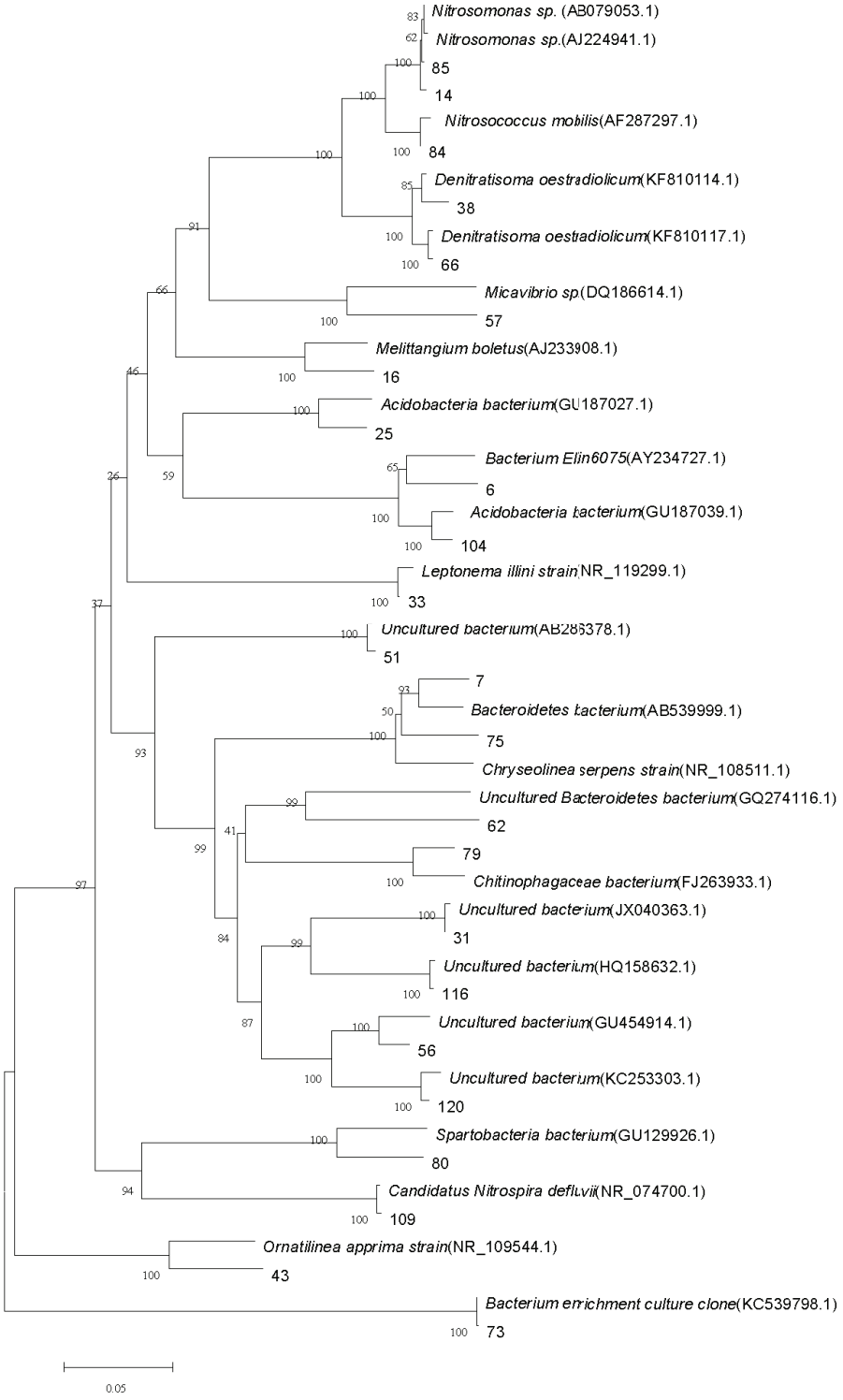

Fig. S1. Phylogenetic tree of total bacteria for R1 (without organic carbon).

Table S2
16SrDNA cloning results of total bacteria for R2 (with organic carbon)

Clone	Clone numbers of OTU	Proportion of OTU (%)	Sequence length (bp)	Accession number	The most similar bacteria (NCBI)	Similarity (%)	Phylum	Proportion of phylum (%)
15	1	1	1,433	KP663386	*Brevundimonas* sp. (DQ413152.1)	99	*α-Proteobacteria*	2
31	1	1	1,434	KP663393	*Hyphomicrobium* sp. (HM124367.1)	95		
21	2	2	1,483	KP663387	*Derxia gummosa* strain (NR_114127.1)	92	β-*Proteobacteria*	18
23	1	1	1,500	KP663388	*Burkholderiales* bacterium (KM083133.1)	94		
26	4	4	1,491	KP663391	*Denitratisoma oestradiolicum* (KF810118.1)	99		
36	5	5	1,477	KP663395	*Thauera phenylacetica* strain (NR_027224.1)	99		
42	1	1	1,483	KP663399	*Hydrogenophaga* sp. (KF441572.1)	97		
47	1	1	1,481	KP663400	*Azonexus hydrophilus* strain (EF158391.1)	95		
55	4	4	1,469	KP663402	*Comamonadaceae* bacterium (KC252871.1)	99		
40	2	2	1,489	KP663398	*Dokdonella* sp.(GQ281768.1)	91	γ-*Proteobacteria*	7
87	4	4	1,477	KP663406	*Cellvibrio japonicus* strain (NR_074804.1)	91		
100	1	1	1,469	KP663408	*Dokdonella immobilis* strain (NR_108377.1)	98		
77	3	3	1,454	KP663404	*Bdellovibrio bacteriovorus* strain (AF148941.1)	89	δ-*Proteobacteria*	3
24	5	5	1,485	KP663389	*Ferruginibacter* sp. (KF360051.1)	94	*Bacteroidetes*	17
38	1	1	1,467	KP663397	*Bacteroidetes* bacterium (FN658701.1)	87		
95	3	3	1,439	KP663407	*Bacteroidetes* bacterium (JQ683777.2)	88		
102	1	1	1,465	KP663409	*Tuber borchii* symbiont (AF233292.1)	88		
104	4	4	1,479	KP663410	*Parabacteroides chartae* strain (NR_109439.1)	99		
116	3	3	1,438	KP663412	Uncultured *Flexibacter* sp. (FN668187.2)	86		
60	2	2	1,465	KP663403	*Planctomyces* sp. (X81954.1)	87	*Planctomycetes*	2
25	8	8	1,458	KP663390	*Prosthecobacter vanneervenii* (AJ966883.1)	98	*Verrucomicrobia*	8
6	1	1	1,441	KP663383	Uncultured bacterium (HE646324.1)	98	*uncultured bacterium*	43
11	14	14	1,481	KP663384	Uncultured bacterium (JX564510.1)	99		
14	3	3	1,477	KP663385	Uncultured *Bacteroidetes* bacterium (CU925607.1)	99		
28	12	12	1,442	KP663392	Uncultured bacterium (KC551654.1)	86		
35	2	2	1,471	KP663394	Uncultured bacterium (KC471253.1)	90		
37	6	6	1,485	KP663396	Uncultured bacterium (EU104322.1)	99		
52	1	1	1,414	KP663401	Bacterium K-4b6 (AF524858.1)	94		
86	2	2	1,429	KP663405	Uncultured bacterium (JN609348.1)	99		
115	2	2	1,494	KP663411	Uncultured bacterium (AM490689.1)	89		

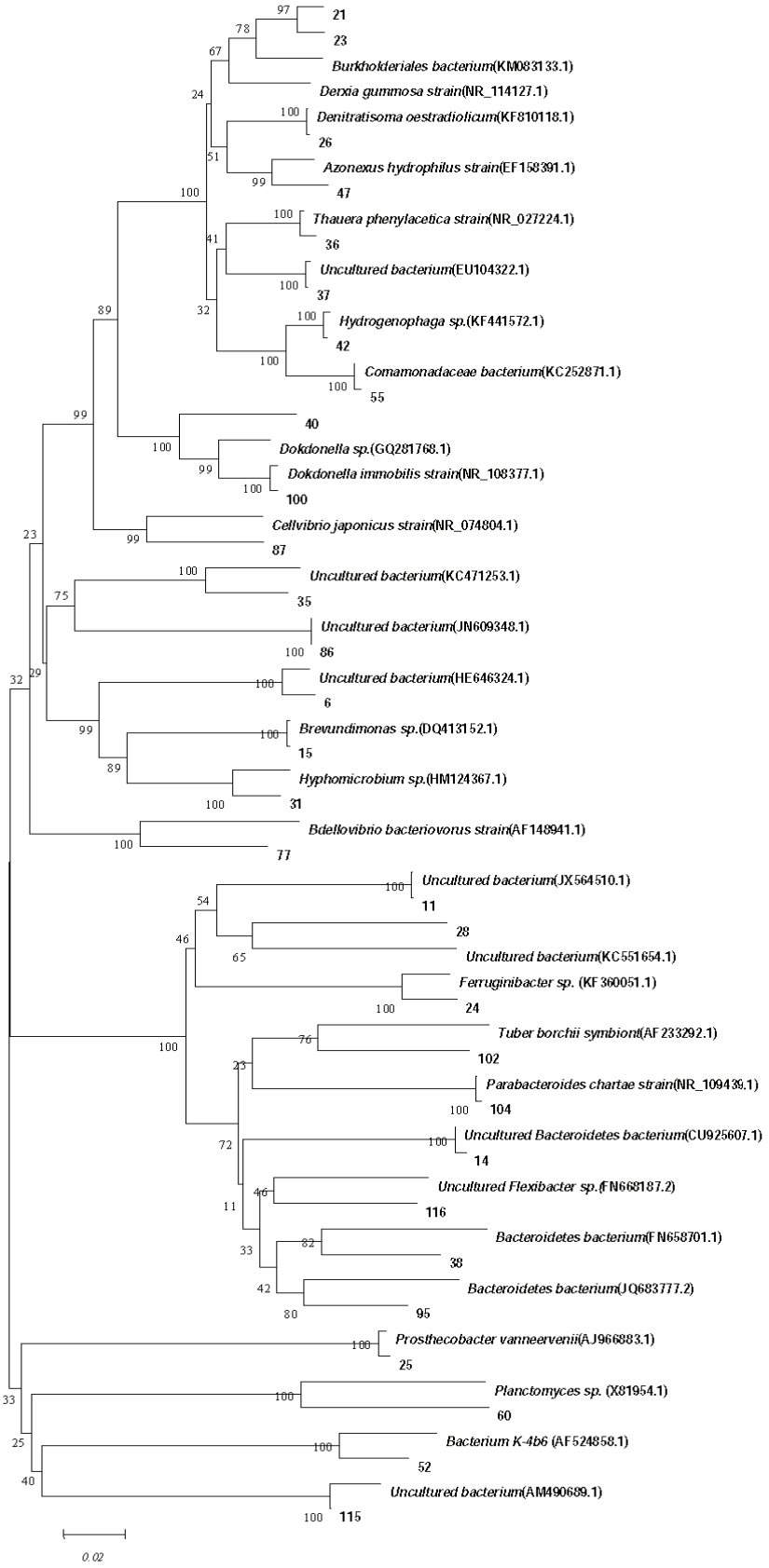

Fig. S2. Phylogenetic tree of total bacteria for R2 (with organic carbon).

Table S3
16SrDNA cloning results of AOB for R1 (without organic carbon)

OTU	Clone numbers of OTU	Proportion of OTU (%)	The most similar bacteria (NCBI)	Similarity (%)	Phylum
1	76	96.20	*Nitrosomonas* sp. (JN367456)	99	β-*Proteobacteria*
2	1	1.27	*Nitrosomonas* sp. (JN367453)	99	
3	1	1.27	*Nitrosomonas* sp. (AF272407)	99	
4	1	1.27	*Nitrosomonas* sp. (DQ228469)	89	

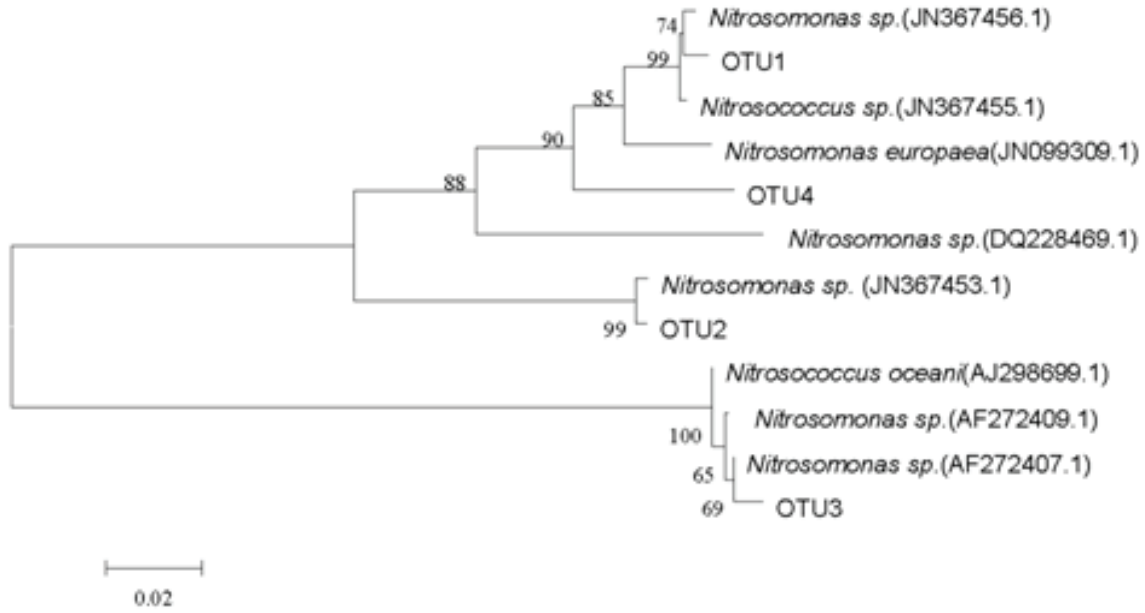

Fig. S3. Phylogenetic tree of AOB for R1 (without organic carbon).

Table S4
16SrDNA cloning results of AOB for R2 (with organic carbon)

OTU	Clone numbers of OTU	Proportion of OTU (%)	The most similar bacteria (NCBI)	Similarity (%)	Phylum
1	31	48.438	*Nitrosomonas* sp. (JN367456.1)	99	*β-Proteobacteria*
2	18	28.125	*Nitrosomonas* sp. (JN367453.1)	99	
3	12	18.75	*Nitrosomonas europaea* (JN099309.1)	97	
4	2	3.125	*Nitrosomonas* sp. (AY958703.1)	96	
5	1	1.563	*Nitrosomonas* sp. (AB079055.1)	93	

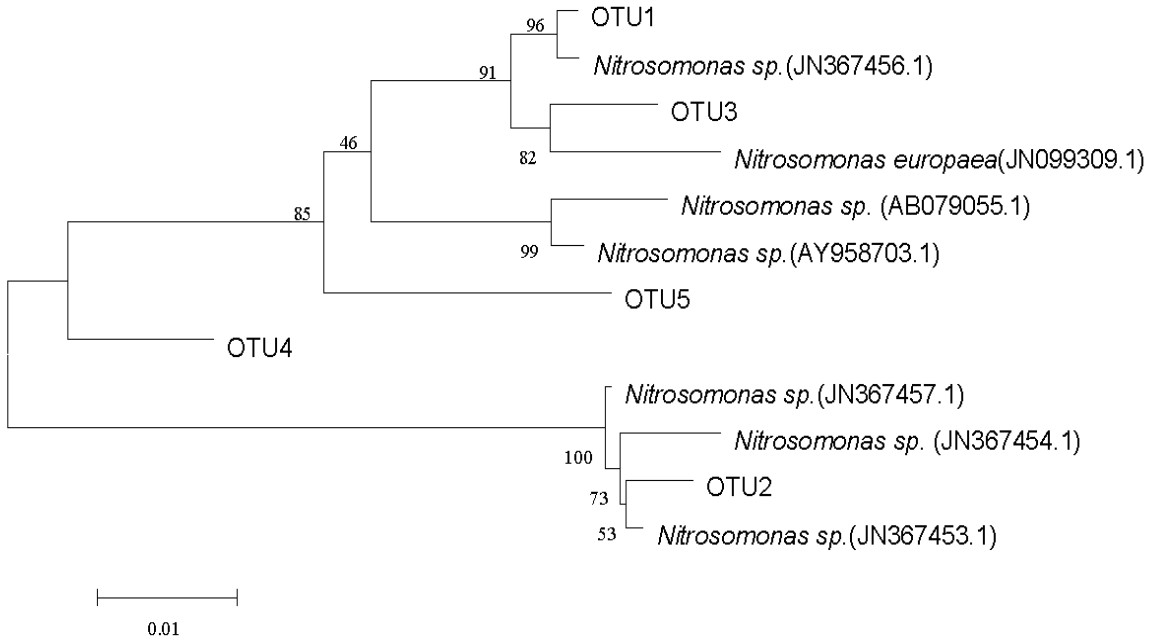

Fig. S4. Phylogenetic tree of AOB for R2 (with organic carbon).