African Journal of Biotechnology Vol. 10(29), pp. 5635-5647, 22 June, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2009
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Nitrification and nitrifying bacteria in the Chengdu section of middle Min River (China)

Jie Zhang[1,2,3], Da-Ping Li[1]*, Ping Gao[2], Yong Tao[1], Xiao-Mei Wang[1] and Xiao-Hong He[1]

[1]Chengdu Institute of Biology, Chinese Academy of Sciences, No.9, Section 4, Renmin Nan Road, 610041, China.
[2]School of Life Science, Sichuan University, No.24, South Section 1, Yihuan Road, Chengdu, 610065, China.
[3]School of Life Science, Shanxi Normal University, No.1, Gongyuan street, Linfen, Shanxi 041004, China.

Accepted 2 May, 2011

The Min River is one of the most important tributaries of the upper Yangtze River. Its middle reach, below Chengdu city, receives large numbers of effluents from Sanwayao wastewater treatment plant (WWTP), where ammonia concentration raises significantly. In this study, the situation of ammonia pollution, nitrification and nitrifying bacteria in the Min River was studied during the year 2007 to 2008. Nitrifying activity was assessed under optimum conditions based on the background ammonia concentration and abundance of nitrifying bacteria was measured by the real time quantitative-polymerase chain reaction (RTQ-PCR). The community composition of ammonia-oxidizing bacteria (AOB) was investigated with polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S ribosomal DNA fragments. The results showed that the ammonia contamination of the Chengdu section of the middle Min River was severe. Nitrification mainly takes place in the 30 km reach of the lower river, where the numbers of nitrifying bacteria are relatively more than those in the upper river do. The sequence analysis of the DGGE bands reveals that the majority of AOB presenting in the river belong to lineage 2, represented by *Nitrosomonas oligotropha*-and *Nitrosomonas ureae*-like bacteria. The rest of the AOB population is represented in minor proportions by *Nitrosospira*-like species.

Key words: Nitrifying activity, nitrifying bacterium, Min River, real time quantitative-polymerase chain reaction (RTQ-PCR), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

INTRODUCTION

City waters contaminated by ammonia from large wastewater discharges is a widespread phenomenon in densely populated watersheds (Brion and Billen, 2000; Féray and Montuelle, 2003; Drolc et al., 2007). The ammonia (NH_3), the un-ionized form of ammonium, has a toxic effect on the aquatic organisms in the receiving water (Arthur et al., 1987; Torres et al., 2009). Large inputs of nitrogen (NH_4^+-N) also promote algal growth and lead to eutrophication in seas and lakes (Ryther and Dunstan, 1971). Nitrification, a two-step process, is the main way by which ammonium can be eliminated from polluted river water (Chestérikoff et al., 1992; Xia et al., 2009). Ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), the two groups of highly specialized organisms, carry out these steps respectively. The physiologically defined group of autotrophic AOB carries out the first step, which is the oxidization of ammonia into nitrite in freshwater environments. These bacteria are located within the β-class of *proteobacteria* and encompass the members of the genera *Nitrosomonas* (such as *Nitrosococcus mobilis*), genus *Nitrosospira* (such as *Nitrosovibrio*) and *Nitrosolobus* (Purkhold et al., 2000; Purkhold et al., 2003; Spieck et al., 2005). The microscopic images reveal that those bacteria are dominant nitrifying bacteria in the biofilm treating the inorganic ammonium wastewater and occupy above 60%of the total bacteria number. AOB of the γ-*Proteobacteria* exist only in halophilic and marine environments (Koops et al., 1990). The second step, the oxidization of nitrite into nitrate, is carried out by lithotrophic NOB located within the four phylogenetic distinct groups. The genus *Nitrobacter* belongs to the α-class of the *Proteobacteria* (Stackebrandt, 1988), whereas *Nitrococcus* is affiliated with the

*Corresponding author. E-mail: lidp_cib@yahoo.com.cn. Tel: +86 28 85235149. Fax: +86 28 85222753.

γ-Proteobacteria. The two known species (*Nitrospira moscoviensis* and *Nitrospira marine*) of the genus *Nitrospira* are located in a distinct phylum (Ehrich et al., 1995). *Nitrococcus mobilis* and *Nitrospina gracilis*, the two marine species, are members of the γ- and δ-classes of the *Proteobacteria* (Teske et al., 1994), respectively.

A few methods has been used for the investigation of nitrifying bacteria in the field. The most-probable-number technique and selective plating are both time-consuming methods, because of the prolonged generation time of nitrifier. In the last ten years, based on the 16S rRNA sequence information for *in situ* analyses, several molecular ecology techniques for ecology have been developed. Primers in the polymerase chain reactions (PCRs) for amplification based on 16S rRNA gene have been used for detecting AOB in environmental samples (Speksnijder et al., 1998; Nakamura et al., 2006). A modified PCR technique named real-time PCR (Heid et al., 1996) can determine the initial template concentration and deduce the cell numbers more accurately from the AOB in the environment (Hermansson and Lindgren, 2001). Moreover, the denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments analysis has been another good tool for analyzing the biodiversity of complex nitrifying bacterial populations (Kowalchuk et al., 1997; Stephen et al., 1998).

The middle reach of the Min River flowing through the urbanized area of Chengdu receives large numbers of effluents discharged mainly from WWTP. Moreover, the effluents containing high level of ammonia has polluted the river severely in the last several years. The plans for the pollution control of the middle Min River require further information about the pollution status of nitrogen and microbial transformation characteristics. This study focuses on assessing ammonia nitrogen, nitrifying activities, abundance and community composition of AOB in the Chengdu section of middle Min River from 2007 to 2008 year.

MATERIALS AND METHODS

Research area

Located in Sichuan Province, southwest China, the Min River is a 750 km long river, with drainage area of about 46 000 km^2. Its upper stream is 341 km long, with drainage area of 2.3×10^3 km^2 above the Dujiang Dam. It is an important part of the ecological barriers for the upper reaches of the Yangtze River and a main lifeline of water resource for the Chengdu plain, supporting the sustainable development of the Chengdu Plain and the upper reaches of the Yangtze River. The middle reach of Min River is 235 km long, located between the Dujiang Dam and Leshan, where it flows through the Chengdu city.

In this study, the Chengdu section of the middle Min River from Jiuyan Qiao to Pengshan was studied. Fourteen transects were designated (Figure 1). Jiuyan Qiao is at km 0, and the other stations are designated by their distances from Jiuyan Qiao. The Chengdu section of middle Min River receives pollution inputs from a drainage basin characterized by high population density. At present, all the domestic effluent of the 5.57 million inhabitants, ranging from 900 000 to 1 000 000 m^3 day^{-1} in the center district except a fraction of wastewater discharged directly into the river, is treated by five WWTPs. The Sanwayao WWTP was the most important, treating daily about 800 000 m^3 of sewage with a classical activated sludge process. The discharge of treated effluents results in a significant increase of the ammonium concentration downstream the outlet of WWTP in the river. It is far above the threshold value of 1 mg l^{-1} considered as a major pollution by the Water Bureau of Chengdu city (CDBWR) according to the National Environmental Quality Standards for Surface Water in the People's Republic of China (National Environmental Protection Agency, 2002). Therefore, the lower river is enriched in nutrients, especially ammonium, which is known to be incompletely converted into nitrate even in the last station through preliminary investigations.

Samples collection

Water samples were collected at 14 stations along the middle Min River, from Jiuyan Qiao to Pengshan, in April, July, October 2007 and January 2008. The three longitudinal patterns contained samples collected in the upstream and downstream of the WWTP outlet (at the stations of Maliuwan and Sanwayao, at km 5 and 8, respectively) (Figure 1). At each station, the temperature and DO of water were measured *in situ* with one Model 52 oxygen probe (Yellow Spring Instruments, Ohio, USA). An eight liters, water sample was collected at a depth of 0.4 m in the middle of the river with a bucket for chemical, bioactivity and molecular analyses and was brought to the laboratory within 3 to 4 h.

Chemical analysis

Concentrations of NH_4^+-N, NO_2^--N and NO_3^--N in water samples were examined. Aliquots of water samples were filtered through 0.45 μm filters before the analysis. The Nesslerization colorimetric method was applied to the NH_4^+-N analysis, NO_2^--N was determined by colorimetric method through formation of a reddish purple azo dye produced at pH 2.0 to 2.5 by coupling diazotized sulfanilamide with *N*-(1-naphthyl)-ethylenediamine. NO_3^--N was determined by the phenol disulfonic acid ultraviolet spectrophotometric method. Their detection limits were 0.02, 0.003 and 0.08 mg l^{-1}, respectively (National Environmental Protection Agency, 2004).

Measurement of nitrifying activities

80 ml water samples were added to a series of 150 ml sterilized flasks and were then cultivated in the air-bath shaker with 150 rpm for a week at 30°C to ensure oxygen saturation and to promote the growth of indigenous nitrifying bacteria. Aliquots were withdrawn and examined for determining concentrations of ammonia in cultivated water systems every 24 h. The nitrifying activity was evaluated based on the level of ammonia decrease.

DNA extraction

All water samples were filtered separately through the 0.22 um-pore-size micro-cellulose membranes. Then, all the dregs were eluted with sterile water and centrifuged at 10 000 *g* for 20 min at 4°C. According to the manufacturer's instructions, the genomic DNA of water samples was extracted from the collected precipitation with the soil DNA Kit (Bioteke Corporation, Beijing, China). DNA concentration was determined spectrophotometrically at 260 nm with UV transillumination (Gene Company, Hong Kong, China).

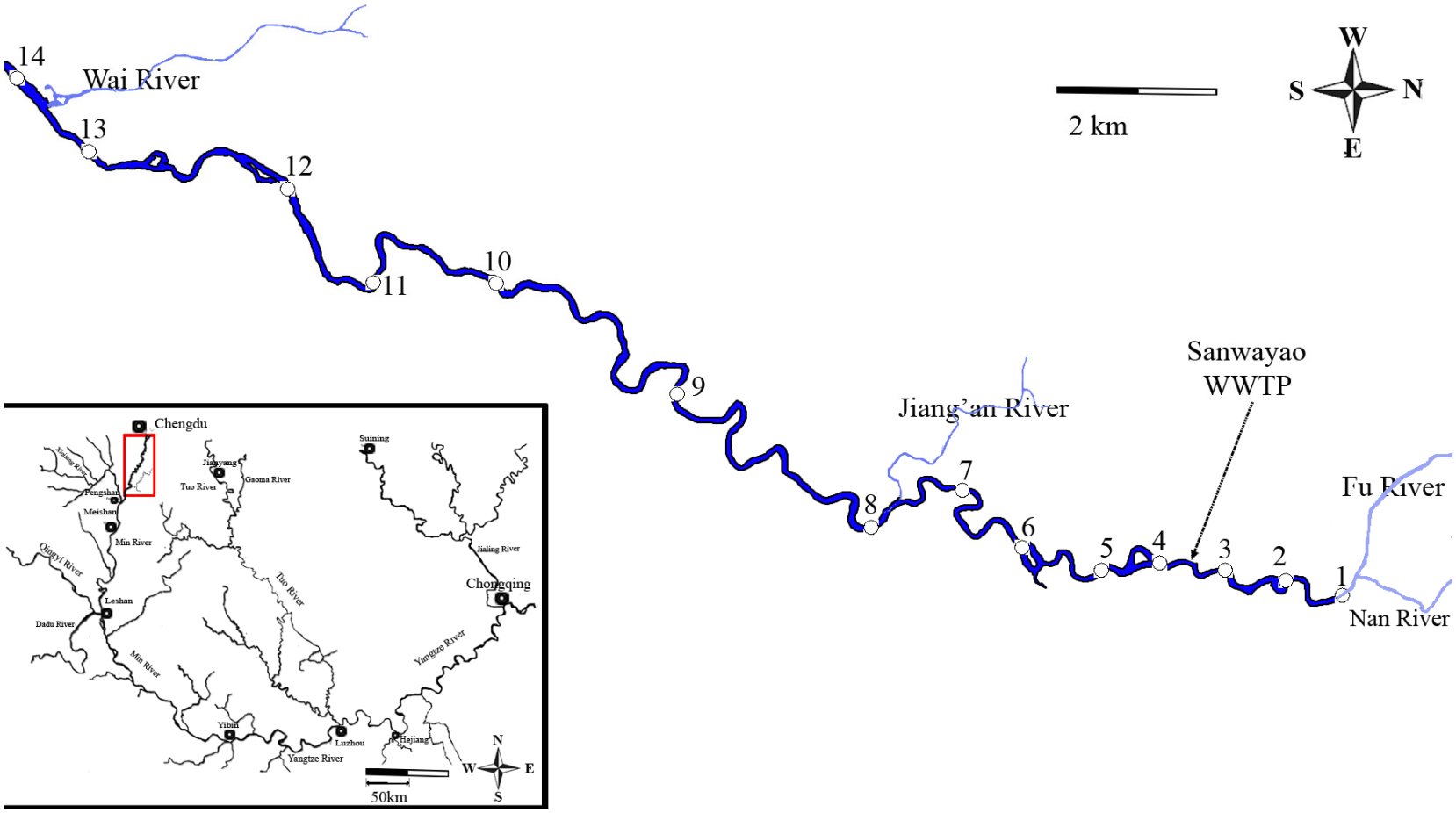

Figure 1. Description of the sampling sites: the Chengdu section of middle Min River from Jiuyan Qiao to Pengshan. Numbers 1 to 14 represent the sampling stations, as follows: 1. Jiuyan Qiao; 2. Donghu (km 2); 3. Maliuwan (km 5); 4. Sanwayao (km 8); 5. Fubin (km 12); 6. Zhonghe (km 18); 7. Huayang (km 23); 8. Jiang′an (km 30); 9. Maojiawan (km 40); 10. Huangfo (km 50); 11. Huanglongxi (km 55); 12. Xiajiadu (km 60); 13. Jiangkou (km 70); 14. Pengshan (km 75).

RTQ-PCR for AOB

Abundance of AOB in the river water were investigated by RTQ-PCR with two forward primers CTO189fA/B (5'-GGAGRAAAGCAGGGGATCG-3'[R is A or C]) and CTO189fC (5'-GGAGGAAAGTAGGGGATCG-3') at a 2:1 ratio, one reverse primer RT1r (5'-CGTCCTCTCAG ACCARCTACTG-3') as described by Hermansson and Lindgren (2001). The reaction mixture contained 12.5 µl of SYBR® Premix Ex Taq (TaKaRa, Dalian, China) including Taq DNA polymerase, dNTP, $MgCl_2$ and SYBR Green I dye, 1 µl of a 10 mmol l^{-1} solution of sense/anti-sense primer, 2 µl of template DNA(0.2 µmol l^{-1}) and distilled H_2O added to a total of 25 µl. Forty cycles of PCR were performed at 95°C for 5 s, 60°C for 20 s, 72°C for 10 s on a DNA Engine Peltier Thermal Cycler (Bio-RAD, USA). At the end of each cycle, the fluorescent signal was measured with a Chromo 4™ RTQ-PCR Detector (Bio-RAD). The RTQ-PCR program was followed with a melting curve analysis by raising the temperature 0.5°C from 65 to 95°C.

The use of a standard curve based on known

concentrations of DNA makes it theoretically possible to quantify DNA from any source. In this study, three repeats of real-time amplification of purified PCR products of seven different concentrations, from the DNA template in the middle Min River with the primer CTO189fA/B/C-RT1r, were used to construct the standard curves. DNA samples generated slopes of -3.12, -3.10 and -3.18ΔC_t fg of DNA $^{-1}$, respectively. The R^2 values were greater than 0.99 for all of the curves. Quantification of AOB DNA extracted from the freshwater in this study was based on a mean slope value (-3.13 ± 0.04 ΔC_t fg of DNA^{-1}) derived from the standard curves. The PCR mix with a total volume of 25 µl contained 0.2 µmol l^{-1} primers CTO189f and RT1r, 1.1×10^4 to 1.1×10^{10} copies of the standard ranging from 0.03 fg to 33.9 pg.

PCR amplification for DGGE

All PCRs for AOB DGGE were conducted with an equimolar mixture of two forward primers each with a GC clamp to increase the PCR sensitivity, according to the work of Kowalchuk et al. (1997). The forward primers CTO189fA/B-GC (5'-CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGRAAAGCAGGGGATCG-3'[underlining indicates GC clamp; R is A or C]) and CTO189fC-GC (5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGGAAAGTAGGGGATCG-3') were synthesized separately and collectively referred to as CTO189f-GC. The reverse primer sequence was CTO654r (5'-CTAGCYTTGTAGTTTCAAACGC-3') (Y is C or T). These primers were designed to amplify partial 16 ribosomal DNA sequences (465 bp) from the AOB of β-class *proteobacteria*. The conditions used for the first round of amplifying reaction were as follows: 5 min at 95°C, followed by 35 cycles of 60 s at 95°C, 60 s at 57°C and 90 s at 72°C, with a 5-min final extension at 72°C.

A second round of PCR was performed on the PCR products obtained from CTO189f-GC primers with a second primer pair allowing the amplification of an internal fragment of 196 bp (Muyzer et al., 1993). These primers 357f-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3') were used to target a stretch in the variable V3 region of the 16S ribosomal DNA under the following PCR conditions: 2 min at 94°C, followed by 30 cycles of 45 s at 94°C, 35 s at 65°C and 35 s at 72°C, with a 5 min final extension at 72°C. PCR products were examined by standard agarose gel electrophoresis (1.2% agarose, 1×Tris-acetic-EDTA) with ethidium bromide staining to confirm the product size and estimate the DNA concentration.

PCR products were loaded for DGGE analysis according to the protocol described by Muyzer et al. (1993) and modified by Kowalchuk et al. (1998). Polyacrylamide gels (8% polyacrylamide, 1.5 mm thick, 16 by 16 cm) were run in a 1×TAE buffer (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA [pH 8.3]). The denaturant gradient ranged from 30 to 60% denaturant (100% denaturant was 7 mol l^{-1} urea and 40% formamide in the 1×TAE buffer). Gels were run at 60°C on a DCode^{TM} universal mutation detection system (Bio-RAD, California, USA) for 16 h at 75 V. The DNA was stained with ethidium bromide and visualized by UV transillumination. Double-band formation due to heteroduplex formation had been checked and confirmed by reamplification of the two bands with CTO primers.

DGGE band excision and phylogenetic analysis

The target bands for sequence analysis were excised from DGGE gel. Each gel fragment was put into a 0.2-ml sterile EP tube, added into 50 µl of sterile ultrapure water and placed at 4°C overnight. Five µl of these DNA samples were used as templates for subsequent PCR, using the same PCR primer sets (CTO189f-654r or 357f-518r) without a GC clamp. The ABI PRISM 3700 DNA sequencer (California, USA) at Shanghai Sangon Bio-Tech Company in China performed bi-directional sequencing.

The sequences were aligned with the published 16S rRNA gene sequences of AOB and related non-AOB sequences from GenBank of NCBI by software Clustal X (version 1.83). Phylogenetic trees were generated by neighbor-joining method with 1 000 resampling bootstrap using the Mega 4.0 package (Kumar et al., 2008). Phylogenetic distance trees were generated with γ-class of *proteobacteria* AOB sequences as an outgroup.

Accession numbers of nucleotide sequences

The sequences determined in this study were deposited in the GenBank database under accession number: GQ227644 to GQ227669.

RESULTS

Nitrifying activities in the Chengdu section of the middle Min River

The samples collected in April, July, October 2007 and January 2008 correspond to mean temperature of 20.8, 21.5, 16.8 and 8.2°C, respectively (Figure 2a). Two significantly oxygen-depleted sectors was observed along the sampling sites (Figure 2b). The first DO-depletion occurred downstream from the outlet of Sanwayao WWTP and probably related with the decomposition of organic matter by heterotrophic bacteria. The second depletion of DO appeared in the lower Chengdu sections (Maojiawan-Jiangkou) and were linked to the progressive increase of nitrification activity (Figure 2c and 3a).

As nitrite is rarely found to accumulate in most systems, ammonia oxidization is thought to be the rate-limiting step for nitrification (Rotthauwe, 1995). Hence, it is very important to study the conditions of ammonia oxidization for the control of river nitrogen pollution in the middle Min River. The longitudinal profiles for ammonium and nitrate concentrations resulting from the large input of treated wastewater show similar patterns for each sampling date (Figure 2c). The effluents from Sanwayao sewage treatment plant increase spectacularly ammonium concentration in four different seasons in a year. As for nitrate, the increasing trend of 2 to 2.5 mg N L^{-1} in the first and fourth quarters is obvious, whereas the nitrate increase in another two quarters is not obvious, just 1 mg N L^{-1}. The concentrations of ammonium and nitrate then remain constant down to Huayang, about 15 km from Sanwayao. Since the branch flow from the Jiang'an River is significant relative to the upstream, a dilution was observed. In addition, the slight increase of nitrate and decrease of ammonium concentration in the lower stretch of the river, between Maojiawan (station 9, km 40) and Jiangkou (station 13, km 70) is observed systematically in a year. The net nitrate production represented approximately 0.7 to 0.9 mg l^{-1} of N-NO_3^- produced, which is less than the

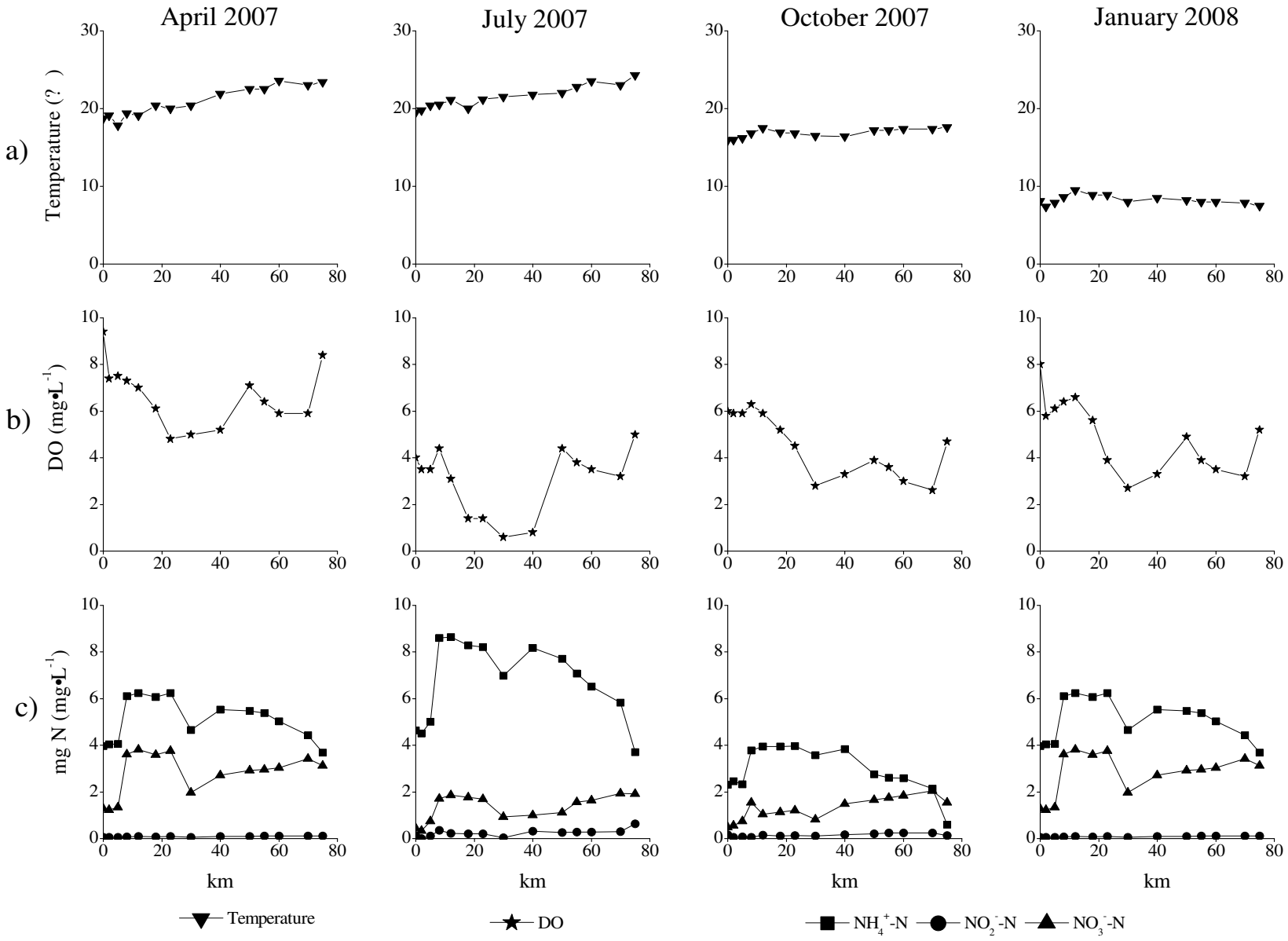

Figure 2. Variations in temperature (a), DO (b), NH_4^+-N, NO_2^--N and NO_3^--N (c) along a longitudinal profile of the Chengdu section in middle Min River during April 2007, July 2007, October 2007 and January 2008.

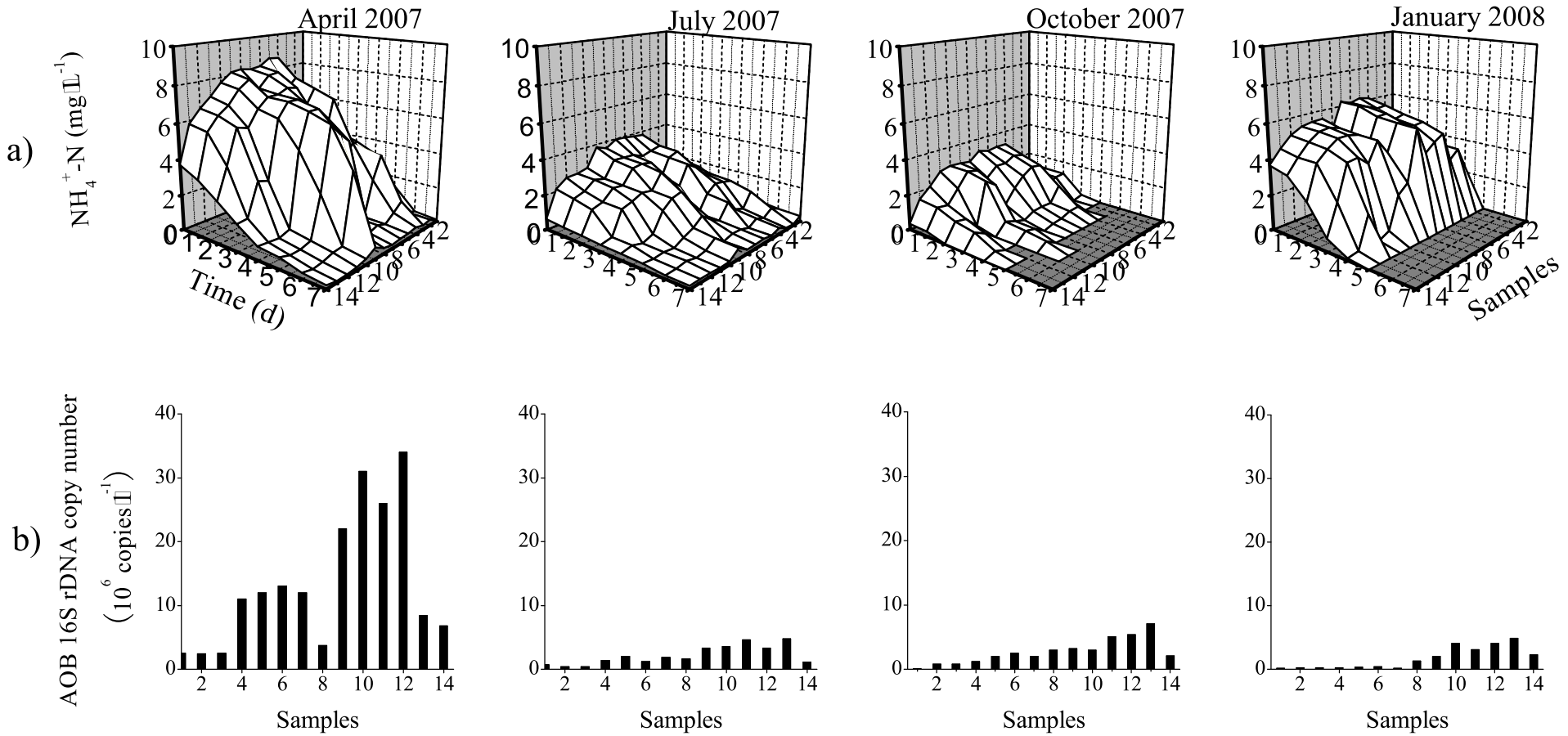

Figure 3. (a) Laboratory simulation of nitrifying activities of raw water column in April 2007, July 2007, October 2007 and January 2008. X-axis: Culture time of one week; Y-axis: raw water samples in 14 sites; Z-axis: Ammonium background concentration; (b) AOB numbers in raw water in April 2007, July 2007, October 2007 and January 2008.

total ammonium consumed. This was probably due to (1) dilution by the tributary of the Jiang'an River and (2) denitrification at the water-sediment interface (Chestérikoff et al., 1992). At the last station (Pengshan, km 75), the nitrate and ammonium concentrations decreased rapidly, mainly due to dilution by the branch of the Wai River. The ammonium mainly supplied by the treated wastewater was not completely consumed in the role of nitrification until the last station, also illustrating that ammonia pollution has far exceeded its own purification capacity of river. Nitrifying activities assessed in the laboratory help us to learn the state of nitrification in the middle Min River itself. The patterns of nitrifying activity in the raw water for four different sampling seasons along the longitudinal profiles were similar to each other (Figure 3a). These patterns, however, differed from others because of the differences in the terms of ammonia background concentration and number of nitrifying bacteria among the four

sampling dates. Based on the decreased trend and rate of ammonium concentration under ideal laboratory conditions, the nitrifying activity of raw water samples in the lower river, especially the river reach between Maojiawan and Jiangkou, is higher than that in the upper river. In January 2008, background ammonium existing in all water samples was fully consumed after 4 to 5 days of culture time, whereas ammonium of all water samples in October 2007 could be completely oxidized under the role of indigenous nitrifying bacteria of raw water in 3 to 4 days. The NH_4^+-N pollution in middle Min River in April 2007 was the most serious because of the dry season. Although, the number of nitrifying bacteria was slightly higher than that in the other three quarters (Figure 3b), the higher ammonium concentration was not down to zero after a week culture. In the wet season of July 2007, ammonium was also not fully consumed after a week culture because of the small number of nitrifying bacteria (Figure 3b). Moreover, this earlier mentioned nitrifying activity through the year is exactly required for explaining the observed decrease of ammonium and increase of nitrate in the reach Maojiawan-Jiangkou (Figure 2c), showing that nitrification in the water explains the transformations of inorganic nitrogen in the river.

Abundance of AOB species in raw water samples

The AOB-specific primer set was used to target a stretch of the 16S rDNA. Application of this primer set generated a 116 bp DNA fragment for all water samples collected along the longitudinal profile. Estimate of AOB cell numbers was based on the following assumption that all AOB have only one *rrn* operon per genome, as shown for all the strains studied so far (Aakra et al., 1999). Quantification of AOB 16S rRNA gene copies in the middle Min River led to a pattern fairly similar to the ones for nitrifying activity (Figure 3). Upstream WWTP and down to Jiang'an, the AOB abundances were low except one in April 2007. An increase of the AOB cell numbers was observed in the lower river for the four sampling dates (Figure 3b). The AOB number of the raw water in April 2007 was significantly above that in other three quarters because of the dry season and higher water temperature of 20.8°C, increasing ~14-fold (from 2.5×10^6 to 3.4×10^7 16S rDNA copies L^{-1}) between Maliuwan (km 5, upstream of the Sanwayao effluent output) and Xiajiadu (km 60) -Jiangkou (km 70). In January 2008, the AOB 16S rDNA copies number increased ~25-fold (from 0.24×10^6 to 4.9×10^6 16S rDNA copies L^{-1}). The AOB 16S rDNA copies number in June 2007 was the lowest because of the wet season. The AOB 16S rDNA copies number increased ~9-fold (from 0.8×10^6 to $7.1\times10^6$16S rDNA copies L^{-1}) in October 2007. Invariably, the difference was not very significant in the term of magnitude order which focuses on the 10^6 to 10^7 in the four different seasons of a year. The nitrifying activity increased slightly in the lower Min River, where population number of AOB was at the maximum. Progressive nitrification activities and ammonia consumption between Maojiawan and Jiangkou in the lower Min River were associated. The delay in the development of ammonia oxidizer following the increase of ammonium concentration at the station of WWTP is consistent with the observation that no increase in nitrate nor decrease in ammonium are observed in the first 20 km below the outfall of the Sanwayao WWTP.

Characterization of the AOB communities in the middle Min River

Since DGGE analysis revealed that the community composition of AOB in the water among the four sampling seasons was quite similar, results are shown only for the January 2008 samples (Figure 4). We identified seven different bands (a1 to a7 (Figure 4a)). It seems that bands a1 and a2, which were very weak in the upper Min River and relative intense in the lower Min River, were present in all the sites. Bands a3 and a4 were also observed at all sampling stations, but presented different intensity compared with bands a1 and a2 in the different samples. Band a6 only appeared in the station 6. Bands a5 and a7 were not well determined in the stations 9 to 14 in the downstream of Min River. The DGGE profiles produced from the second PCR products showed that banding patterns obtained from the four sampling seasons were rather similar. Results with the dominant bands (from A to H) are shown only for the January 2008 samples, showing the same overall distribution from Jiuyang Qiao to Pengshan (Figure 4b). These results suggest that the diversity of the AOB varied little over time. Band E (double bands due to the heteroduplex formation) was present in all the sampling stations. Bands A, B and C were also omnipresent. Bands G and D were only appeared in stations 5 and 6, respectively. Band F was determined between station 3 and 6. Band H was limited in the stations 4 and 5.

According to the methods described earlier (see DGGE band excision), one to three repeats of each band in the same position were sequenced. Sequence analyses of 406 bp 16S rDNA fragments for CTO PCR products and 160 bp 16S rDNA fragments for nested-PCR products allowed us to build two phylogenetic trees (Figures 5 and 6). The result of sequences analysis based on the two different DGGE supports each other as follows: band a1 corresponds to band A, a2 to B, a3 to C, a4 to E, a5 to G, a6 to D, a7 to H.

The sequence analysis shows that the majority of bands are not affiliated with known AOB from the β-*Proteobacteria*. Bands a3 and a4 are closely related to *Methylophilus* sp. enrichment culture clone MWE C44 (97% homology), a5 and a7 show high homology with *Dechloromonas* sp. A34 (98%), respectively. Similarly, bands C, E, G and H are not affiliated with AOB from the

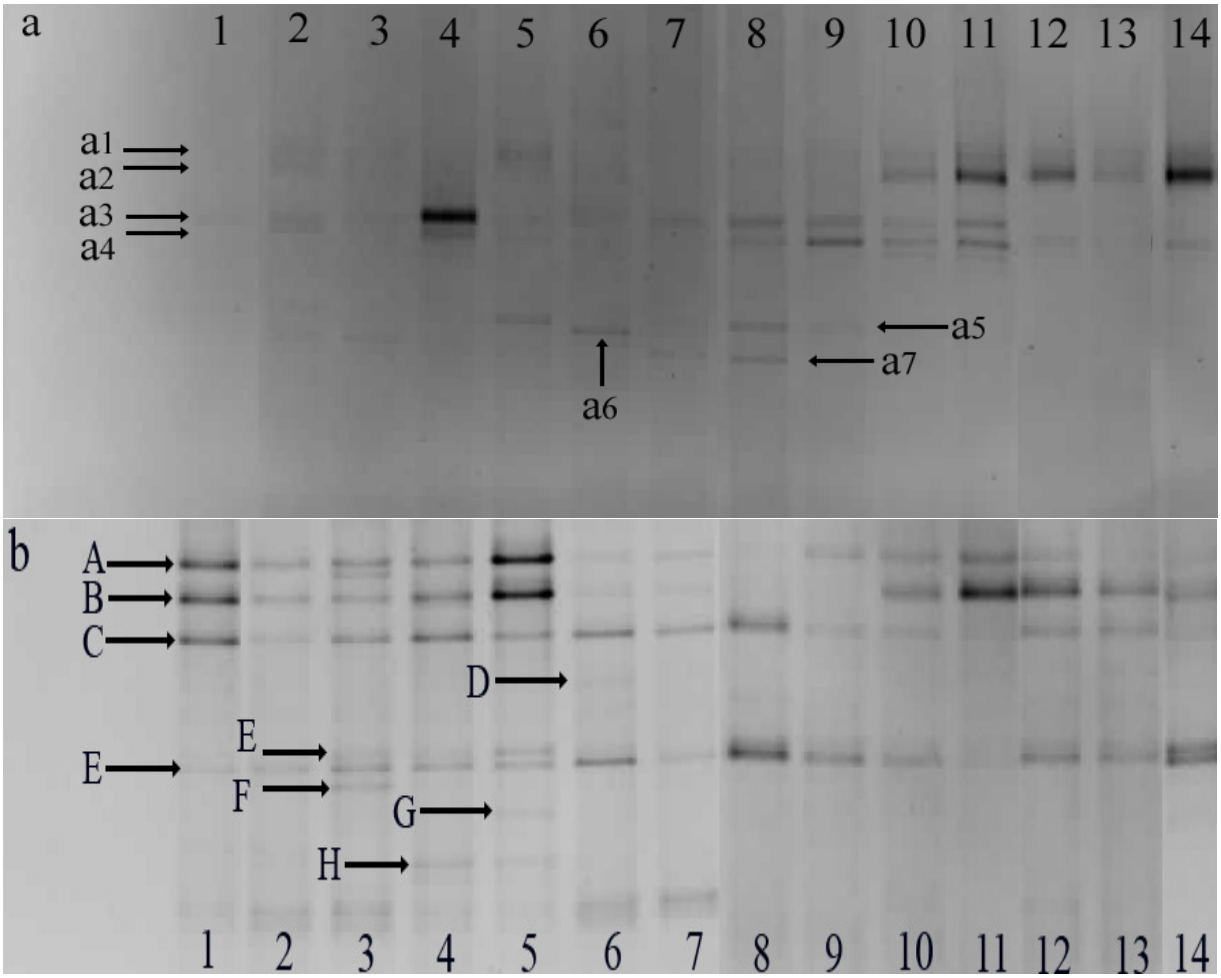

Figure 4. (a) DGGE profiles of AOB of the β-*Proteobacteria*, obtained by CTO PCR on January 2008. Lanes 1 to 14 correspond to 14 different sampling stations. The principal bands are labeled a1 to a7 (bands that were cut and sequenced); (b) DGGE profiles of AOB of the β-*Proteobacteria*, obtained by nested PCR on January 2008. Lanes 1 to 14 correspond to the 14 different sampling stations. The principal bands observed are labeled with capital letters (A to H).

β-class of the *Proteobacteria*. All these bands will be considered artifacts, because none of the CTO primers used presently to target all the beta-class AOB showed the 100% specificity (Purkhold et al., 2000). The other bands are affiliated with ammonia-oxidizing lineages belonging to the β-class of the *Proteobacteria*. Band a1 and a2 (band A and B) are affiliated with lineage 2, represented by *N. oligotropha*- and *N. ureae*-like bacteria (Spieck et al., 2005). Band a6 belonged to the *Nitrosospira*-like bacteria. Band F does not correspond to any CTO DGGE band. We recovered one band a1 (CJ8-a1-10) and three different bands A (NJ8-A-1, NJ8-A-5, and NJ8-A-11) from three different stations that showed 100% homology in the 160 bp 16S rDNA sequences. Sequences of two bands a2 (CJ8-a2-10 and CJ8-a2-11) and three different bands B (NJ8-B-1, NJ8-B-5 and NJ8-B-11) in different stations show 100% homology in the 160-bp 16S rDNA sequences. Band a6 and D (CJ8-a6-6 and NJ8-D-6) are also completely homologous in the 160 bp sequence. Although, bands a1 and a2 show 97% sequence similarity with *Nitrosomonas* sp. Nm84, there is a three-base difference between them.

DISCUSSION

In the upstream sampling stations 1, 2 and 3, ammonium concentration in a year was above the limit of 1 mg l^{-1} (about 2 mg l^{-1} in July and October 2007 and 4 to 4.7 mg l^{-1} in January 2008 and April 2007, respectively). This presumably results from a small part of untreated effluent in the upper river discharged into the receiving water. Nitrate concentration was approximately 1 mg l^{-1} in a year. Immediately in downstream from the Sanwayao WWTP, there is a significant increase in ammonium level from 1.5 to 6.9 to 8.7 mg of $N\text{-}NH_4^+$ L^{-1} and nitrate level increase from 0.7 to 1.5 to 3.6 mg l^{-1} due to the effluent input. After dilution by the Jiang'an River at km 30, the ammonium

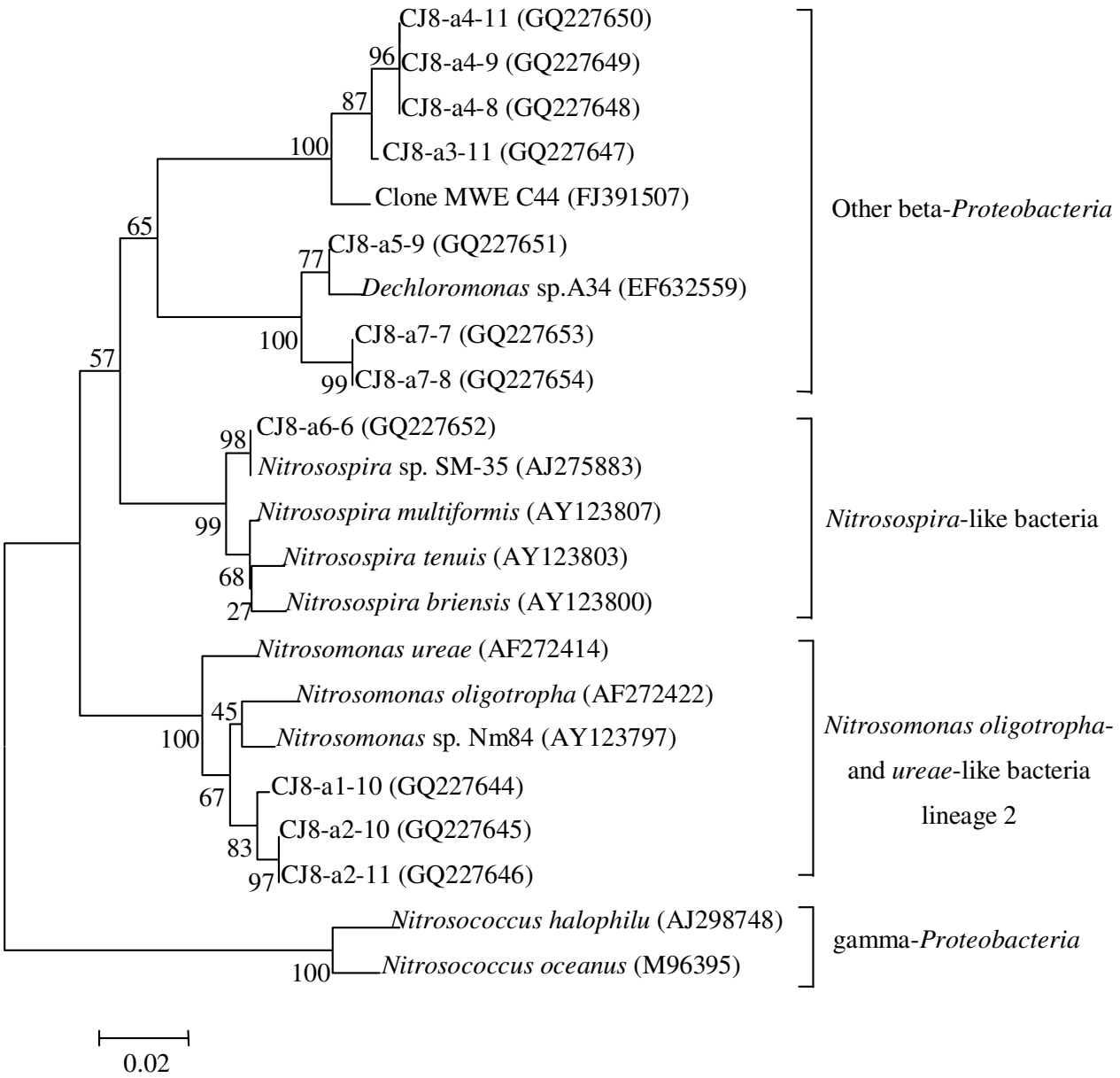

Figure 5. Phylogenetic neighbor-joining tree based on a comparison of 406 bp of 16S rRNA gene sequences from AOB of the β-class of the *Proteobacteria* and some other β-*proteobacteria*. The tree is rooted with two sequences of AOB belonging to the γ-class of the *Proteobacteria*. Their designations in our DGGE sequences are composed of C, for CTO PCR products; J8 for a sampling date of January 2008; a1 to a7, for the band as labeled in Figure 4a; and the number of the sampling station from which the sequence originated.

concentration slowly decreases down to the lower river, presumably consumed by nitrifying bacteria. As indicated by the laboratory simulation of nitrifying activities in raw water samples, the transformation of ammonium into nitrate mainly focuses on stations between Maojiawan and Jiangkou in the lower river (Figure 3), where the population numbers of nitrifying bacteria are higher than that in the upstream river.

Two significant dissolved oxygen depletions were monitored in the Chengdu section of the middle Min River. It is probably a constant feature of the ecological functioning of the system strongly impacted by domestic effluents (Figure 2b). The oxygen budget exhibited for the whole part investigated here showed that oxygen was consumed in the degradation course of organic matter by heterotrophic bacteria below the outlet of WWTP; whereas nitrification was a major influencing factor of oxygen depletion in the downstream river.

The treated domestic effluents not only contain large numbers of organic matter, but also are sources of

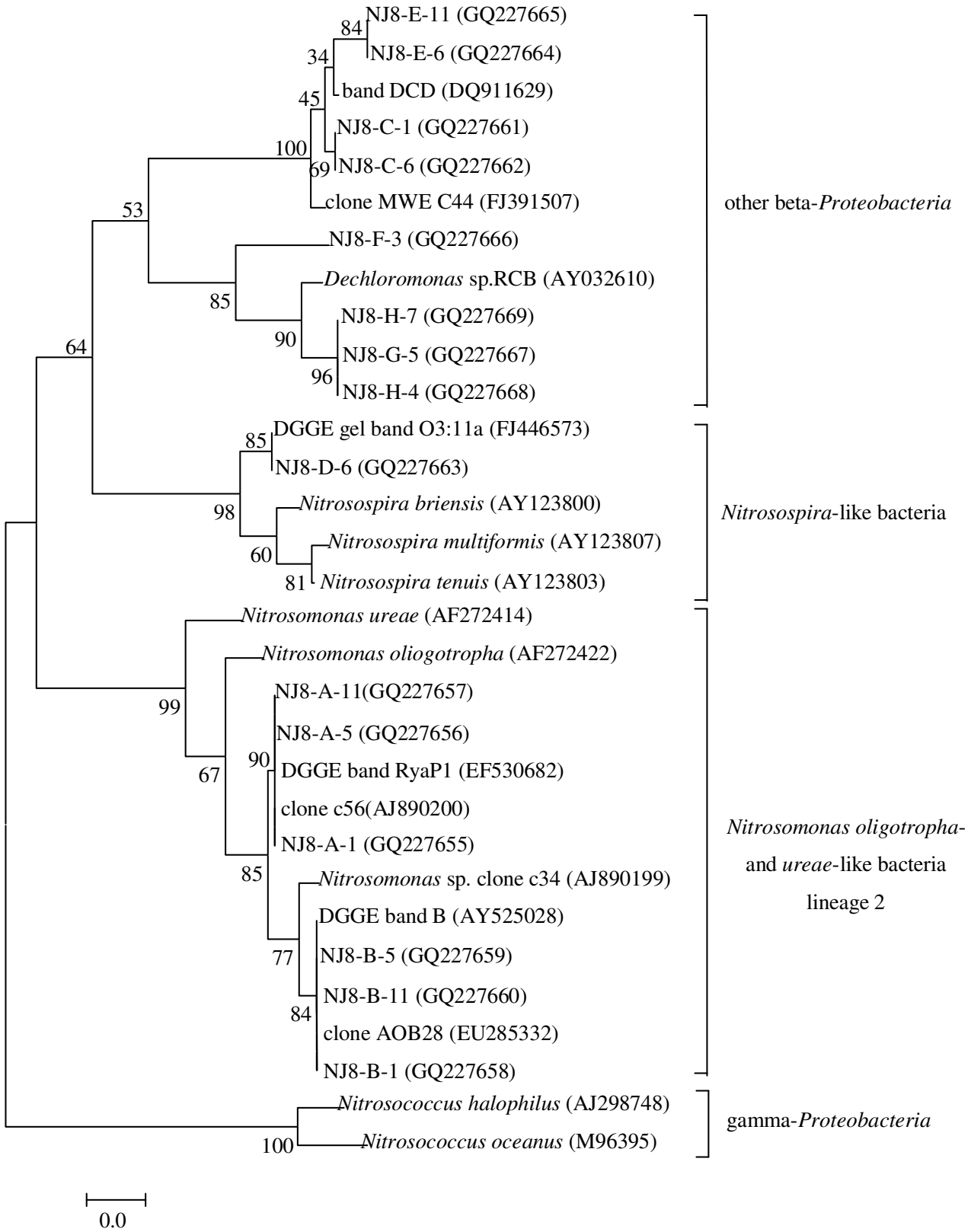

Figure 6. Phylogenetic neighbor-joining tree based on a comparison of 160 bp of 16S rRNA gene sequences from AOB of the β-class of the *Proteobacteria* and some other β-*proteobacteria*. The tree is rooted with two sequences of AOB belonging to the γ-class of the *Proteobacteria*. Their designations in our DGGE sequences are composed of N, for Nested PCR products; J8 for a sampling date of January 2008; A to H, for the band as labeled in Figure 4b; and the number of the sampling station from which the sequence originated.

microorganisms, such as heterotrophic bacteria, lithoautotrophic nitrifying bacteria, etc. (Brion and Billen, 2000). They may play a basic role in the ecological functioning of the system (Goni-Urriza et al., 2000). Heterotrophic bacteria deplete the oxygen immediately downstream from the WWTP effluent input, whereas the nitrifying bacteria deplete O_2 just in the lower river. Upstream WWTP and down to Jiang′an, the phenomenon that the abundance of AOB was low could be explained by the fact that the condition of DO in the water column was not conducive to the growth of AOB, expect the April 2007 because of the low water flow and high temperature.

Therefore, only low numbers of nitrifying bacteria are present in the upper river. In the lower river, where conditions seemed to be different from the one below Sanwayao WWTP, it could allow the AOB population to build up an increasing biomass. With the increase of AOB abundance, the simulated nitrifying activity in water bodies increased slowly. Hence, although, ammonium is present below the outfall of Sanwayao at concentrations far above the requirement for optimal growth of nitrifying bacteria, they take several days to reach a level of population at which the nitrifying bacteria are able to carry out a significant activity of ammonium oxidation in the lower river.

It is very important to investigate the microbial diversity in order to know the relationship between ecological functioning of microbes and corresponding niche (Torsvik and Ovreas, 2002). The PCR-DGGE in this study was used to analyze the diversity of the AOB in the middle Min River. The CTO primer set (Kowalchuk et al., 1997; Cébron et al., 2004) was shown not to be completely specific to AOB of the β-*proteobacteria.* It also can match the *Methylophilus* sp. and *Dechloromonas* sp. of beta-class of *proteobacteria* beside the AOB. Cébron et al. (2004) also described the similar results. So far, none of the *in situ* detection primers for all beta-class AOB in the environment showed both 100% sensitivity (targeting all beta-class AOB) and 100% specificity (excluding all non beta-class AOB) (Purkhold et al., 2000). One of the most important uncertain factors is that the degree of 16S rRNA gene divergence is very low within some AOB groups comprising closely related species (Spieck et al., 2005). In addition, the fact that it is not always possible to separate DNA fragments, which have a certain amount of sequence variation with the DGGE technique, has been demonstrated by Vallaeys et al. (1997). The adjacent bands of the same lane, such as CJ8-a1-10 and CJ8-a2-10 in this study, represent the bacteria belong to the same genus because of the presence of a so-called *wobble base* (either a C or a T) in the reverse primer (Kowalchuk et al., 1997). Hence, the number of bands generated by DGGE may not accurately reflect the number of AOB present in the middle Min River. To confirm the identity of these bands, one to three bands that located in the same position on the DGGE patterns were sequenced for the four sampling dates. Results showed that all the bands at the same position had the same nucleotide composition. So, the method used herein can be reasonably considered to lead to the same bacterium for any sample and sampling date. Although, some PCR-amplified products based on the CTO primers in this study was not the target AOB, they reflected indirectly that the microbiological community homologous to AOB in different orders of beta class of *proteobacteria* exists in water column and the heterogeneity between the samples at the same time.

The described species in the genus *Nitrosomonas*, together with other cultured but undescribed species of this genus and *"Nitrosomonas mobilis"* (*"N. mobilis"*), represent six distinct lines of descent (Spieck et al., 2005). As far as is known, these lineages reflect distinctly different ecophysiological groupings (Koops and Pommerening-Röser, 2001), that is, lineage 2 includes the salt-sensitive and oligotrophic species *"N. ureae"* and *"N. oligotropha"*. Most isolates of these species originate from oligotrophic freshwater environments, such as rivers or lakes. The sequences (bands a1 and a2) obtained by PCR amplification from water samples, illustrated by DGGE patterns, suggest that the *Nitrosomonas oligotropha*-like AOB appeared in all the sampling stations of Min River. The major difference between the investigated situations was spatial shifts in the abundance of AOB species. Cébron et al. (2004) also have demonstrated that the diversity of AOB community in Seine River varied little over time. In this study, the same AOB species composition at four different time points was observed along the river transect. This result shows a temporal stability in the genetic diversity of the functional bacterial population, which indicates that long-term and similar hydrological (residence time), meteorological (temperature) and nutritional (NH_4^+ concentrations) conditions have led to the same selection and adaptation of AOB species in the middle Min River (Cébron et al., 2004). The *Nitrosospira*-like sequences are typical AOB populations presenting in soil fertilized with amended water (Bhuiya and Walker, 1977; Oved et al., 2001), which supports our results, that is, the presence of *Nitrosospira* (band a6) only in the station 6, where agricultural activities distributed around the river frequently interfered with the river. The *Nitrosospira* (band a6) is probably introduced by soil leaching. In addition, *Nitrosospira* is generally regarded as the most ubiquitous genus in forest soils (Hastings et al., 2000) and it dominates in grasslands and agricultural ecosystems (Stephen et al., 1998; Bruns et al., 1999; Kowalchuk et al., 2000).

Conclusions

This study presents preliminarily ammonia contamination, nitrification and nitrifying bacteria in the Chengdu section of Min River; major conclusions are: (1) the ammonium, mainly supplied by the treated wastewater of Sanwayao

WWTP, deteriorate seriously the water quality in the lower river. This kind of pollutant cannot be completely eliminated from the water body in the role of nitrification. Also, illustrating that ammonium contamination has far exceeded routine level recovered by self-purification of river microbes; (2) the nitrification mainly focuses on the 30 km river way between Maojiawan and Jiang′kou in the lower river, where the abundance of AOB are relatively higher than those in the upper river because of the gradual recovery of dissolved oxygen; (3) the hydrological, meteorological and nutritional conditions lead to the similar AOB community composition, whereas the difference of AOB species abundance in spatial scale is probably resulted from the conditions of rivers itself.

ACKNOWLEDGEMENTS

The National High Technology Research and Development Program (863 Programs, No. 2007AA06-2324) of China funded the research. We are grateful to Jie Hu, Qi Zhong, Ji-Jun Hu, Yuan-Fen Chen, Wei-Qiang Guo, Jin-Lian Zhang, Ning Fu, Guo-Qiang Zhan, Lan Wang, Jian-Min Deng, Zhi-Guo Jia, Hua-Dong Jiang, Liang Zhang, Juan Dong and Nuan Yang from the Center of Applied And Environmental Microbiology for their kind help in the course of samples collection in the field and analysis in the laboratory.

REFERENCES

Aakra Å, Utåker JB, Nes IF (1999). RFLP of rRNA genes and sequencing of the 16S-23S rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. Int. J. Syst. Bacteriol. 49: 123-130.

Arthur JW, West CW, Allen KN, Hedtke SF (1987). Seasonal toxicity of ammonia to 5 fish and 9 invertebrate species. B. Environ. Contam. Tox. 38: 324-331.

Bhuiya ZH, Walker N (1977). Autotrophic nitrifying bacteria in acid tea soils from Bangladesh and Sri Lanka. J. Appl. Microbiol. 42(2): 253-257.

Brion N, Billen G (2000). Wastewater as a source of nitrifying bacteria in river systems: the case of the river Seine downstream from Paris. Water Res. 34: 3213-3221.

Bruns MA, Stephen JR, Kowalchuk GA, Prosser JI, Paul EA (1999). Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Appl. Environ. Microbiol. 65: 2994-3000.

Cébron A, Coci M, Garnier J, Laanbroek HJ (2004). Denaturing gradient gel electrophoretic analysis of ammonia-oxidizing bacterial community structure in the lower Seine River: impact of Paris wastewater effluents. Appl. Environ. Microbiol. 70: 6726-6737.

Chestérikoff A, Garban B, Billen G, Poulin M (1992). Inorganic nitrogen dynamics in the river Seine downstream from Paris (France). Biogeochemistry, 17: 147-164.

Drolc A, Koncan JZ, Tisler T (2007). Evaluation of point and diffuse sources of nutrients in a river basin on base of monitoring data. Environ. Moni. Assess. 129: 461-470.

Ehrich S, Behrens D, Lebedeva E, Ludwig W, Bock E (1995). A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, Nitrospira moscoviensis sp. nov. and its phylogenetic relationship. Arch. Microbiol. 164: 16-23.

Féray C, Montuelle B (2003). Chemical and microbial hypotheses explaining the effect of wastewater treatment plant discharges on the nitrifying communities in freshwater sediment. Chemosphere, 50: 919-928.

Goni-Urriza M, Capdepuy M, Arpin C, Raymond N, Caumette P, Quentin C (2000). Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and Aeromonas spp. Appl. Environ. Microb. 66: 125-132.

Hastings RC, Butler C, Singleton I, Saunders JR, McCarthy AJ (2000). Analysis of ammonia-oxidizing bacteria populations in acid forest soil during conditions of moisture limitation. Lett. Appl. Microbiol. 30: 14-18.

Heid CA, Stevens J, Livak KJ, Williams PM (1996). Real time quantitative PCR. Genome Res. 6: 986-994.

Hermansson A, Lindgren PE (2001). Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. Appl. Environ. Microbiol. 67: 972-976.

Koops HP, Böttcher B, Möller UC, Pommerening-Röser A, Stehr G (1990). Description of a new species of Nitrosococcus. Arch. Microbiol. 154: 244-248.

Koops HP, Pommerening-Röser A (2001). Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol. 37: 1-9.

Kowalchuk GA, Bodelier PLE, Heilig GHJ, Stephen JR, Laanbroek HJ (1998). Community analysis of ammonia-oxidizing bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridization. FEMS Microbiol. Ecol. 27: 339-350.

Kowalchuk GA, Stephen JR, Boer WD, Prosser JI, Embley TM, Woldendorp JW (1997). Analysis of ammonia-oxidizing bacteria of the β subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. 63: 1489-1497.

Kowalchuk GA, Stienstra AW, Heilig GHJ, Stephen JR, Woldendorp JW (2000). Molecular analysis of ammonia-oxidizing bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). FEMS Microbiol. Ecol. 31: 207-215.

Kumar S, Nei M, Dudley J, Tamura K (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinform. 9: 299-306.

Muyzer G, De Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59: 695-700.

Nakamura Y, Satoh H, Kindaichi T, Okabe S (2006). Community structure, abundance, and in situ activity of nitrifying bacteria in river sediments as determined by the combined use of molecular techniques and microelectrodes. Environ. Sci. Technol. 40:1532-1539.

National Environmental Protection Agency (2002). Environmental Quality Standards for Surface Water (GB-3838-2002). Department of the scientific and technological standard, Beijing, China.

National Environmental Protection Agency (2004). Analyzing Method for Water and Waste Water. China Environmental Science Press, Beijing, China.

Oved T, Shaviv A, Goldrath T, Mandelbaum RT, Minz D (2001). Influence of effluent irrigation on community composition and function of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol. 67: 3426-3433.

Purkhold U, Pommerening-Röser A, Juretschko S, Schmid MC, Koops HP, Wagner M (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. 66: 5368-5382.

Purkhold U, Wagner M, Timmermann G, Pommerening-Röser A, Koops HP (2003). 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. Int. J. Syst. Evol. Microb. 53: 1485-1494.

Rotthauwe JH, Boer WD, Liesack W (1995). Comparative analysis of gene sequences encoding ammonia monooxygenase of *Nitrosospira* sp. AHB1 and *Nitrosolobus multiformis* C-71. FEMS Microbiol. Lett. 133: 131-35.

Ryther JH, Dunstan WM (1971). Nitrogen, phosphorus, and eutrophication in the coastal marine environment. Science, 171: 1008-1013.

Speksnijder AG, Kowalchuk GA, Roest K, Laanbroek HJ (1998). Recovery of a Nitrosomonas-like 16S rDNA sequence group from freshwater habitats. Syst. Appl. Microbiol. 21: 321-330.

Spieck E, Bock E (2005). Nitrifying bacteria. *In* Bergey's Manual of Systematic Bacteriology. Edited by DJ Brenner, NR Krieg and JT Staley. Springer press, New York. pp. 137-154.

Stackebrandt E (1988). Phylogenetic relationships vs. phenotypic diversity: how to achieve a phylogenetic classification system of the eubacteria. Can. J. Microbiol. 34: 552-556.

Stephen JR, Kowalchuk GA, Bruns MAV, McCaig AE, Phillips CJ, Embley TM, Prosser JI (1998). Analysis of β-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. Appl. Environ. Microbiol. 64: 2958-2965.

Teske A, Elm E, Regan JM, Toze S, Rittmann BE, Stahl DA (1994). Evolutionary relationships among ammonia and nitrite-oxidizing bacteria. J. Bacteriol. 176: 6623-6630.

Torres AI, Gil MN, Amín OA, Esteves JL (2009). Environmental characterization of a eutrophicated semi-enclosed system: nutrient budget (Encerrada Bay, Tierra del Fuego Island, Patagonia, Argentina). Water Air Soil Poll. 204(1-4): 259-270.

Torsvik V, Ovreas L (2002). Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol. 5: 240-245.

Vallaeys T, Topp E, Muyzer G, Macheret V, Laguerre G, Soulas G (1997). Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. FEMS Microbiol. Ecol. 24: 279-285.

Xia XH, Yang ZhH, Zhang XQ (2009). Effect of suspended-sediment concentration on nitrification in river water: Importance of suspended sediment-water interface. Environ. Sci. Technol. 43: 3681-3687.