

Nitrification and Nitrifying Bacteria in the Lower Seine River and Estuary (France)

Aurélié Cébron,* Thierry Berthe,† and Josette Garnier

UMR Sisyphe 7619, Université Pierre et Marie Curie (Paris 6), 75005 Paris, France

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The Achères wastewater treatment plant, located just downstream of Paris, discharges its effluents into the lower Seine River. The effluents contain large numbers of heterotrophic bacteria, organic matter, and ammonium and are a source of nitrifying bacteria. As a result, degradation of organic matter by heterotrophic bacteria and subsequent oxygen depletion occur immediately downstream of the effluent outlet, whereas nitrifying bacteria apparently need to build up a significant biomass before ammonium oxidation significantly depletes the oxygen. We quantified the potential total nitrifying activity and the potential activities of the ammonia- and nitrite-oxidizing communities along the Seine River. In the summer, the maximum nitrifying activity occurs in the upper freshwater estuary, ~200 km downstream of Achères. The quantities of nitrifying bacteria, based on *amoA* gene copy numbers, and of *Nitrobacter* organisms, based on 16S rRNA gene copy numbers, were correlated with the potential nitrifying activities. The species composition of ammonia-oxidizing bacteria was investigated at two sites: the Triel station just downstream from Achères (km 84) and the Seine freshwater estuary at the Duclair station (km 278). By means of PCR primers targeting the *amoA* gene, a gene library was created. Phylogenetic analysis revealed that the majority of the analyzed clones at both sites were affiliated with the genus *Nitrosomonas*. The *Nitrosomonas oligotropha*- and *Nitrosomonas urea*-related clones represented nearly 81% of the community of ammonia-oxidizing bacteria at Triel and 60% at Duclair. Two other ammonia-oxidizing clusters of the β subclass of the *Proteobacteria*, i.e., *Nitrosomonas europaea*- and *Nitrosospira*-like bacteria, were found in smaller numbers. The major change in the ammonia-oxidizing community between the two stations along the Seine River-upper estuary continuum was the replacement of the *N. oligotropha*- and *N. urea*-related bacteria by the *Nitrosospira*-affiliated bacteria. Although the diversities of the ammonia oxidizers appear to be similar for the two sites, only half of the restriction patterns are common to both sites, which could be explained by the differences in ammonium concentrations, which are much lower in the upper estuary than in the river at the effluent outlet. These results imply a significant immigration and/or selection of the ammonia-oxidizing bacterial population along the continuum of the Seine River from Paris to the estuary.

The Seine River downstream of Paris is greatly affected by the discharge of treated effluents from the Achères wastewater treatment plant (WWTP) (8.5 million inhabitant equivalent from Paris and its suburbs, subjected only to a secondary treatment). A typical feature of the ecological functioning of the lower Seine River is a high summer oxygen deficit immediately downstream of the Achères effluent outlet due to degradation of organic matter (18, 19, 48) and another depletion in the freshwater estuary due to nitrification of ammonium that is also contained in the effluent (41). Since 1989, studies of the Seine River have extended the knowledge of heterotrophic organic matter degradation (18, 48) and nitrification (8), as well as of the characteristics of wastewater effluents (49) and oxygen budgets (19). Moreover, nitrifying bacteria had previously been shown to originate from wastewater effluent that was not subjected to tertiary treatments (8). The plans for a tertiary-treatment facility (including nitrification) at the Achères WWTP in 2005, therefore, required further studies of

the nitrification process and its associated bacteria in the continuum of the Seine River.

Nitrification, mainly due to autotrophic bacteria, is the oxidation of ammonia into nitrite and subsequently into nitrate. In freshwater environments, the first step is carried out by chemolithotrophic ammonia-oxidizing bacteria (AOB), which form a tight cluster within the β subclass of the *Proteobacteria* and contain members of the genera *Nitrosomonas* (as well as *Nitrosococcus mobilis*) and *Nitrosospira* (as well as *Nitrosolobus* and *Nitrosovibrio*) (23, 58, 60); AOB of the γ subclass of the *Proteobacteria* are only found in halophilic and marine environments. The second step of nitrification is carried out by chemolithotrophic nitrite-oxidizing bacteria (NOB) that belong to four phylogenetically distinct groups. One group, belonging to the α subclass of the *Proteobacteria*, is represented by the genus *Nitrobacter*. The two known species of the genus *Nitrosospira* are members of a distinct phylum. Finally, the two marine species *Nitrosococcus mobilis* and *Nitrosospira gracilis* are assigned to the γ and the δ subclasses of the *Proteobacteria*, respectively. Several methods can be used to investigate nitrifying bacterial populations in situ; cultivation-dependent analysis often leads to significantly underestimated cell counts due to the presence of unculturable species, and the very low growth rate of the nitrifiers (62) makes it time-consuming. Measurements of potential nitrifying activities by the ^{14}C

* Corresponding author. Mailing address: UMR Sisyphe 7619, Université P. et M. Curie—Paris 6, BP 123, Tour 56, Étage 4, 4 Place Jussieu, 75005 Paris, France. Phone: (33)-(1)-44 27 51 34. Fax: (33)-(1)-44 27 51 25. E-mail: aurelie.cebron@ccr.jussieu.fr.

† Present address: Laboratoire de Microbiologie du Froid, Université de Rouen, Mont-Saint-Aignan, 76000 Rouen, France.

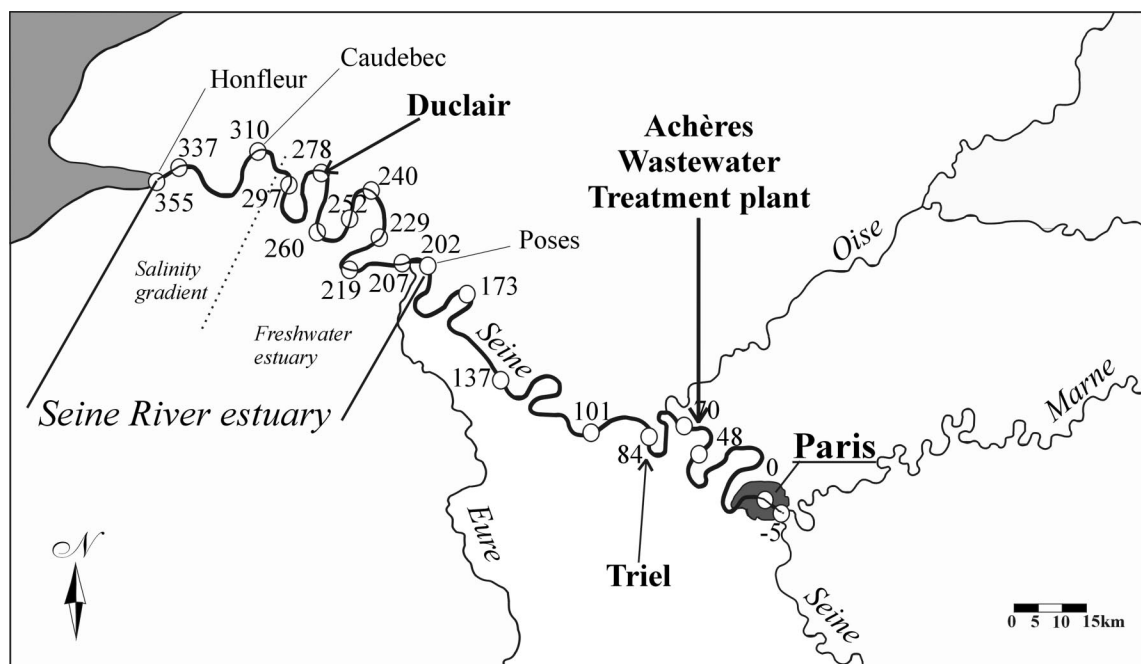


FIG. 1. Representation of the Seine River from Paris to the estuary. The numbered circles represent sampling points for process work. The two sites in boldface type are where the molecular characterization was done.

method can be used to estimate biomass (7) but do not identify the bacterial populations involved in the two steps of the process. However, in the last 5 years, molecular techniques have proved to be well suited to studying these bacteria, both for quantifying the nitrifiers by competitive or real-time PCR and for assessing the composition of the nitrifying population by the construction of a specific clone library and subsequent sequencing. Primers targeting part of the *amoA* gene, which encodes ammonia monooxygenase, may serve to target the AOB by the use of PCR (42). PCR primers targeting the whole NOB functional group do not exist at present, but 16S ribosomal DNA (rDNA) primers are used to target a specific genus. The genus *Nitro bacter*, which can be targeted according to the method of Degrange and Bardin (12), was chosen as a representative of the nitrite-oxidizing population in the system studied here.

The study was carried out in summer, at low flow, from Paris to the coastal zone (Honfleur) along the aquatic continuum of the lower Seine River and its estuary, both of which are strongly impacted by the effluent inputs. In addition to determining the relative contribution of the nitrifying activity to the oxygen budget (19), the aims were (i) to quantify the bacterial populations involved in the two steps of the process in order to pursue quantitative modeling of the ecological functioning of the Seine River and estuary (4) and (ii) to identify the composition of the AOB community by molecular methods in order to investigate potential species selection or adaptation of the populations along the Seine River. The population composition was therefore studied at two sites: one immediately downstream of the effluent outlet of the Achères treatment plant and the other in the upstream part of the freshwater estuary, where nitrification generally reaches its maximum.

MATERIALS AND METHODS

Description of research area. The lower Seine River extends from Paris (km 0) to the weir at Poses (km 202), which prevents tidal water from moving upstream and consequently forms the upstream limit between the river and the estuary (Fig. 1). Paris is at km 0, and the other stations are designated by their distances from Paris. The fluvial sector of the estuary extends up to Caudebec (km 310), which is the limit of saline intrusion. From Caudebec to Honfleur (km 356), at the mouth of the estuary, there is a continuous salinity gradient influenced mainly by tides and river flow.

The lower Seine River and estuary receive all the inputs from a drainage basin characterized by great industrial activity, intensive agriculture, and a high population density, including, in particular, the city of Paris (Fig. 1). The domestic effluent of the 8.5 million inhabitants of Paris and its suburbs is treated by activated sludge in the WWTP of Achères, 70 km downstream from Paris. The lower Seine River is therefore enriched in organic matter and nutrients, especially ammonium, which is known to be completely converted into nitrate in the upstream estuary.

Sample collection and analytical methods. Water samples were taken along the lower Seine River and estuary, from upstream of Paris to Honfleur (the mouth of the estuary), in September 1997, September 1998, and July 1999. The three longitudinal profiles included samples collected upstream and just downstream of the treatment plant outlet (at the stations of Maisons-Laffitte and Conflans-Ste Honorine, at km 48 and 70, respectively) (Fig. 1). At each station, a 10-liter water sample was collected for all laboratory analyses of water quality variables (especially suspended matter and nitrogen) and was stored at 4°C during transfer to the laboratory. DNA was extracted from the same samples but only from those collected in September 1998; an aliquot of 500 ml was immediately poured into sterile polypropylene centrifugation bottles (Nalgene) and adjusted to 0.1% sodium dodecyl sulfate and 1 mM EDTA. The samples were then stored at -20°C until the nucleic acids were extracted. Four hundred fifty milliliters of each water sample was used for the measurement of potential nitrifying activity and ammonia and nitrite oxidation rates. Ten milliliters was fixed with formaldehyde (final concentration, 2%) to determine the total bacterial abundance by DAPI (4',6'-diamidino-2-phenylindole) staining and epifluorescence microscopy (35).

Measurement of potential nitrifying activity. The potential nitrifying activities of all the water samples brought to the laboratory were measured according to the method described by Brion and Billen (7). The measurements were made

under optimum conditions for oxygen ($7.5 \text{ mg} \cdot \text{liter}^{-1}$) and ammonium concentration ($2 \text{ mM NH}_4\text{Cl}$) and at a constant temperature of 20°C . The potential nitrifying activities were determined by the differences between the amounts of $\text{H}^{14}\text{CO}_3^-$ incorporated after 20 to 24 h of incubation in the samples with and without added specific nitrification inhibitors (7, 51). N-Serve (2-chloro-6-trichloromethyl pyridine [also called Nitrapyrin]; $5 \text{ mg} \cdot \text{ml}^{-1}$) and sodium chlorate (NaClO_3 ; 10 mM) were used in order to specifically inhibit ammonia and nitrite oxidation, respectively (1, 26, 56). Carbon incorporation is proportional to ammonia oxidation, with 0.11 mol of carbon incorporated per mol of NH_4^+ oxidized into nitrate (7). Under these optimal conditions, the nitrifying activity is roughly proportional to the nitrifying biomass (1, 7).

In order to separately estimate the ammonia and nitrite oxidation rates, we measured nitrite concentration changes in subsamples containing either N-Serve or NaClO_3 , which inhibit the oxidation of ammonia and nitrite, respectively. The samples were incubated in the dark, at room temperature, under optimum conditions for oxygen and with the addition of ammonium (NH_4Cl ; 2 mM) and nitrite (KNO_2 ; 0.36 mM). The nitrite concentrations were measured at the beginning and end of the 24-h incubation period, during which the oxidation rates were shown to be linear. The ammonia or nitrite oxidation rates were calculated from the increase (ammonia oxidation) or decrease (nitrite oxidation) of nitrite concentrations during the incubation (3).

Measurements of water quality variables. Ammonium and nitrite were measured by spectrophotometry in water filtered through a glass fiber membrane (GF/F; Whatman) according to the method of Slavyck and McIsaac (50). Nitrate was assessed spectrophotometrically in filtered water after Cd reduction to nitrite (40). Suspended matter was weighed on GF/F filters dried at 450°C .

DNA extraction and purification. DNA was extracted from the Seine River water samples (only samples from September 1998) according to the protocol developed by Petit et al. (33). This method allows extraction of nucleic acids from particle-attached and free-living bacteria in the water. The DNA was further purified through an Elutip-d column (Schleicher & Schuell) according to the recommendations of the manufacturer. The purified DNA was then precipitated with ethanol, resuspended in Tris-EDTA buffer, and stored at -20°C until it was used.

Detection and quantification of ammonia oxidizers and *Nitrobacter* species by PCR and cPCR. PCR amplification of a 491-bp fragment of the *amoA* gene was carried out by using the *amoA*-1F and *amoA*-2R primer set specific for ammonia oxidizers belonging to the β subclass of *Proteobacteria* (42). *Nitrobacter*, one of the most studied genera for which the tools to target the population were available, was chosen as a representative of the NOB for a quantitative study (2). *Nitrobacter* cells were detected by amplification of a 397-bp fragment of the *Nitrobacter* 16S rDNA gene with the FGPS 872 and FGPS 1269' primers (12). All PCR amplifications were carried out in a total volume of $50 \mu\text{l}$ in 0.2-ml tubes by means of a DNA thermocycler (GeneAmp 2400 PCR system; Perkin-Elmer Cetus). The reaction mixtures were prepared in $1\times$ buffer [75 mM Tris , pH 9.0, $20 \text{ mM (NH}_4)_2\text{SO}_4$, $0.01\% \text{ Tween 20}$, 1.5 mM MgCl_2 , 100 ng of DNA, and 0.5 U of *Taq* DNA polymerase (RedGoldtar; Eurogentec). The thermal profiles included an initial denaturing step consisting of 94°C for 45 s followed by 35 cycles of denaturation at 94°C for 45 s , annealing at 55°C with the *amoA* primers and at 50°C with the FGPS primers, and elongation at 72°C for 120 s . The cycle was ended by a final elongation step at 72°C for 10 min . Aliquots ($10 \mu\text{l}$) of the amplification products were analyzed by gel electrophoresis on 2% (wt/vol) agarose gels (Boehringer). Estimates of ammonia oxidizers (*amoA* gene copy numbers) and *Nitrobacter* cell numbers were made by competitive PCR (cPCR) as described by Stephen et al. (55) and Berthe et al. (2), respectively. For cPCR of AOB, we constructed a competitor as described by Stephen et al. (55). One of our clones (Duc27) was used as a DNA template. Amplification products were analyzed by agarose gel electrophoresis, and DNA band intensities were estimated with imaging and analysis software (Bio-Rad).

Construction and analysis of an *amoA* gene fragment library. Two clone libraries were constructed for two sites along the Seine River continuum, Triel (km 84) and Duclair (km 278). The amplified *amoA* PCR fragments were excised from the agarose gel, purified with an agarose gel extraction kit (Sephaglass; Pharmacia), and eluted in Tris-EDTA buffer. The 491-bp *amoA* DNA fragments from the Duclair sample were cloned in a pPCR-Script Amp SK(+) vector (PCR-Script Amp cloning kit; Stratagene) according to the manufacturer's recommendations. The 491-bp *amoA* DNA fragments from the Triel sample were cloned in a pCR2.1 vector (TAcloning kit; Invitrogen), also according to the manufacturer's recommendations. Fifty clones from each library were randomly selected for further analysis. The cloned inserts were reamplified with *amoA* primers and then digested with the *MspI* enzyme. Restriction patterns were analyzed after gel electrophoresis on 2% agarose gels. Clones representative of each restriction pattern were chosen for sequence analysis. Selected plasmids

were then subjected to double-strand service sequencing (Eurogentec). Phylogenetic analyses were conducted by aligning our *amoA* sequences with the *amoA* sequences from the GenBank database using Clustal W version 1.7 (59). Phylogenetic algorithms and trees (DNA-DIST, NEIGHBOR, and SEQBOOT) were operated through the PHYLIP version 3.5 package written by J. Felsenstein (16).

Nucleotide sequence accession numbers. The sequences determined in this study were deposited in the GenBank database under the following accession numbers: Duc1 (AF367461), Duc2 (AF367462), Duc14 (AF367464), Duc17 (AY249149), Duc26 (AF367465), Duc27 (AF367463), Duc31 (AY249150), Duc32 (AF367466), Duc34 (AY249151), Duc47 (AY249152), T15 (AY249153), T21 (AY249154), T22 (AY249155), T27 (AY249156), T38 (AY249157), T42 (AY249158), T45 (AY249159), T47 (AY249160), and T48 (AY249161).

RESULTS

Nitrifying activities in the lower Seine River and estuary.

The samples collected in September 1997, September 1998, and July 1999 corresponded to summer low flows of 290 , 230 , and $240 \text{ m}^3 \text{ s}^{-1}$, respectively. As mentioned above, the two oxygen-depleted sectors were monitored (Fig. 2). The first oxygen depletion (~ 6 to 7 mg of $\text{O}_2 \cdot \text{liter}^{-1}$) was observed immediately downstream from the outlet of the Achères WWTP and was linked to the decomposition of organic matter by heterotrophic bacteria (18, 19). The second occurred in the Seine estuary (with oxygen concentrations as low as $1.2 \text{ mg} \cdot \text{liter}^{-1}$) and was associated with the maximum of potential nitrifying activity (from 0.6 to $2.6 \mu\text{mol N-oxidized} \cdot \text{liter}^{-1} \cdot \text{h}^{-1}$). Despite a regular decrease in ammonium along the studied sector due (i) to dilution by the Oise River at km 100 and (ii) to a progressive nitrification increase, the ammonium supplied by the effluent from the Achères plant was completely oxidized in the estuarine sector. As a consequence, NO_3^- concentrations increased, typically from 4 mg of $\text{N} \cdot \text{liter}^{-1}$ downstream of Achères to 7 mg of $\text{N} \cdot \text{liter}^{-1}$ in the Seine estuary; therefore, low levels of denitrification occurred.

Comparison of potential ammonia and nitrite oxidation rates with the total potential nitrifying activity (by the ^{14}C method) showed strong correlation (Fig. 3a). For the two steps of nitrification, the potential ammonia oxidation rate was ~ 5 to 10 times higher than the nitrite oxidation rate, which can be explained by the much higher specific growth yield (the biomass formed per unit of substrate oxidized) of the former than of the latter (7).

Detection and quantification of ammonia oxidizers and *Nitrobacter* cells. The approaches used for the bacterial populations involved in the two steps of the nitrification process were conceptually different. We used the specific primer set published by Rotthauwe et al. (42) to target a stretch of the *amoA* gene, which encodes the active site of ammonia monooxygenase, a key enzyme in AOB, whereas a primer set targeting only *Nitrobacter* 16S rDNA (12) was used for the detection of a fraction of the nitrite-oxidizing community. As *Nitrobacter* cells have one copy of the 16S rRNA operon (31), the number of 16S rDNA copies determined by cPCR represents a true evaluation of the abundance of *Nitrobacter* cells, whereas the abundance of the AOB can be determined only by a factor of 1 to 3, as the cells contain from one to three copies of the *amoA* gene (28, 32).

Following DNA extraction, application of the bacterial 16S rDNA primers pA and pHr (14) generated a fragment of 1.5 kbp for all samples examined, suggesting that no inhibitory substances (such as humic acids) were present in the DNA

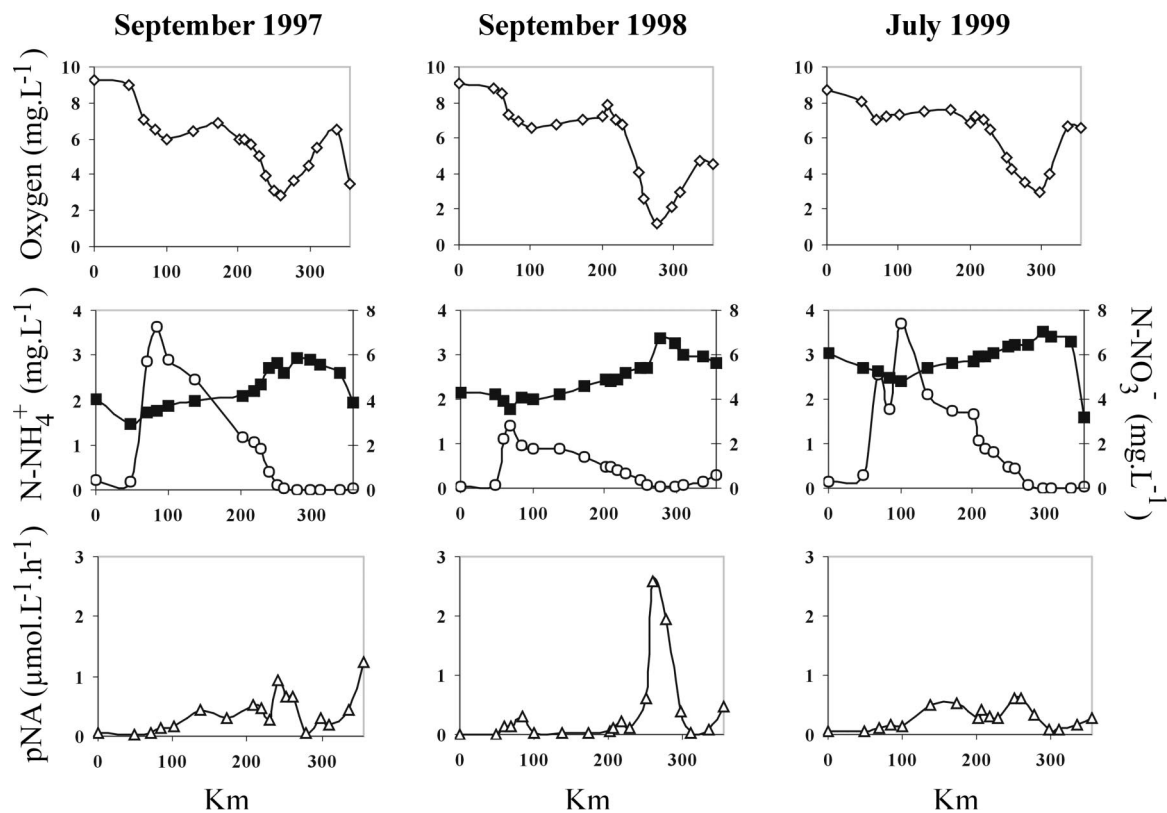


FIG. 2. Variations in oxygen, ammonium (○), and nitrate (■) concentrations and potential nitrifying activity (pNA) along a longitudinal profile of the Seine River (from Paris, km 0, to Honfleur, km 356) during September 1997, September 1998, and July 1999.

samples. Application of the *amoA* primer set generated a 491-bp DNA fragment for nearly all water samples collected along the longitudinal profile, except for those from the Maisons-Laffitte station upstream of the Achères plant. For NOB, amplification of a 397-bp region of the *Nitrobacter* 16S rDNA gene was achieved in all water samples, from Paris to Honfleur, including the station upstream of the WWTP. Quantification of *Nitrobacter* abundances and the numbers of *amoA* gene copies (by cPCR) along the longitudinal profile led to a pattern fairly similar to the one for the potential nitrifying activity (Fig. 3). The number of *amoA* gene copies increased ~75-fold (from 9.7×10^5 to 7.3×10^7 *amoA* copies liter⁻¹), and the *Nitrobacter* abundance increased 370-fold (from 9.8×10^4 to 3.6×10^7 *Nitrobacter* cells liter⁻¹) between Maisons Laffitte (km 48, upstream of the Achères effluent output) and La Bouille (km 260)-Duclair (km 278), the sector where the potential nitrifying activity was at its maximum. We noticed an increase in the number of *amoA* gene copies immediately downstream from the Achères WWTP that was not observed for *Nitrobacter*. In the lower estuary, a sharp decrease in both nitrifying populations was observed at Caudebec (km 300), the limit of the saline intrusion; *Nitrobacter* abundance then increased again in the turbidity maximum and salinity gradient.

The concentration of the total bacterial community (by epifluorescence microscopy) showed a rough increase along the continuum, from 8.3×10^9 bacteria liter⁻¹ at the station upstream of Achères (Maisons Laffitte) to 3×10^{10} bacteria liter⁻¹ in the estuary. Considering the whole longitudinal pro-

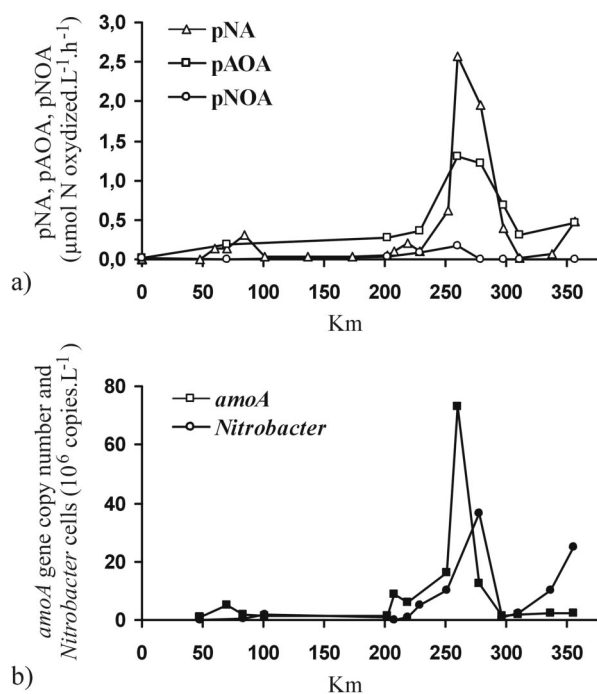


FIG. 3. (a) Potential nitrifying activity (pNA) compared with potential ammonia-oxidizing activity (pAOA) and potential nitrite-oxidizing activity (pNOA). (b) *amoA* gene copy numbers and *Nitrobacter* 16S rDNA gene copy numbers determined by cPCR (samples from September 1998).

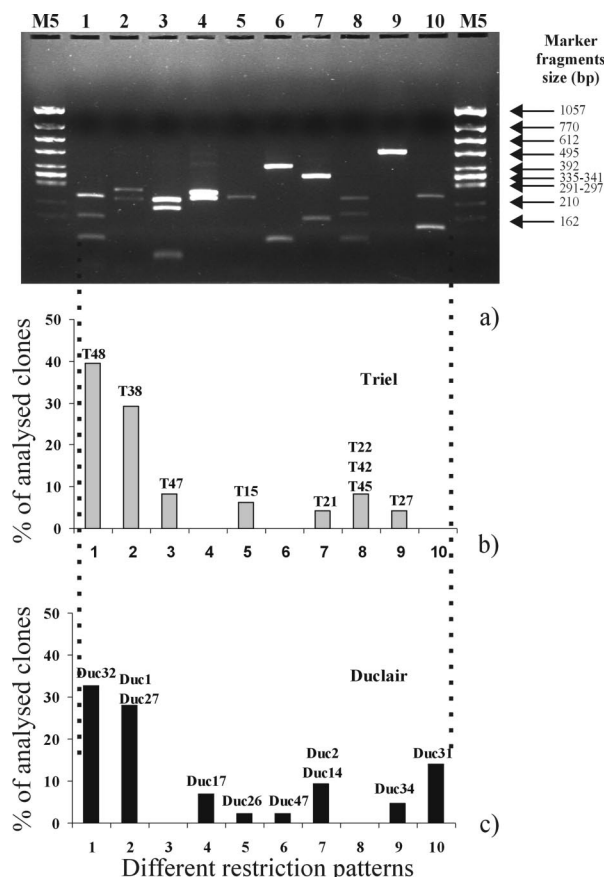


FIG. 4. (a) Gel with 10 different *MspI* restriction patterns of analyzed *amoA* clones from Triel and Duclair; the size marker (M5; Eurogentec) was a *HincII* digest of phage ϕ X174 DNA. (b and c) Proportions of analyzed clones in each restriction pattern for the two clone libraries of Triel and Duclair. The names of the representative clones that were sequenced and used for phylogenetic analyses are indicated above the bars (samples from September 1998).

file, the β -AOB and *Nitrobacter* represented, on average, 0.11 and 0.03%, respectively, of the total bacterial community, with maximum values at La Bouille and Duclair (0.70% for β -AOB; 0.17% for *Nitrobacter*).

Characterization of the communities of AOB at two sites on the Seine River. Evaluations of the natural diversity of *amoA* genes, as obtained by PCR amplification, cloning, and sequence analysis, were conducted at two key stations on the river continuum: at Triel, strongly influenced by the effluent of the Achères WWTP, and at Duclair, in the estuary (Fig. 1), where the highest value for potential nitrifying activity and the lowest concentration of ammonium were measured. The *amoA* PCR products retrieved from these water samples were used for the generation of two *amoA* gene libraries. A restriction enzyme analysis (restriction fragment length polymorphism) was performed on *amoA* fragments from 50 clones from each library.

Digestion with *MspI* revealed 10 different restriction patterns (Fig. 4) among the 100 analyzed clones. The analyzed Triel (T) clones were clustered in seven different patterns, while the analyzed Duclair (Duc) clones were clustered in eight different patterns. The distribution of the analyzed clones

in the different patterns is indicated in Fig. 4. The two major groups were the same at both sites (restriction groups 1 and 2) and represented 60 and 70% of the Duclair and Triel clones, respectively. Two groups were unique to the Triel station (restriction groups 3 and 8), and three were unique to Duclair (restriction groups 4, 6, and 10).

In order to identify the dominant ammonia oxidizers, one clone representative of all pattern groups was chosen for sequence analysis, but for some groups two were selected, i.e., a total of 19 sequences were analyzed (Fig. 4). The similarity among all *amoA* sequences (from the two samples from the Seine River) ranged from 71 to 99%, according to the results from the Edtaln program. BLASTN searches of the GenBank database confirmed that all of the sequenced clones corresponded to *amoA*-like sequences and showed that some clones were very close to sequences available in the databases (Table 1). All of the analyzed clones showed strong homology (from 87 to 99%) with uncultured environmental clones. The majority were retrieved from various nitrifying WWTPs (29), except T27 and T47, which seemed closely related to clones retrieved from freshwater habitats.

A phylogenetic tree was obtained from the analysis of a stretch of 417 bp (within the 491-bp *amoA* PCR product cloned) (Fig. 5). The majority of these *amoA* fragments retrieved from the two sites on the Seine River belonged to three clusters, with a majority belonging to the genus *Nitrosomonas*. The analyzed sequences did not form a monophyletic cluster but were affiliated with different *Nitrosomonas* species. The Duc1, Duc27, Duc32, T21, T22, T38, T42, T45, and T48 clones belonged to the cluster represented by the *Nitrosomonas oligotropha*- and *Nitrosomonas urea*-like sequences. These clones represented 60 and 81%, respectively, of the total analyzed clones from the Duclair and Triel stations (Fig. 5). In the *Nitrosomonas europaea*-like cluster, two clones from Duclair, Duc2 and Duc14 (belonging to the same *MspI* restriction group), formed a common branch with *Nitrosomonas europaea*, Duc47 was closely related to *Nitrosomonas nitrosa*, and Duc34 formed another branch. The last four sequenced clones made up 16% of the total analyzed clones from Duclair. For the Triel station, the proportion of the *N. europaea*-like cluster was roughly the same: clones T27 and T47 represented 13% of the total analyzed clones (Fig. 5) and were in the same separate branch as Duc34. Finally, the third cluster, *Nitrospira*-like bacteria, comprised three sequenced clones from Duclair (Duc17, Duc26, and Duc31) but only one from Triel (T15), making up 23 and 6%, respectively, of the total analyzed clones. These *Nitrospira*-like sequences formed a separate branch within the *Nitrospira* cluster (Fig. 5).

DISCUSSION

The treated effluent discharged into the Seine River sharply increased the ammonium concentration in the river water. After dilution by the Oise River at km 110, the concentration slowly decreases down to the estuary, presumably oxidized by nitrifying bacteria. As indicated by the potential nitrifying activity, the transformation of ammonium into nitrate was highest in the freshwater section of the estuary (Fig. 2), by which point the population of nitrifying bacteria had built up a large biomass. This generally occurred in summer, at a high temper-

TABLE 1. Description of closest relatives of Duclair and Triel clones found in GenBank database

Clone	Closest relative sequence	% of similarity	Environment	Reference
Duc1	Uncultured AOB B2-3 (AF293067) B2-1 (AF293065)	87	Biofilm	21
Duc2	Uncultured AOB BF1-2 (AF272483) SBBR1-5 (AF272426)	98	WWTP	38
Duc14	Uncultured AOB BF1-2 (AF272483) SBBR1-5 (AF272426)	98	WWTP	38
Duc17	Uncultured AOB amoA17 (AF272507)	97	WWTP	38
Duc26	Uncultured AOB amoA17 (AF272507)	97	WWTP	38
Duc27	Uncultured AOB PoII-5 (AF272474)	99	WWTP	38
Duc31	Uncultured AOB amoA17 (AF272507)	97	WWTP	38
Duc32	Uncultured AOB M-20 (AF420299)	99	WWTP	13
Duc34	Uncultured AOB GLII-9 (AF272472)	98	Activated sludge	38
Duc47	<i>N. nitrosa</i> (AF272404)	97	Activated sludge	38
T15	Uncultured AOB amoA17 (AF272507)	96	WWTP	38
T21	Uncultured AOB IA-32 (AF272442)	97	WWTP	38
T22	<i>Nitrosomonas</i> sp. strain Nm47 (AY123830)	88	WWTP	38a
T27	Uncultured AOB pMpC.1 (AJ388579)	97	Freshwater	52
T38	Uncultured AOB PoII-8 (AF272475)	97	WWTP	38
T42	<i>Nitrosomonas</i> sp. strain Nm47 (AY123830)	88	WWTP	38a
T45	<i>Nitrosomonas</i> sp. strain Nm47 (AY123830)	87	WWTP	38a
T47	Uncultured AOB pDtC.1 (AJ388575)	97	Freshwater	52
T48	Uncultured AOB M-20 (AF420299)	98	WWTP	13

ature, when the water residence time was sufficiently long (8, 19).

Summer oxygen depletion in the fluvial part of the Seine estuary is a constant feature of the ecological functioning of the system (Fig. 2); the oxygen budget established for the whole section investigated here showed that nitrification was a major controlling process, probably typical of large river systems strongly impacted by domestic and industrial effluents (19).

Even after treatment, domestic effluents are sources not only of chemical compounds, but also of microorganisms that can be active in the river, and they may play a fundamental role in the ecological functioning of the system (18, 22). Heterotrophic bacteria deplete the oxygen immediately downstream from the input of the effluent (17, 18), whereas the nitrifying bacteria deplete it far downstream in the upper estuary. However, whereas heterotrophic bacteria are massively supplied by the effluent (18), the input of nitrifying bacteria is much weaker (8), which is corroborated by the molecular approaches originally used in the Seine River study. The DNAs of ammonia and nitrite oxidizers were detected even in wastewater at the heads of effluent collectors (20). The presence of nitrifying bacteria in the domestic wastewater suggests that the biofilm

covering the walls of the collectors favors the growth of nitrifying bacteria. It has been reported that *Nitrosomonas* strains can survive in low oxygen concentrations, and they have been observed to constitute about one-fourth of the microbial biomass in an anoxic trickling filter biofilm (44).

Despite the fact that allochthonous heterotrophic bacteria supplied by the effluent were active in the river immediately downstream of the effluent input, they have been shown to disappear rapidly (17, 18). In contrast to observations of the heterotrophs, the Achères effluent was previously seen as seeding the system with nitrifying bacteria, and the allochthonous populations were thought to grow along the aquatic continuum, depending on the residence time of the system, and to build up a large biomass in the estuary (8). The molecular approach has led to a change in this view.

Indeed, the observed decrease in the numbers of *amoA* gene copies immediately after the WWTP input could be explained by the fact that the nitrifying bacteria supplied by the WWTP disappeared from the river water because they were less competitive than the autochthonous bacteria. The total number of *amoA* gene copies remained low until Poses (km 202), the entrance of the freshwater estuary, where conditions seemed to allow an AOB population, possibly different from the one

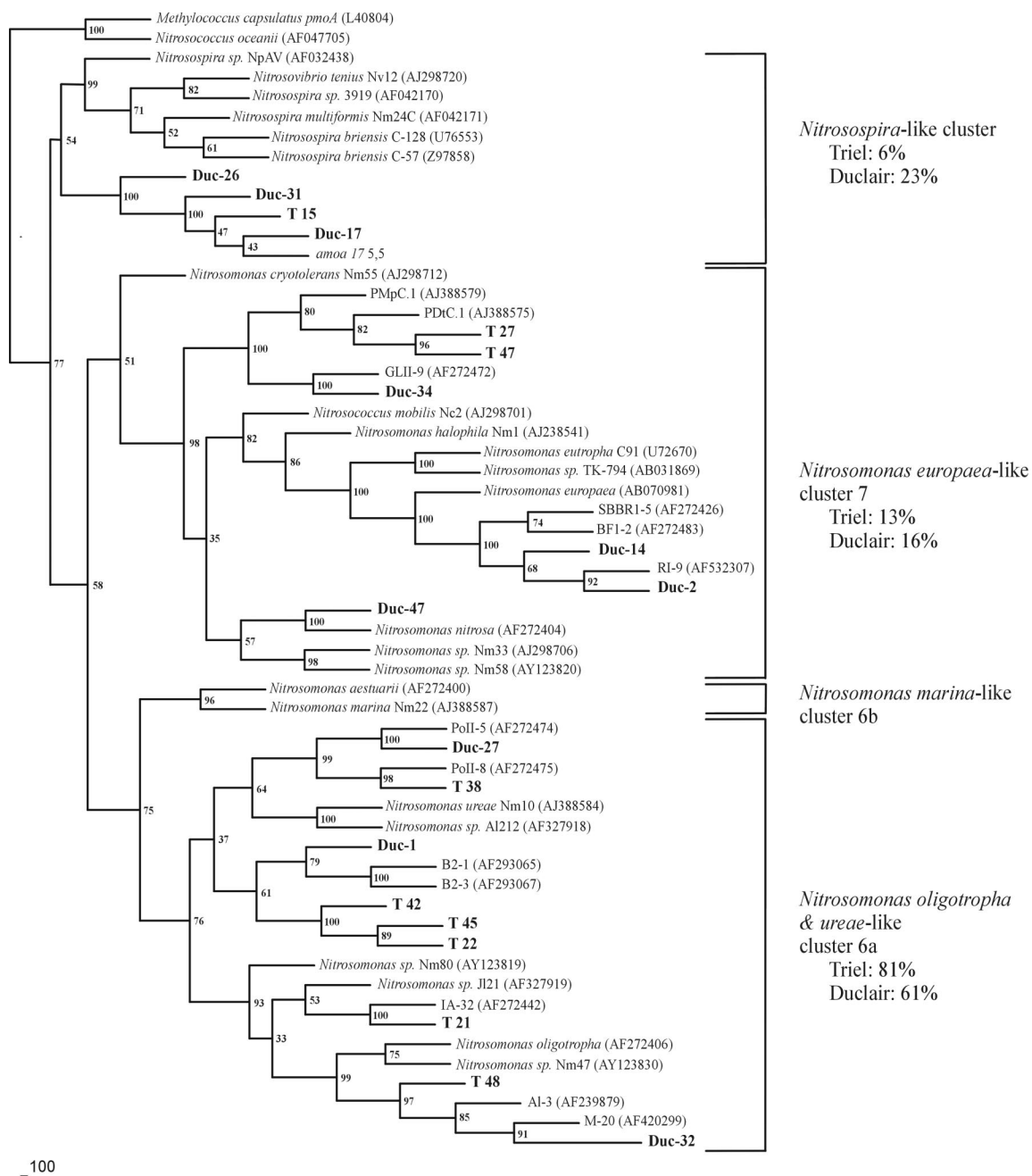


FIG. 5. Fitch-Margoliash tree of β subgroup *Proteobacteria amoA* sequences. The clones obtained in this work (in boldface type) are positioned in relation to cultured or environmental ammonia oxidizers (references are cited in Table 1). The tree was rooted with the *pmoA* sequence of *Methylococcus capsulatus* and the *amoA* sequence of *Nitrosococcus oceanus*, an AOB of the γ subclass of the *Proteobacteria*. The tree scale is in the lower left corner.

from Achères, to build up a large biomass. The number of *amoA* gene copies increased sharply concomitantly with the potential ammonia-oxidizing activity (pAOA) (Fig. 3b), with a peak at the Bouille-Duclair stations and a sudden decrease at the saline intrusion limit. In the salinity gradient, the pAOA increased again, but not the number of β -*amoA* gene copies, which suggests a shift from the freshwater AOB community to another that is better adapted to the saline environment and that was not targeted in this study. In the saline estuarine

sector, halophilic populations, probably belonging to the γ subclass of *Proteobacteria*, might be found.

As for the NOB, the lack of any increase downstream from the Achères WWTP might be due to the limitation of our research to the genus *Nitrobacter* (see Materials and Methods). According to the literature, the NOB of the WWTP belong mainly to the genus *Nitrospira* (13, 27), but they were not investigated here. Note that although the potential nitrite-oxidizing activity was very weak, the performance of the mo-

lecular biology tools still allowed us to detect and quantify the presence of *Nitrobacter* as a representative of this functional group of NOB. The genus *Nitrobacter* is detected in various environments (10, 43, 45). Our results clearly show that *Nitrobacter* cells were able to grow in the Seine River, their abundance increasing noticeably together with the potential nitrifying activity, reaching a maximum at the Duclair station in the estuary. According to the specific-activity values for various species of *Nitrobacter* (from 5 to 42 fmol of NO_3^- produced $\text{cell}^{-1} \text{h}^{-1}$, according to Prosser [37]) and to the estimate of *Nitrobacter* abundance, assuming one operon per cell (12), rough calculations show that *Nitrobacter* might be responsible for the whole nitrite oxidation step in the Seine River. However, the presence of other NOB cannot be excluded, especially in the area directly impacted by the wastewater effluent. As several articles have recently reported a dominance of *Nitrospira* spp. in freshwater systems, activated sludge, and nitrifying fluidized bed reactors (24, 25, 27, 46, 61), further investigations into the NOB of the lower Seine River will be needed. On the basis of their different K_m values, which are lower for *Nitrospira* than for *Nitrobacter* (15, 45), low nitrite concentrations would allow a *Nitrospira* population to dominate the nitrite-oxidizing communities in many environments.

The molecular approach based on restriction fragment length polymorphism that was used to analyze the diversity of the ammonia-oxidizing community led to the identification of 10 different restriction patterns for 100 analyzed clones; this is a relatively high number compared to the four groups obtained from the analysis of *amoA* sequences retrieved from heavy-metal-contaminated soils (55) and continuous culture enrichments (5). This result may indicate a greater diversity of ammonia oxidizers in waters polluted by domestic effluent, such as the lower Seine River. The different lineages in which the sequenced clones were found corresponded to the different restriction pattern groups, except for the clones Duc2, Duc14, and T21, which belonged to the same restriction group but were found in two different phylogenetic clusters. However, all the restriction patterns have been confirmed by the positions of the *MspI* enzyme sites found on the nucleic acid sequences.

The majority of the clones (from Triel and Duclair) have sequences closely related to those of bacteria commonly detected in WWTPs (Table 1) (38). However, this result could be biased, as WWTPs, together with soils, are mostly studied from the point of view of AOB diversity; thus, the majority of sequences available in databases are taken from these two types of environments. Due to the scarcity of studies of nitrifying populations in freshwater systems, we cannot conclude that all the clones originate from the WWTP effluent. As a consequence, the nitrifying community at Triel would represent a mixture of allochthonous bacteria from the Achères WWTP and autochthonous bacteria from the river upstream, whereas the population at Duclair would be the result of growth, between Paris and the estuary, of bacteria that have become adapted to their environment.

The proportions of the analyzed clones must be considered only as indicative, because the phylogenetic study has qualitative rather than quantitative value. We know that the various AOB species can have from one to three *amoA* genes with the same sequence resulting from a duplication phenomenon and not from interspecies transfer (32). *Nitrosomonas* strains usu-

ally carry two gene copies, whereas most *Nitrosospira* strains carry three (28, 32). Therefore, more than one clone might have been isolated from the same bacterium, so the proportions of the different bacterial groups cannot be extrapolated to the environmental populations.

Among the three identified clusters of β -AOB, the proportions of analyzed clones present in the *N. europaea*-like cluster were about the same for the two sites (13 and 16%, respectively, at the Triel and Duclair stations), whereas the 6a cluster (*N. oligotropha*-*N. ureae*) and the *Nitrosospira*-like cluster show noticeable differences between the two stations (81 and 60% for the former and 6 and 23% for the latter at Triel and Duclair, respectively).

The *N. ureae*-*N. oligotropha*-like cluster, to which the majority of the sequenced clones were closely affiliated, has also been found to be the predominant group of ammonia oxidizers in the estuary of the Elbe River (53) and in the freshwater part of the Schelde estuary (11). Bacteria showing affinity with the *Nitrosomonas* cluster 6a (closely affiliated with the cultured species *N. urea* and *N. oligotropha*) have previously been found to be the dominant AOB in soil, freshwater, and freshwater sediment (34, 52, 54) due to input of AOB from untreated wastewater (13, 38, 42). This cluster, well adapted to low ammonium concentrations, can grow in various environments (5, 6). Moreover, isolates of this lineage (e.g., *N. oligotropha*, *N. ureae*, and *Nitrosomonas* sp. strains JL21 and AL212) are inhibited at high ammonium concentrations (exceeding 10 mM) (5, 9, 29, 39, 53, 57). Usually, these bacteria have a high affinity for ammonium, with K_m values ranging from 0.075 to 0.03 mM NH_4^+ (53). The conditions in the Seine River water, therefore, appear to favor their growth, i.e., to have high ammonium concentrations compared to other freshwater systems (0.128 mM N-NH_4^+ downstream from the WWTP, 0.09 mM N-NH_4^+ at Triel, and 3.8 μM N-NH_4^+ at Duclair) but lower concentrations than those (10 mM or more) found in reactors, cultures, or WWTPs.

The *N. europaea*-like clones (cluster 7) were present at both sites on the Seine River, but in small proportions. Due to its low substrate affinities (high K_m values, ranging from 0.4 to 7 mM NH_4^+) (30, 37, 53), this cluster needs high ammonium concentrations for its growth (6, 9, 36). The concentrations found in the Seine River would therefore not favor it. A small proportion (6%) of *Nitrosospira*-like bacteria were found at the Triel station, while at Duclair they represented 23% of the analyzed clones. Like the *Nitrosomonas* cluster 6a, *Nitrosospira* bacteria, based on their low K_m values (40 μM NH_4^+) (39, 47), would be more competitive in an environment with a low ammonium concentration, for example, in the downstream part of the river, where most of the ammonium has been oxidized.

In agreement with Bollmann et al. (6), we suggest that *N. europaea* and other members of *Nitrosomonas* cluster 7 could be classified as r strategists in accordance with their need for a substrate-rich environment (ammonium in this case) and their ability to colonize various environments. On the other hand, *Nitrosomonas* cluster 6a and *Nitrosospira*-like bacteria would both represent K strategists, as defined by their high affinity with the resources required for their growth and their demand for a selective environment.

This study combined a functional approach (i.e., nitrifying

activities) with molecular tools to analyze the nitrifying bacterial population by quantifying the functional groups of both AOB and NOB and by determining the phylogenetic composition of ammonia oxidizers at two key points in the Seine River. The quantitative approach to estimating the nitrifying populations clearly fit the spatial distribution obtained from the potential activity and, furthermore, allowed us to estimate the numbers of *amoA* gene copies and *Nitrobacter* cells in a range from $1 \cdot 10^5$ to $7 \cdot 10^7$ cells liter⁻¹ compared to the 10^9 cells liter⁻¹ commonly found for whole populations of bacteria. These results are fundamental to quantitative ecological studies (see Billen et al. [4] and references therein). Moreover, the studies of community composition tend to modify our previous view of nitrifier dynamics in this sector that is strongly impacted by domestic effluent; nitrifying bacteria originating from the effluents and disappearing in the river would not represent seeding of the Seine River. However, more studies are needed to fully understand the role of allochthonous versus autochthonous nitrifying bacteria.

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