

Exploited Application of Denaturing Gradient Gel Electrophoresis in Analysis of Ammonia Oxidizing Bacterial Community Structure

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Abstract The aim of the present study was to investigate the diversity of the ammonia-oxidizing bacterial population by identifying autochthonous bacteria from allochthonous ammonia-oxidizing bacteria from the WWTP effluents. Measurements of potential nitrifying activity, competitive PCR, and denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal DNA fragments specific to ammonia-oxidizing bacteria (AOB) were used to explore the succession and shifts of the ammonia-oxidizing community. The CTO primers used in this study were shown not to be completely specific to AOB of the β subclass of Proteobacteria. We further demonstrated that when DGGE patterns are interpreted, all the different bands must be sequenced, as one major DGGE band proved to be affiliated with a group of non-AOB in the β subclass of Proteobacteria. The majority of AOB (75 to 90%) present in the Amla Khadi river downstream of the effluent output belong to lineage 6a, represented by *Nitrosomonas oligotropha*- and *Nitrosomonas ureae*-like bacteria. This dominant lineage was represented by three bands on the DGGE gel. The major lineage-6a AOB species, introduced by the WWTP effluents, survived and might have grown in the receiving medium far downstream, in the estuary; it represented about 40% of the whole AOB population. The other two species belonging to lineage 6a seem to be autochthonous bacteria. One of them developed a few kilometers downstream of the WWTP effluent input in an ammonia-enriched environment, and the other appeared in the freshwater part of the estuary and was apparently more adapted to estuarine conditions. The rest of the AOB population was represented in equal proportions by *Nitrosospira*- and *Nitrosococcus mobilis*-like species.

Keywords: DGGE, ammonia, *nitrospira*, *nitrosococcus*

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1. Introduction

Effluents discharged from wastewater treatment plants (WWTPs) into rivers may have detrimental environmental effects, because they are a source of high levels of nutrients, organic matter, and bacteria [10,20,51]. The Amla Khadi is greatly affected by the discharge of the effluents from the WWTP, which treats the wastewater from 6.5 million inhabitant equivalents. These effluents contain large amounts of ammonium (NH_4^+), which is completely oxidized into nitrite (NO_2^-) and subsequently converted to nitrate (NO_3^-) by the nitrification process. Autotrophic nitrification is accomplished in two stages by two distinct groups of bacteria: ammonia oxidizers and nitrite oxidizers. Ammonia oxidation due to chemolithotrophic ammonia-oxidizing bacteria (AOB) is the first and often the rate limiting step of nitrification; it is essential for the removal of nitrogen from the environment [44]. Aerobic autotrophic ammonia-oxidizing bacteria are found within two phylogenetic

groups based on comparative analyses of 16S rRNA sequences [45,62]. One group comprises strains of *Nitrosomonas* and *Nitrosospira* spp. within the class of β -Proteobacteria, and the other contains *Nitrosococcus oceanus* and *Nitrosococcus halophilus* within the class of gamma-Proteobacteria. All β -proteobacterial ammonia oxidizers belong to a phylogenetically coherent group, within which all the organisms have the same basic physiology as far as we know. A continually expanding database of AOB 16S ribosomal DNA (rDNA) gene sequences has produced descriptions of distinct lineages and clusters within the genera *Nitrosomonas* and *Nitrosospira* of the β -Proteobacteria [31,45]. Pure culture representatives have been isolated for all groups, except for *Nitrosospira* cluster 1 and *Nitrosomonas* lineage 5, where only clone sequences are available. A number of studies suggest that there are physiological and ecological differences between the different AOB genera and lineages and that environmental factors such as salinity, pH, and concentrations of ammonia and suspended particulate matter (SPM) select for certain species of AOB [16,30,34,43,60]. These external factors, which include

the impact of WWTP effluents, may therefore influence the range of AOB diversity and consequently the structure and function of the ammonia-oxidizing community. Most research has focused on AOB community composition in WWTP activated sludge [15,17,28,29,48,52]. Only a few studies on AOB diversity in freshwater environments or estuaries are available [7,16,26,27,56,57]. *amoA* gene library analysis showed that a majority of AOB at the two sampling stations were affiliated

with lineage 6a, represented by *Nitrosomonas oligotropha*- and *Nitrosomonas ureae*-like bacteria, while the other AOB lineages are present in smaller proportions. However, the differences in AOB composition between the two stations did not allow any conclusion to be drawn either on the variety of AOB input by the WWTP effluents or on their fate in the receiving medium. AOB, generally found in small numbers in natural environments, have low growth rates and low biomass yields and are therefore difficult to isolate in a pure culture [44]. Molecular techniques allow a more complete understanding of the diversity and distribution of AOB in natural environments than that offered by cultivation-based methods alone [2,28,64]. Therefore, we combined PCR of 16S rRNA (rDNA) gene fragments using AOB-selective primers with denaturing gradient gel electrophoresis (DGGE) to allow comparative analysis of the dominant AOB populations. Moreover, we complement the use of competitive PCR (cPCR) and the determination of the nitrification rates. The aims of this study were (i) to investigate the proportion of the AOB from upstream that is autochthonous and/or the proportion from the WWTP effluents that is allochthonous, (ii) to better understand the successions of these bacteria in the River, highlighting possible shifts in the AOB populations, and (iii) to analyze the temporal and spatial functioning of the system with respect to ammonium oxidation by using observations made on several sampling dates.

2. Sample Collection

Five to 10 liters of water were collected for chemical, biochemical, and molecular analyses and were brought to the laboratory within 2 to 3 h. The water was filtered through glass fiber membrane filters (GF/F; Whatman) and frozen until it could be analyzed for inorganic nitrogen compounds. Ammonium, nitrite, and nitrate levels were determined spectrophotometrically; ammonium and nitrite concentrations were determined according to the procedure of Slawycck and MacIsaac [54], and nitrate levels were determined after Cd reduction to nitrite [46]. Suspended matter was weighed on GF/F filters dried at 450°C. Potential nitrifying activities were determined by two methods directly after the sampling (see below). For molecular analysis, 150 to 250 ml of water (depending on the amount of SPM) was filtered through 0.22- μ m-pore-size nitrocellulose filters (diameter, 45 mm; Durapore) in triplicate, and the filters were frozen (-20°C) until DNA extraction.

2.1. Measurement of Potential Nitrifying Activity and Potential Ammonia Oxidation Rate

Potential nitrifying activities (i.e., the sum of ammonia and nitrite oxidation rates) were measured according to the methods described by Cébron et al. [12]. Potential nitrifying activities were determined by the difference in $H_{14}CO_3^-$ incorporation between samples with and without specific nitrification inhibitors, all of which were incubated under optimal conditions, i.e., 7.5 mg of O_2 d liter⁻¹, 20°C, and 2 mM NH_4Cl [9]. The inhibitors were allylthiourea (10 mg liter⁻¹) and sodium chlorate (10 mM), which inhibit the oxidation of ammonia and nitrite, respectively. Determination of ammonia oxidation rates was based on measurements of the changes in nitrite concentration in samples containing chlorate as an inhibitor that were incubated under optimal conditions.

2.2. DNA Extraction

DNA was extracted from the filters by a bead-beating method with the Fast DNA spin kit for soil (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions. DNA extracts were then stored at -20°C until purification on a Sephadex G-200 column and ethanol precipitation. Nucleic acids were quantified by comparison between 1 μ l of an undiluted sample and a range of known DNA concentrations on an agarose gel stained with ethidium bromide. To obtain suitable PCR amplicons, 10- to 100-fold dilutions of crude DNA were used as templates for subsequent PCRs. DNA was visualized by UV transillumination (Gel Doc 200; Bio-Rad Laboratories; Calif.). Digital images of the gels were obtained with a charge-coupled device camera controlled by Quantity One software (Bio-Rad).

2.3. cPCR for AOB Quantification

Ammonia oxidizers were estimated as *amoA* gene copy numbers by cPCR as described by Stephen et al. [58] and Cébron et al. [12]. PCR amplification of a 491-bp fragment of the *amoA* gene and a 428-bp fragment of a DNA competitor was carried out by using primer set *amoA*-1F (5'-GGG GTT TCT ACT GGT GGT-3')-*amoA*-2R (5'-CCC CTC KGS AAA GCC TTC TTC-3'), which is specific to ammonia oxidizers belonging to the β subclass of Proteobacteria [47]. PCR amplifications were carried out in a total volume of 50 μ l in 0.2-ml tubes by using a DNA thermocycler (GeneAmp 2400 PCR system; Perkin-Elmer Cetus), according to the thermal profiles described in Table 1. Reaction mixtures were prepared in a 1x buffer [75 mM Tris (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20] with 1.5 mM $MgCl_2$, 10 ng of DNA, and 0.5 U of Taq DNA polymerase (RedGoldtar; Eurogentec). Aliquots of amplification products were analyzed by gel electrophoresis on 2% (wt/vol) agarose gels (Eurogentec). DNA band intensities were estimated with imaging and analysis software (Quantity One; Bio-Rad). The cPCR for *amoA* was performed in triplicate for 10 samples to establish the experimental variability of the method and to determine standard deviations for the results.

2.4. PCR amplification for DGGE

All PCRs for DGGE were conducted according to the work of Kowalchuk et al. [32] with an equimolar mixture of three forward primers, each with a GC clamp, to increase the PCR sensitivity (CTO189fA-GC, CTO189fB-

GC, and CTO189fCGC [5'-CCG CCG CGC GGC GGG CGG GGC GGG GGCACG GGG GGA GRA AAG YAG GGG ATC G-3'] [underlining indicates GC clamp; Y is C or T, and R is A or C]) and with the reverse primer CTO654r (5'-CTA GCY TTG TAG TTT CAA ACG C-3'), containing a single ambiguous base. These primers were designed to amplify partial 16S rDNA sequences (465 bp) from beta-subclass AOB. The PCR was run under the conditions described in Table 1. A nested PCR was performed on the PCR products obtained from CTO primers with a second primer pair allowing the amplification of an internal fragment of 196 bp. These universal primers (357f-GC [5'-C GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG CCT ACG GGA GGC AGC AG-3'] and 518r [5'-ATT ACC GCG GCT GCT GG-3']) were used for the PCR under the conditions described by Muyzer et al. [38] (Table 1). PCR products were examined by standard agarose gel electrophoresis (1.2% agarose, 0.5x Tris-borate-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. [38] and modified by Kowalchuk et al. [32]. Polyacrylamide gels (8% polyacrylamide, 1.5 mm thick, 16 by 16 cm) were run in a 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.3]). The denaturant gradient ranged from 30 to 55% denaturant (100% denaturant was 7 M urea and 40% formamide in the 1x TAE buffer). Gels were run at 60°C on a PROTEAN II xi system (Bio-Rad Laboratories) for 16 h at 75 V. The DNA was stained with ethidium bromide and visualized by UV transillumination. DGGE patterns were visually analyzed, and the intensity of the bands was estimated by imaging and software analysis (Quantity One; Bio-Rad). Double-band formation due to heteroduplex formation had been checked and confirmed by reamplification of the two bands with CTO primers.

Table 1. Summary of primer characteristics and PCR conditions

Primers	Target (specificity)	PCR conditions	Refereces
amoA-1F- amoA-2R	amoA gene (AOB Beta-Proteobacteria)	5 min at 94°C, followed by 42 cycles of 1 min at 94°C, 1 min 30 s at 60°C, and 1 min 30 s at 72°C, followed by a 10-min final extension at 72°C	27
CTO189F-GC or CTO189F-CTO654r	16S rRNA gene (AOB Beta-Proteobacteria)	3 min at 94°C, followed by 38 cycles of 30 s at 92°C, 30 s at 57°C, and 45 s at 72°C, followed by a 5-min final extension at 72°C	40
357F-GC or 357F-518R	16S rRNA gene (universal)	5 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min 30 s at 65°C (-1°C each 2 cycles and 5 cycles at 55°C), and 1 min at 72°C, followed by a 5-min final extension at 72°C	33

2.5. DGGE Band Excision and Sequence analyses

For subsequent DNA reamplification, the central sections of selected DGGE bands were excised. Each gel fragment was placed overnight, at 4°C, in a 2-ml screw-cap tube containing 50 μ l of MilliQ H₂O. Tenfold dilution of these DNA band samples were used as templates for subsequent PCR, using the same PCR primer sets. From each PCR, 5 μ l was subjected to DGGE as described above to confirm the recovery of the desired band, thus obtaining a pure sample for the sequencing step. The PCR products submitted to sequencing resulted from amplification with CTO189F-CTO654R or CTO357F-CTO518R without a GC clamp according to the conditions described in Table 1. The name of each sequence consists of C or N (for the result of PCR with CTO primers or nested PCR); J2, S2, or S3 for the sampling date (July 2012, September 2012, or September 2013); b1 to b8 or A to L for the corresponding DGGE band letter; and finally, the name of the sampling station from which the band has been extracted.

2.6. Phylogenetic Analysis

The partial 16S rRNA gene sequences from amples were aligned with published 16S rRNA gene sequences from β -proteobacterial AOB and related non-AOB sequences by use of Clustal X, version 1.8. Phylogenetic algorithms and tree design (DNA-DIST, NEIGHBOR, and SEQBOOT) were operated using the PHYLIP 3.5 package, written by J. Felsenstein [19]. Bootstrap analysis was

based on 1,000 replicates. Phylogenetic distance trees were generated with non-AOB sequences as an outgroup.

3. Results

Variations in inorganic nitrogen, potential nitrifying activities, and amoA gene copy numbers. Immediately downstream from the WWTP effluent input, there was a significant increase in ammonium levels, from less than 0.25 to 4 to 6 mg of N-NH₄ d liter⁻¹ (Figure 1a). Depending on residence times and temperatures, these amounts were totally consumed by nitrification at distances of 240, 190, and 130 km downstream of the effluent input in July 2012, September 2012, and September 2013, respectively. The net nitrate production represented approximately 2.5 mg of N-NO₃⁻ produced d liter⁻¹. The nitrate concentration decreased rapidly, mainly due to dilution by seawater rather than to denitrification activity [49]. The longitudinal profiles resulting from measurements of potential nitrification activities (14^C technique) and of ammonium oxidation rates showed patterns similar to each other for each sampling date (Figure 1b). These patterns, however, differed among the three sampling dates (Figure 1b). In July 2012, potential nitrifying activities averaged 0.1 μ mol of N d liter⁻¹ d h⁻¹ when impacted by WWTP effluents and reached a maximum of 1.8 μ mol of N d liter⁻¹ d h⁻¹ in the upper freshwater estuary, whereas in September 2012, the maximum potential nitrifying activity and ammonium oxidation rate of 0.7 μ mol of N d liter⁻¹ d h⁻¹ occurred at 278 km. In September 2013, the values were quite constant along the whole transect, between 0.1 and 0.3

$\mu\text{mol of N d liter}^{-1} \text{ h}^{-1}$, from the effluent output to the estuary. Invariably, the potential nitrifying activities increased slightly in the saline estuarine sector, where turbidity was maximal. Maximum potential nitrifying activities within the freshwater estuary were associated with a significant increase in suspended particulate matter concentrations, which then increased drastically at the turbidity maximum of the saline estuary (Figure 1c). An increase in the *amoA* gene copy number as determined by cPCR was observed just downstream of the WWTP input for the three sampling dates (Figure 1d). Although the

amoA gene copy number increased at the maximum of the potential activities, no significant correlation could be found. The standard deviation calculated for *amoA* gene copy number results led to variation coefficients of 1.75 to 5.1% at stations with high *amoA* gene copy numbers and 11.1 to 14.5% at stations with low *amoA* gene copy numbers, so that the observed spatial changes are significant. However, further knowledge of the diversity, physiology, and gene regulation of ammonium-oxidizing bacteria is needed in order to interpret these temporal and spatial patterns in detail.

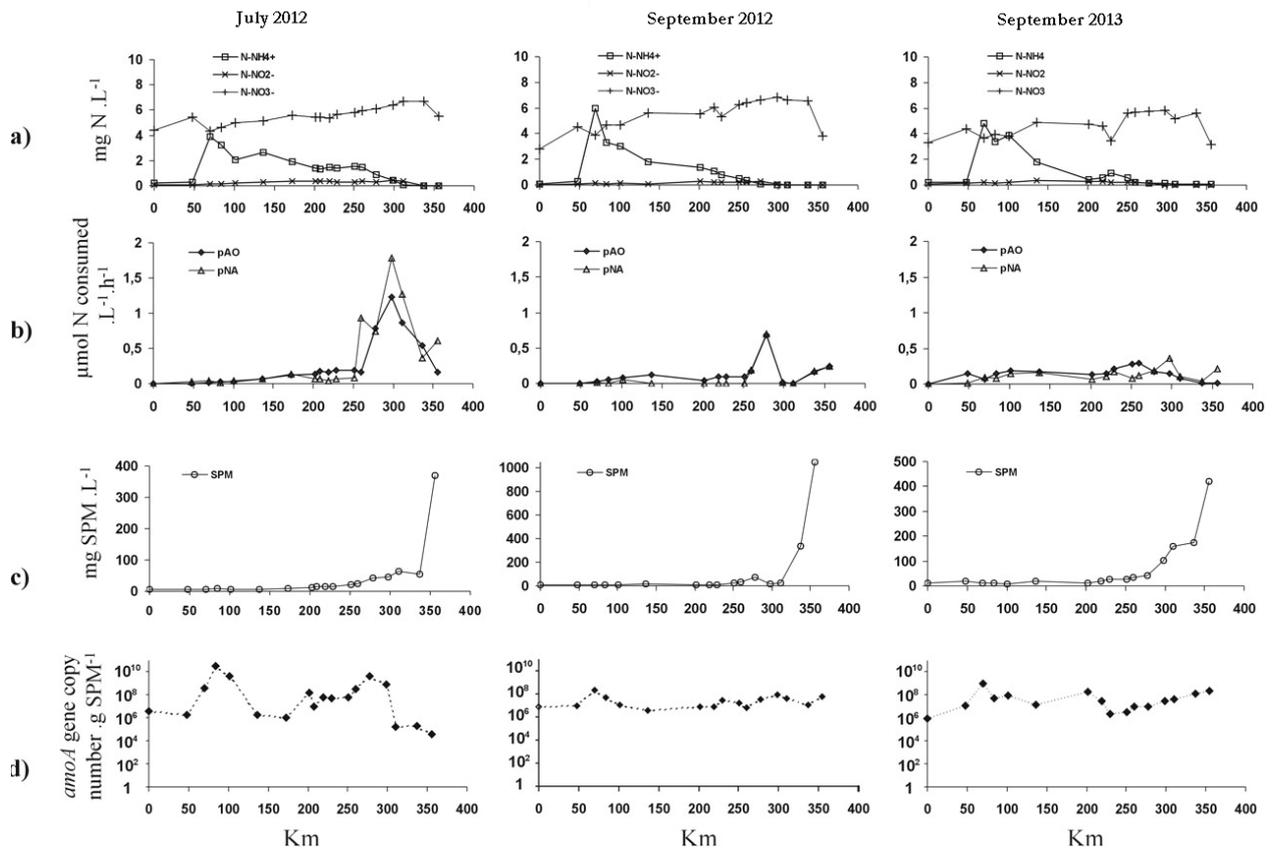


Figure 1. Variations along the river from Amla Khadi (km 0) to the mouth of the estuary (km 356) in (a) ammonium (NH_4^-), nitrite (NO_2^-), and nitrate (NO_3^-) levels, (b) potential ammonia oxidation rates (pAO) and potential nitrification activities (pNA), (c) SPM concentrations, and (d) *amoA* gene copy numbers determined by cPCR for three sampling dates (July 2012, September 2012, September 2013)

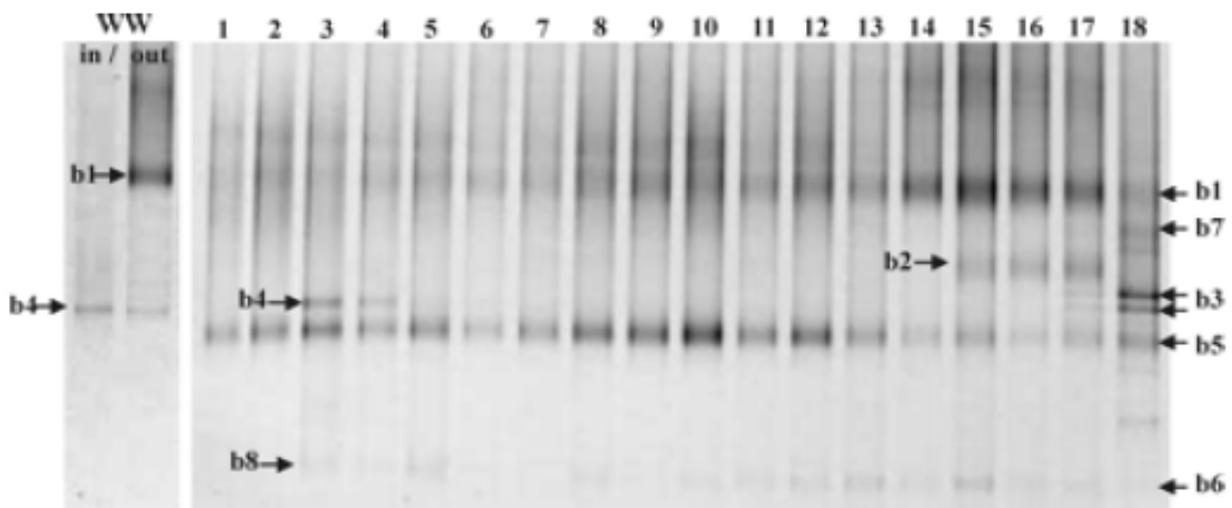


Figure 2. DGGE profiles of ammonia-oxidizing bacteria of the β -subclass of the *Proteobacteria*, obtained by CTO PCR on July 2012 samples. Lanes 1 to 18 correspond to the 18 different sampling stations; lanes "WWin" and "WWout" are samples from domestic wastewater before and after treatment, respectively. The principal bands are labeled b1 to b8 (bands that were cut and sequenced). The denaturing gradient is from 39 to 52%

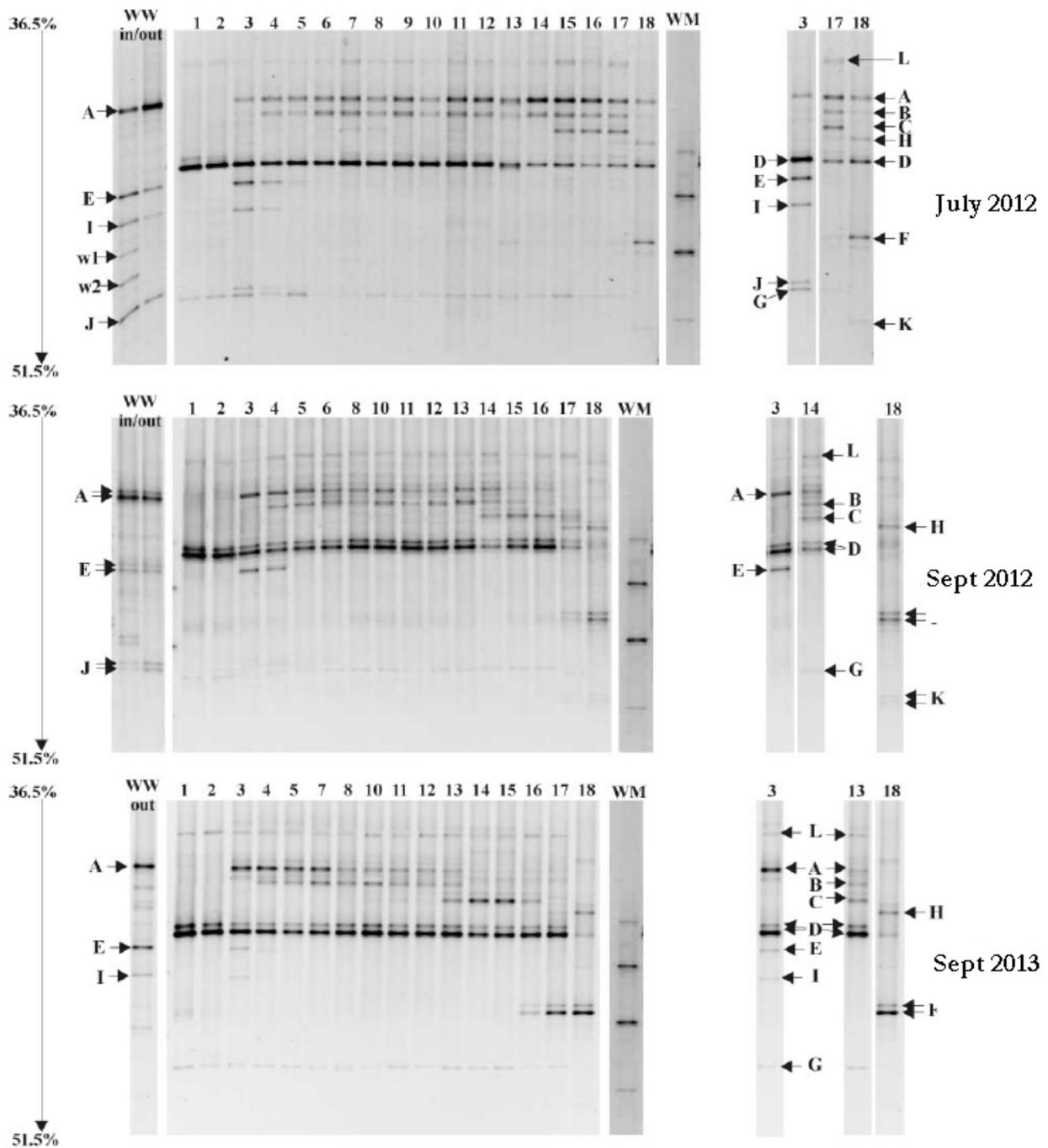


Figure 3. DGGE profiles of ammonia-oxidizing bacteria of the β -subclass of the *Proteobacteria*, obtained by nested PCR on July 2012, September 2012, and September 2013 samples. Lanes 1 to 18 correspond to the 18 different sampling stations; lanes “WW in” and “WW out” represent samples from domestic wastewater treatment plants before and after treatment. Lanes WM, weight markers. The principal bands observed are labeled with capital letters (A to L). All these bands have been cut and sequenced to determine the phylogeny of each ammonia-oxidizing bacterium and to compare the three sampling dates. The denaturing gradient is the same for the three DGGE gels; only 36.5 to 51.5% is shown here

3.1. Analysis of CTO and Nested PCR Products by DGGE

Because analysis of the CTO PCR products by DGGE revealed that the profiles for the three sampling dates were quite similar, results are shown only for the July 2012 samples (Figure 2). We identified eight different bands (b1 to b8 [Figure 2]). It seems that bands b5 and b6 (Figure 2) were present in all the samples; b5 was very intense, and b6 was very weak. Band b1 was not well defined upstream of stations 4 and 5 but was very intense in the treated wastewater sample (WWout) and even more so toward the downstream sector. Band b4, which was

clearly present in both wastewater samples, was also observed at station 3, apparently due to seeding of bacteria from WWTP effluents. Band b8 is probably also provided by WWTP effluents; these bands disappeared again 30 km downstream. In the estuary, a new band, b2, appeared from station 15 to station 17; this bacterial group is probably adapted to particular estuarine conditions. At the last station (station 18), which is strongly influenced by seawater, two additional bands appeared (b3 and b7), leading to a DGGE profile quite different from those of the upstream sites and probably characterizing a new community of halotolerant and halophilic ammonia-oxidizing bacteria. The DGGE profiles produced after nested PCR on CTO PCR products showed that banding

patterns obtained from the three sampling dates were rather similar, with the dominant bands (from A to L) showing the same overall distribution from Paris to the estuary (Figure 3). These results show that the diversity of the bacteria varied little over time. Band D (double band due to heteroduplex formation) was present in all the samples from station 1, to station 18. It dominated everywhere, except in some estuarine samples. Bands G and L were also omnipresent, but at lower intensities. The impact of the WWTP effluents was visible on all three sampling dates. Immediately downstream from the WWTP input at station 3, four new bands (bands A, E, I, and J) appeared, which were also present in the wastewater from the treatment plant. Bands E, I, and J quickly disappeared again in the river, indicating that these bacteria are well adapted to the conditions in the

WWTP but do not survive in the receiving medium. In contrast, band A persisted much longer in the river and was present as far as stations 18, 16, and 13 in July 2012, September 2012, and September 2013, respectively. Band B, which was not present in the effluents but appeared at the station, 15 km downstream from the effluent output, might represent an autochthonous bacterium that thrived on the massive input of ammonia. Finally, band C was present only in the upper freshwater estuary, at stations 13, 14, and 15 in September 2013, September 2012 and July 2012, respectively. This ammonia oxidizing bacterium seems well adapted to low ammonia concentrations and large amounts of suspended particulate matter. At the last marine station in the mouth of the estuary, new bands, H, F, and K, appeared on the three sampling dates, confirming the presence of halotolerant or halophilic AOB.

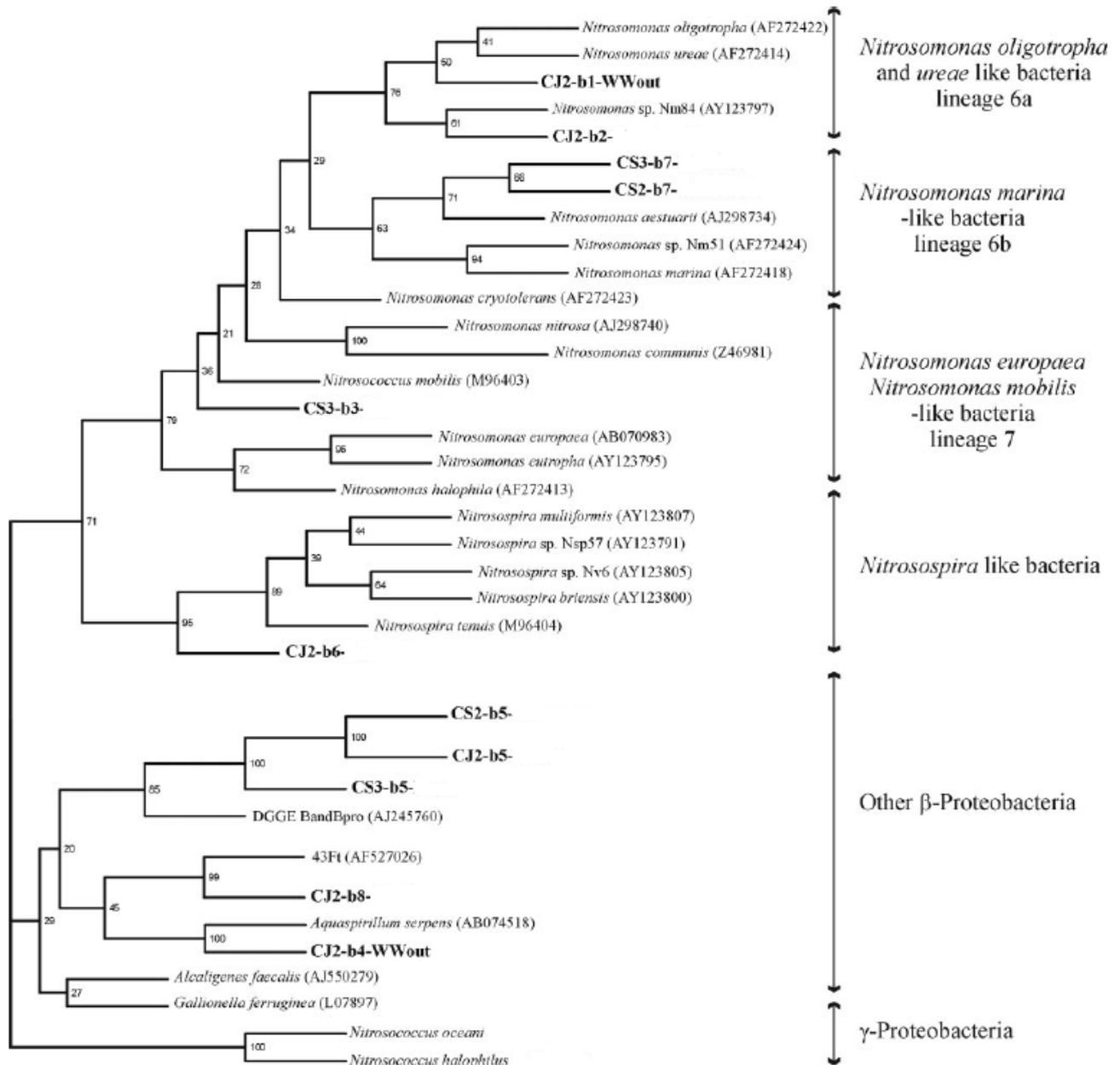


Figure 4. Phylogenetic neighbor-joining tree based on a comparison of 404 bp of 16S rRNA gene sequences from AOB of the β -subclass of the *Proteobacteria* and some other β -proteobacteria. The tree is rooted with two sequences of AOB belonging to the β -subclass of the *Proteobacteria*. Our DGGE sequences are boldfaced. Their designations are composed of C, for CTO PCR products; J2, S2, or S3, for a sampling date of July 2012, September 2012, or September 2013; b1 to b8, for the band as labeled in Figure 2; and the name of the sampling station from which the sequence originated

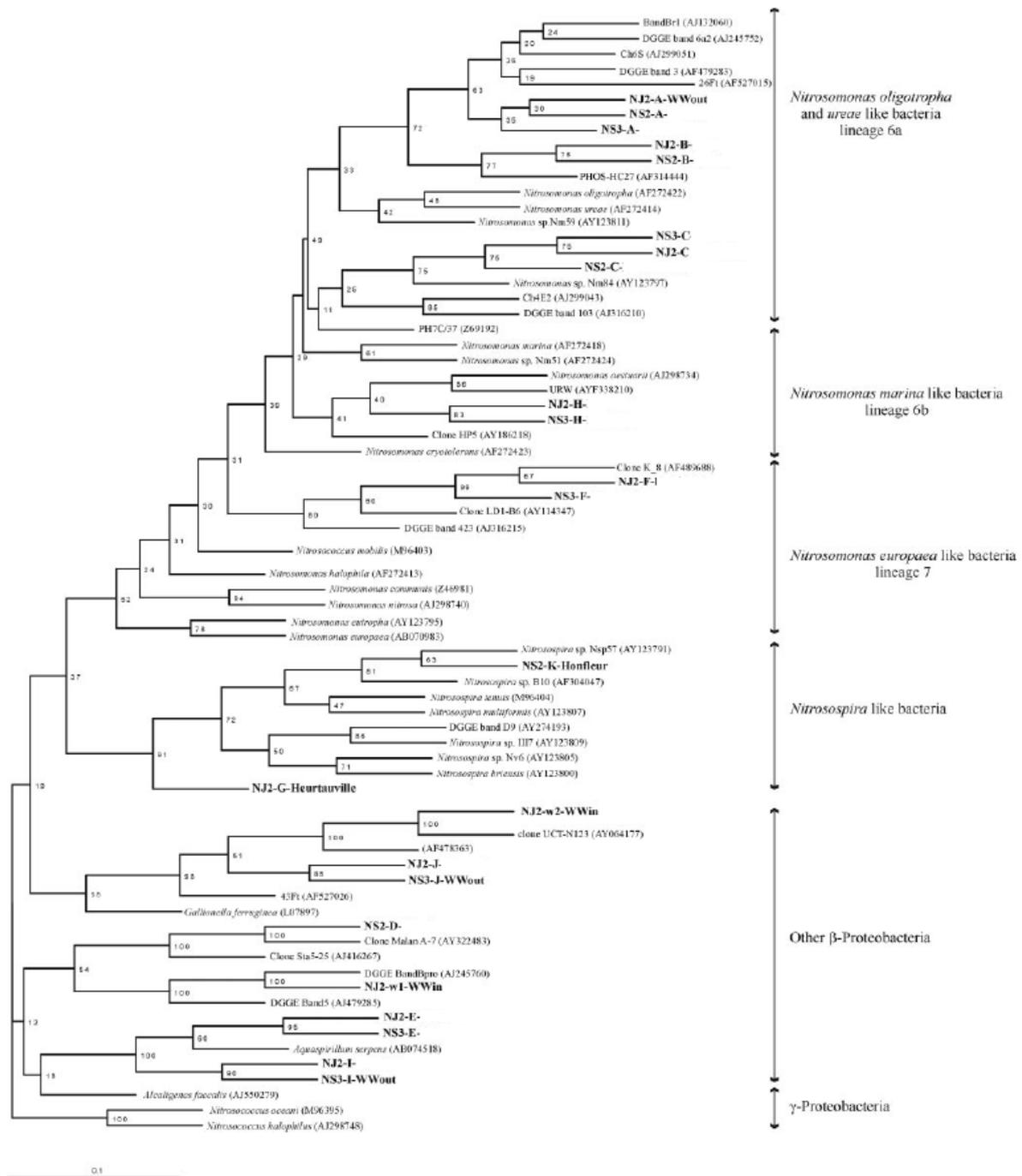


Figure 5. Phylogenetic neighbor-joining tree based on a comparison of 152 bp of 16S rRNA gene sequences from AOB of the β -subclass of the *Proteobacteria* and some other β -proteobacteria. The tree is rooted with two sequences of AOB belonging to the β -subclass of *Proteobacteria*. Our DGGE sequences are boldfaced. Their designations are composed of N, for nested PCR products; J2, S2, or S3, for a sampling date of July 2012, September 2012, or September 2013; A to L, w1, or w2, for the band as labeled in Figure 3; and, at the end, either the name of the sampling station from which the sequence originated or WWin or WWout (explained in the legend to Figure 3).

3.2. Phylogenetic Analyses

After confirmation of the purity of the bands (see Materials and Methods), one to three copies of each band were sequenced. Sequence analyses of 404-bp 16S rDNA fragments for CTO PCR products and 152-bp 16S rDNA fragments for nested-PCR products allowed us to build two phylogenetic trees (Figure 4 and Figure 5). The sequences of most of the bands retrieved from the two different DGGE analyses correspond to each other as follows: band b1 corresponds to band A, b2 to C, b3 to F, b4 to E, b5 to D, b6 to G, b7 to H, and b8 to J. The sequence analysis shows that several bands were not

affiliated with known AOB from the beta subclass of the *Proteobacteria*. Bands b4, b5, and b8, which belong to the beta-Proteobacteria, are closely related to *Aquaspirillum serpens* (98% homology with b4), *Methylophilus leisingeri* (94% homology with b5), and a *Dechloromonas* sp. (97% homology with b8). Similarly, bands D, E, I, J, w1, and w2 are not affiliated with AOB from the beta subclass of the *Proteobacteria*. The majority of these bacteria were found in the wastewater samples, except for band D, which was present only in all river samples. All these bands will be considered artifacts, due to the fact that the CTO primers apparently are not completely specific to AOB [45]. All the other bands are affiliated

with ammonia-oxidizing lineages belonging to the beta-subclass of the Proteobacteria. Band b1 (band A) is affiliated with lineage 6a, represented by *N. oligotrophum* and *N. ureae*-like bacteria. We recovered one band b1 (CJ2-b1-WWout), and three different bands A from the three sampling dates and different stations that showed 100% homology in the 152-bp 16S rDNA sequences. Two of these sequences were from treated-effluent samples (WWout), and two others were from the river; this means that the WWTP effluents have inoculated with the ammonia-oxidizing species represented by band A. We analyzed two different band B sequences that belong to lineage 6a and have a mutual homology of 100% in the 152-bp 16S rDNA sequence but a three-base difference from the band A sequence. In low abundance, this ammonia oxidizer may originate from the upstream part but would not be observed as a DGGE band B in the first three samples of the longitudinal profile. Band B could also originate which joins between stations 3 and 4. CTO band b2, which appeared only in the freshwater part of the estuary, has 100% homology with the three bands C in the 152-bp sequence. This ammonia oxidizer belongs to lineage 6a and is closely related to *Nitrosomonas* sp. strain Nm84 (98% homology) and other uncultured beta-proteobacterial AOB sequences [6,7]. Its source was not identified, but as in the case of band B, it might be an autochthonous bacterium present in very low proportions in the upper part, from other wastewater inputs, or from freshwater estuarine mudflat exchanges [24]. Bands b7 and H were also completely homologous in the 152-bp sequence. The corresponding bacteria, present only in samples with high salinity at the mouth of the estuary, were related to lineage 6b, represented by *Nitrosomonas marina*- and *Nitrosomonas aestuarii*-like bacteria, known to be halophilic. These bacteria, isolated from the marine environment, are definitely not specific but are introduced by the seawater. The homologous bands b3 and F correspond to an AOB closely related to *Nitrosococcus mobilis*; phylogenetically, the sequences are situated between the typical halophilic lineages 6b and 7, the latter represented by *Nitrosomonas europaea*-like bacteria. *N. mobilis*, probably introduced by tidal cycles, was found in samples taken within the salinity gradient (1 to 80/00) and was characteristic of the downstream estuary at low tide. The closest relatives reported were some uncultured AOB such as clone K_8 (99% homology), collected in surface sediments from Denmark Fjord, where the salinity value was 140/00 [40]; clone LD1-B6 (98% homology), found in anoxic marine sediments [21]; and DGGE band 423 (98% homology), obtained from the Schelde estuary water with a low ammonia level and high salinity [7]. Band L did not correspond to any CTO DGGE band. The NJ2-L-Poses and NS2-L-Elbeuf sequences (100% homology) were close to *N. mobilis* (97% homology). This band, found in all river samples, may belong to an autochthonous AOB that has become adapted to environmental changes. Band G, similar to CTO band b6 (100% homology between CS2-b6-LaBouille and NJ2-G), belonged to *Nitrosospira*-like bacteria and displayed environmental behavior identical to that of band L. These two species probably represent the upstream bacterial community, because no other AOB band was visible by DGGE analysis for stations 1 and 2. The fact that these bacteria are invariably present in the same low proportion

at all sampling stations and dates argues that these species are only weakly dependent on environmental variations.

4. Discussion

4.1. Nonspecificity of CTO Primers

The CTO primer set described by Kowalchuk et al. [35] was designed from a large database of sequences from cultured representatives and cloned sequences from various environments. Purkhold et al. [45], have clearly demonstrated that there is no perfect primer set for studying AOB, since none of the published primers that are intended to target all betasubclass AOB shows both 100% sensitivity (targeting all betasubclass AOB) and 100% specificity (excluding all non-betasubclass AOB). Consequently, some of the bands in the DGGE patterns were not related to AOB, as revealed by sequencing aimed at confirming the phylogenetic affiliations of the DGGE bands. Many bands, initially thought to represent AOB, were in fact identified as uncultured members of other branches of beta-Proteobacteria; these bands were identical to many sequences obtained in previous studies using the same primers (for example, clone ME-15 [AJ003772] [56] is similar to band b5, and DGGE band 5 [AF479285] [8] is similar to band w1). Since the AOB sequence that is closest to these bands is the sequence of *Nitrosospira* sp. strain III7, with less than 84% homology, we considered that none of these bands represented ammonia oxidizing bacteria.

4.2. Limits of the PCR-DGGE Technique

The limitations inherent in any electrophoresis technique also apply to DGGE. The number of bands generated by DGGE may not accurately reflect the number of different sequences present in a given mixture. Indeed, DNA of bacteria that are present in relatively low numbers in the environment might escape amplification by PCR. Furthermore, one DGGE band might represent several species, or several bands can be generated by the same bacterium due to degenerate primers [35], heteroduplex formation, or multiple heterogeneous rRNA operons [13]. Consequently, sequencing must be used to confirm the identity of the bands. We sequenced one to three bands that had the same position on the DGGE gel for the three sampling dates and showed that all the bands at the same position had the same nucleotide composition. The strategy adopted here can reasonably be considered to lead to the same bacterium for any sample and sampling date. Determination of the intensities of DGGE bands by image analyses enables us to calculate the relative proportion and the percentage of each band and AOB lineage within the whole AOB community. This result is only indicative: the double amplification (nested PCR) can increase the bias, and the presence of band D (non-AOB), representing nearly half of the products of PCR in all the samples, can bias the band intensities. Although Kowalchuk et al. [35] demonstrated that the ratio of the products from the CTO primers was in good agreement with the concentrations in the starting template, Suzuki and Giovannoni [61] argued that this equivalence would not hold for all primer-template combinations in more-complex mixtures. As a result, the intensity of the bands

in a DGGE pattern would provide only limited insights into the microbial community diversity [18]. Typical features of nitrification and ammonium-oxidizing bacteria in the lower River. The temporal and spatial differences observed in potential nitrifying activity profiles and amoA gene copy numbers cannot be explained by changes in the AOB populations. At three summer time points, the same AOB species were identified in the lower Seine, and the same bacterial shifts and successions were observed along the river transect with banding patterns very close to each other. This result shows a temporal stability in the genetic diversity of the functional bacterial population, which indicates that similar hydrological (residence time), meteorological (temperature), nutritional (NH_4^+ concentrations), and bacterial WWTP input conditions have led to the same selection and adaptation of AOB species in the environment. The major difference between the situations investigated was spatial shifts in the presence or absence of AOB species, which can be explained by slight differences in the controlling factors mentioned above.

4.3. Analyzing the Diversity of AOB

To understand the relationship between environmental variables and ecosystem functioning, it is essential to investigate microbial diversity [63]. The relative abundances of sequences obtained by PCR amplification from environmental samples, illustrated by DGGE patterns, suggest that the distribution of different bands might be linked to environmental factors. For example, Suwa et al. [60] showed that various *Nitrosomonas* strains isolated from activated sludge could be either sensitive or insensitive to high ammonium concentrations. Temperature may also affect the community structure of ammonia oxidizers [3] either directly or indirectly. From upstream to downstream in the River, the first change in DGGE patterns appeared at station 3, due to AOB seeding (mainly band A) by WWTP effluents. The corresponding bacterial strain represented a large proportion (about 40%) of the AOB community in the river and persisted down to the freshwater estuary, showing that it could survive or adapt to a variety of ammonium concentrations, suspended-matter levels, and other environmental conditions. This feature distinguishes it from most heterotrophic bacteria, which, when released from domestic wastewater, rapidly decreased [22]. Unfortunately, no DGGE patterns were studied to ascertain whether the band B species, initially in very low proportions, was favored by increased substrate concentrations or was already present in high numbers. Furthermore, in the freshwater estuary, a new AOB (band C) was selected in the sector of maximum nitrifying activity where oxygen and ammonia concentrations decrease and suspended-matter concentrations increase. Whereas many studies have shown that oxygen has no effect on the composition of the AOB community [4,7,32], the "band C" population may thrive on freshly produced ammonia, organic compounds, or new mineral ions [14] specific to the estuary, where the organic matter turnover is generally high [23,50]. This niche differentiation may be due to physiological differences such as the ability to produce extracellular polymeric substances [57], allowing an attached versus free-living AOB selection [42]. Finally,

the shift from freshwater to halophilic bacteria, related to *N. marina* and *N. aestuarii* (bands F and H), at the mouth of this macrotidal estuary (stations 17 and 18) can easily be explained by a permanent mixing with seawater, where such species are selected [21,27,37]. An additional and/or alternative source for these halophilic bacteria could be the lateral saline mudflats, which are permanently dynamically interacting with the turbidity maximum within the channel. All three major nested DGGE bands (A, B, and C), i.e., 75 to 90% of the AOB community from the WWTP output to the estuary, belong to lineage 6a, represented by *N. oligotropha*- and *N. ureae*-like bacteria. The fact that identical PCR conditions are able to detect lineage 6a sequences in soil, rhizosphere, wastewater, and brackish water samples [32,36,40,48,59] suggests that the frequent detection of *Nitrosomonas* lineage 6a sequences in the River and estuary was not simply due to a bias in the PCR. This population might have a selective advantage over the other populations. Speksnijder et al. [56] found that *Nitrosomonas* lineage 6a was the only sequence lineage of beta-subclass ammoniaoxidizing bacteria to be found at all water and sediment sites. *Nitrosomonas* lineage 6a is well adapted to low ammonia concentrations because of its low K_m for ammonia [5,57,60]. These bacteria outcompeted other AOB at low ammonia concentrations [5,6]; note that these authors also found that *N. oligotropha*- and *N. ureae*-like bacteria were dominant in the freshwater part of the estuary and that *N. marina* and *N. aestuarii* were present in the brackish zone. Lineage 6a is known to be inhibited at ammonia concentrations exceeding 10 mM, which is 20 to 30 times higher than those found in the River downstream of the WWTP effluent discharge [6,31,57,60]. *Nitrospira*-like sequences are typical AOB populations present in soil fertilized with amended water [41], while *Nitrosomonas*-like sequences are typically found in effluents of treated urban sewage. This supports our results, i.e., the presence of *Nitrospira* in low proportions in all the samples. The *Nitrospira* sequence (band G) might come from upstream in the River and is probably introduced by soil leaching. *Nitrospira* is generally regarded as the most ubiquitous genus in forest soils [25] and it dominates in grasslands and agricultural ecosystems [11,36,59]. In the transect of the River that was studied, an *N. mobilis*-like sequence (band L) belonging to lineage 7 was also present in all the samples and had the same behavior as *Nitrospira*. Species belonging to lineage 7 are often found in rich media with high ammonia concentrations such as activated sludge [28,48] and would have been introduced by upstream WWTPs. However, these bacteria have also been found in both oligotrophic and eutrophic lakes [26,55]. It is worth mentioning that many other AOB species in very low numbers might exist in the river without being detected, since approximately 1% of the target community is below the PCR-DGGE detection limit [39]. Because no major temporal change in AOB community composition occurred during our study (July and September 2012, September 2013), these results were compared with those obtained in September 1998 [12], where the amoA gene diversity was obtained from cloning and sequencing of two gene libraries from station 4 (Triel), immediately downstream of the WWTP input, and from station 14, in the freshwater part of the estuary. The comparison is

relevant because the phylogeny of the *amoA* gene, which encodes the subunit of the ammonia monooxygenase enzyme, was found to correspond largely to the phylogeny of the 16S rRNA gene in ammonia oxidizers [1,34,45]. The two studies in the River showed the same major lineage, i.e., 60 to 80% and 75 to 90% of AOB, respectively, belonging to lineage 6a, but a lower diversity was found in the samples taken in 2012 to 2013 and analyzed by DGGE [12]. Whereas three different sequence types (bands A, B, and C) were found here within lineage 6a, four different sequences were previously found at the station (compared to the two different sequences here) and three were found in both studies. To increase the knowledge of the functional community of AOB in the River, its active bacteria could be investigated by RNA DGGE. In reality, analysis of 16S rDNA does not distinguish between metabolically active and quiescent cells. Boon et al. [8] reported that some very active populations visible in the RNA DGGE patterns can hardly be detected in the DNA DGGE fingerprints, and they concluded that whereas an RNA DGGE analysis is appropriate for a rapid examination of changing microbial communities, DNA DGGE analysis is suited to slowly evolving, stable communities, such as those in the river system. Furthermore, analysis of environmental 16S rRNA by using reverse transcription-PCR would provide greater sensitivity because of the higher target copy number and might indicate which members of the community are more metabolically active, i.e., have a high ribosome content [21,33,65]. An alternative approach could be the use of a translated functional gene as a molecular marker [53]. The primer pair (*amoA*-1F-*amoA*-2R) developed by Rotthauwe et al. [47] amplifies a fragment of the *amoA* gene -subunit. Recently Oved et al. [41] and Nicolaisen and Ramsing [40] have applied *amoA* amplicons to DGGE analysis using newly designed PCR primers. Although these authors succeeded in the optimization of the assay, application of their *amoA* PCR-DGGE approach to our samples led to patterns with too many bands, not separable as single bands; furthermore, any changes in the technical approach failed to give valid results (M. Coci, unpublished data). Moreover, the fact that Oved et al. [41] found a band (w2406) that was not an *amoA* sequence tends to support the statement that this technique must be used with caution. As a whole, besides demonstrating the surprisingly stable AOB community patterns in the lower River which are strongly impacted by the WWTP effluents, this study identified the ammonia-oxidizing bacteria introduced by the effluents. In addition, in contrast to many typical allochthonous heterotrophic bacteria from WWTPs, such as coliforms, that are not adapted to surface water, the AOB introduced by the effluents seem to be able to maintain their biomass in the receiving medium and even to grow, probably favored by the ammonia pollution in the river or by the estuarine conditions.

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