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Nitrobacter and *Nitrospira* genera as representatives of nitrite-oxidizing bacteria: Detection, quantification and growth along the lower Seine River (France)

Aurélie Cébron[1], Josette Garnier*

UMR Sisyphe 7619, Université P. et M. Curie, Paris 6, BP 105, Tour 56-55, Étage 4, 4 place Jussieu, 75005 Paris, France

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Abstract

Pollution from agriculture and urban effluents influences the ecology and biochemical functioning of the Seine River. Nitrification dominates nitrogen transformations downstream of the effluents of the Paris wastewater treatment plant (WWTP) at Achères, treating, by activated sludge the wastewater of 6.5 million inhabitant equivalents from Paris and its suburbs, without nitrification and denitrification treatment. It discharges effluents containing large amounts of nitrogen, ammonium mostly ($\sim$30 mg L^{-1} N-NH_4^+ L^{-1}), on average 45 mg L^{-1} of suspended particulate matter, high quantities of total organic carbon ($\sim$30 mg C L^{-1}) largely biodegradable (40%), and high concentration in total phosphorus ($\sim$3 mg Tot P L^{-1}), as well as microorganisms. Ammonium, brought into the river system, is slowly nitrified in the lower Seine River and especially in the freshwater estuary. The nitrifying activities can be observed by measuring inorganic nitrogen compound concentrations and potential activities. To understand the contributions of the WWTP effluents, the upstream agricultural runoff water and the Seine tributaries, it is useful to investigate the bacterial community. Whereas ammonia oxidation has been widely studied, the second step, i.e. nitrite oxidation, is less well understood. We have previously analysed the ammonium-oxidizing bacterial (AOB) community in the Seine (Cébron, A., Berthe, T., Garnier, J., 2003. Nitrification and nitrifying bacteria in the lower Seine River and estuary (France). Appl. Environ. Microbiol. 69, 7091–7100; Cébron, A., Coci, M., Garnier, J., Laanbroek, H.J., 2004. DGGE analysis of the ammonia oxidizing bacterial community structure in the lower Seine River: impact of the Paris wastewater effluents. Appl. Environ. Microbiol. 70, 6726–6737), and focus here on the composition of the nitrite-oxidizing bacterial (NOB) community. As no general molecular probe targeting all known NOBs is currently available, we chose to target and quantify (by competitive PCR) the two genera *Nitrobacter* and *Nitrospira* assumed to be the major players in nitrite oxidation in freshwater environments. *Nitrobacter* species were dominant in the upstream Seine River basin but *Nitrospira* was the dominant NOB downstream of the WWTP. These two genera were equally represented in WWTP effluents. In the Seine River estuary, especially in the salinity gradient, the *Nitrobacter* proportion increases and that of *Nitrospira* disappears, possibly due dilution by seawater.

Keywords: Nitrification; Nitrite-oxidizing bacteria; *Nitrobacter*; *Nitrospira*; River water

*Corresponding author. Tel.: +33 1 44 27 70 27; fax: +33 1 44 27 45 88.
E-mail address: josette.garnier@ccr.jussieu.fr (J. Garnier).
[1]Present address: Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK.

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1. Introduction

The Seine River is heavily impacted by the input of effluents from the Achères wastewater treatment plant (WWTP), immediately downstream Paris (France). This plant treats about 2 millions cubic meter of wastewater from 6.5 million inhabitant equivalents by an activated sludge process and its effluents contain large quantities of nutrients (phosphorus, ammonium), organic matter and bacteria. Ammonium is gradually oxidized to nitrate via the nitrite along the lower Seine River continuum down to the estuary. Previous studies have discussed the distribution of nitrifying activity along this transect; a peak in potential nitrifying activity (pNA) is invariably observed in the upper freshwater estuary together with a minimum oxygen concentration (Brion et al., 2000; Garnier et al., 2001; Cébron et al., 2003).

Nitrification is a two-step process performed by two different functional bacterial groups. The oxidation of ammonia into nitrite by ammonia-oxidizing bacteria (AOB) has been extensively studied in the lower Seine River (Cébron et al., 2003, 2004). As the AOB present in freshwater environments belong to a monophyletic group from the β subclass of Proteobacteria, it is possible to investigate the whole ammonia oxidizing population with molecular tools by targeting part of the ammonium monooxygenase (*amoA*) gene (Rotthauwe et al., 1997). However, the nitrite oxidizing bacteria, which are responsible for the second step in the nitrification process (oxidation of nitrite into nitrate), have been less studied. The genus *Nitrobacter* was previously thought to be the main nitrite oxidizer (Bock and Koops, 1992). However, a variety of recently developed techniques made it possible to explore the composition of the nitrite-oxidizing community in the environment and to enhance our knowledge of its functioning (Degrange and Bardin, 1995; Aamand et al., 1996; Wagner et al., 1996; Berthe et al., 1999; Bartosch et al., 1999; Daims et al., 2001; Dionisi et al., 2002; Maron et al., 2003).

All isolated chemolithoautotrophic nitrite-oxidizing bacteria (NOB) belong to one of the following four genera: *Nitrobacter* (α subclass of Proteobacteria), *Nitrococcus* (γ subclass of Proteobacteria), *Nitrospina* (δ subclass of Proteobacteria) and *Nitrospira* (distinct phylum) (Bock and Koops, 1992). *Nitrobacter* strains are ubiquitous in nature and have been found in several environments including soil, freshwater and sewage sludge (Chartrain et al., 1983; Bock et al., 1990). They can be detected by targeting a specific fragment of 16S rDNA (Degrange and Bardin, 1995). As opposed to the genus *Nitrobacter*, it was assumed until recently that the other three genera were confined to marine environments (Bock and Koops, 1992). Only two *Nitrospira* species *N. marina* and *N. moscoviensis,* are available in pure culture. However, bacteria related to the genus *Nitrospira* (uncultured cloned sequences) were also found to occur in other habitats: *Nitrospira* have been detected by immunological techniques in various soils (Bartosch et al., 2002) and numerous related bacterial 16S rDNA sequences have been obtained from nitrifying bioreactors and biofilms (Burrell et al., 1998; Juretschko et al., 1998; Schramm et al., 1998; Gieseke et al., 2001), WWTPs (Daims et al., 2001), freshwater aquaria (Hovanec et al., 1998) and groundwater contaminated with livestock wastewater (Cho and Kim, 2000). Overall, the *Nitrobacter* and *Nitrospira* genera are widely distributed in nature and both of them might significantly contribute to global nitrite oxidation.

In this study, the aim was to investigate the distribution and quantify the proportion of *Nitrobacter* and *Nitrospira* genera along the lower Seine River continuum, strongly impacted by wastewater effluents. We used competitive PCR specific to these two genera, and determined their activity in order to identify the sources of NOB, as already done for the ammonia oxidizing bacteria in the lower Seine system (Cébron et al., 2003, 2004). A further objective was to better understand the factors regulating nitrite-oxidizing populations in the Seine River, from its headwaters to the estuary, as an example of a large river strongly impacted by domestic pollution. The inclusion of samples from the WWTP effluent and the two major tributaries of the lower Seine River also made it possible to compare the contribution of the effluent and that of the lateral inputs with the initial upstream conditions.

2. Materials and methods

2.1. Description of the study site

A 356 km transect of the Seine was studied from upstream of the city of Paris (St. Maurice at the outlet of the Marne River, set at 0 km), to the mouth of the estuary (Honfleur, 356 km), in which the weir at Poses (202 km) represents the physical limit between the riverine and estuarine sections (Fig. 1). There is a salinity gradient between Caudebec (310 km) and Honfleur (356 km), which is influenced by both tides and river flows.

The lower Seine River and estuary receive the pollution from the whole drainage basin, and is greatly impacted by industrial activity, intensive agriculture and a population density concentrated in the Paris region (Fig. 1). Domestic effluents from 6.5 million inhabitant equivalents of Paris and its suburbs undergo a secondary activated sludge treatment in the WWTP of Achères, the remaining Parisian effluents (around 2 million inhabitant equivalents) being treated in three other WWTPs and discharged upstream Achères WWTP. Raw waters are submitted to (i) a pre-treatment (elimination of

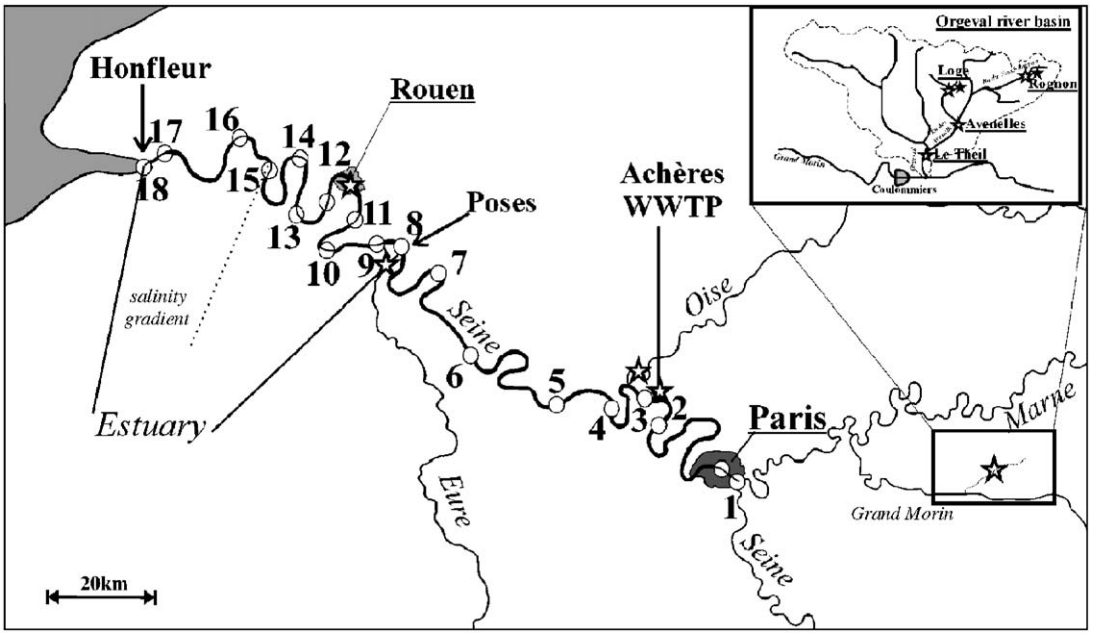

Fig. 1. The lower Seine River continuum and tributaries. The numbers (1–18) represent the sampling stations (1: Saint Maurice, 0 km; 2: Maison Laffitte, 48 km; 3: Conflans, 70 km; 4: Triel, 84 km; 5: Porcheville,101 km; 6: Vernon, 137 km; 7: Les Andelys, 173 km; 8: Poses, 202 km; 9: Pont Arche, 208 km; 10: Elbeuf, 219 km; 11: Oissel, 230 km; 12: Bassin des Docks, 251 km; 13: La Bouille, 260 km; 14: Duclair, 278 km; 15: Heurtauville, 298 km; 16: Caudebec, 310 km; 17: Tancarville, 337 km; 18: Honfleur, 356 km). The stars represent the sampling stations in the upstream basin of the Orgeval River (black stars represent soil samples at the Loge and Rognon stations), on the tributaries (Oise and Eure) and at the WWTPs (Rouen and Achères).

rubbish, large particles, oils, etc.), (ii) to a primary decantation, followed by (iii) an activated sludge biological treatment for organic pollution abatement. Solid retention time is about 2–5 days depending on the temperature, whereas the hydraulic retention time is about 2–5 h depending on the size of the basins. The treated, but ammonium-rich Achères effluents are discharged 70 km downstream from Paris.

2.2. Sample collection and treatments

Water samples were collected at 18 stations in July 2002, at 16 stations in September 2002 (except Les Andelys, st. 7 and Pont Arche, st. 9) and at 16 stations in September 2003 (except Vernon, st. 6 and Pont Arche, st. 9) along the Seine River from Paris (0 km, immediately upstream of Paris, at St. Maurice) to Honfleur (356 km, in the estuary) (Fig. 1); the sampling stations are indicated by the numbers 1–18. Samples of raw and treated water from the Achères WWTP were also collected (these samples are designated by Achères IN and OUT). Treated water from the Rouen WWTP, collected in July 2002, was also analysed. We collected some samples from upstream headwaters, not affected by effluents: the Orgeval River (a tributary to the Grand Morin River in the Marne River sub-basin) at the stations Le Theil, Avenelles, Rognon and Loge and two samples of soil from cultivated fields close to the stream (Fig. 1). Finally, we analysed samples from the Oise and Eure Rivers (main tributaries of the lower Seine River) from stations situated just upstream of their confluence with the Seine.

During the period from July to September, nitrifying activity reaches a maximum when water flows are low ($185–250\,m^3\,s^{-1}$) and temperatures high (18–21 °C) (Brion et al., 2000; Garnier et al., 2001; Cébron et al., 2003). Five to ten liters of water were collected and brought to the laboratory within 2–3 h for chemical, biochemical and molecular analyses.

In the laboratory, the water was filtered through glass-fibre membranes (Whatman, GF/F) and frozen until analyzed for inorganic nitrogen compounds. Ammonium, nitrite and nitrate were determined spectrophotometrically, ammonium and nitrite according to Slavyck and McIsaac (1972) and nitrate after Cd-reduction to nitrite (Rodier, 1984). The suspended matter was weighed on GF/F filters dried at 450 °C. For the molecular analyses, between 150 and 250 mL of water (depending on the amount of suspended particulate matter (SPM)) were filtered through 0.22 μm nitrocellulose filters (Durapore, 45 mm diameter) in triplicates and the filters were frozen (−20 °C) until the

DNA extraction. Soil samples were then storred at −20 °C.

2.3. Measurement of potential nitrifying activities and nitrite oxidation rates

The pNAs were determined by the difference in $H^{14}CO_3^-$ incorporation between the samples with and without specific nitrification inhibitors, incubated under optimal conditions, i.e. 7.5 mg O_2 L^{-1}, 20 °C and 2 mM NH_4Cl, (Brion and Billen, 1998). The inhibitors were allylthiourea (10 mg L^{-1}) and sodium chlorate (10 mM), which respectively inhibit the oxidation of ammonia and nitrite (Billen, 1976; Bianchi et al., 1994).

The potential nitrite oxidizing (pNO) activities were measured according to the methods described in Cébron et al. (2003). The pNO rates were based on measurements of the changes in nitrite concentration (consumption) in the samples incubated under optimal conditions (i.e. in the dark, under agitation to maintain high dissolved oxygen concentration, and with addition of nitrite as KNO_2, at a final concentration of 0.06 mM); allylthiourea (10 mg L^{-1}) was added to inhibit ammonium oxidation. These activities were measured in water samples (containing in situ SPM) except for the four upstream river samples where the activities were measured on a river water suspension of sediment (20 g L^{-1}, i.e. 1 g in the 50 mL "slurry incubation") because the nitrite oxidizing activities were not detectable in the river water sample. The pNO of the soil samples was made on 1 g of soil (dry weight determined independently by drying and weighing a soil sample) made into 50 mL of slurry with the river water. To be able to compare the potential activities from these various samples, we express the activity according to the particulate content (SPM), the volumetric potential activity values being divided by the amount of SPM.

2.4. DNA extraction and purification

DNA was extracted from the filters or from a 500 mg soil sample by a bead-beating method with the FastDNA spin Kit for soil (Bio 101, Lajolla, CA, USA) according to the manufacturer's instructions. The DNA extracts were then stored at −20 °C until purification on a Sephadex G-200 column and precipitation with ethanol. Nucleic acids were quantified by comparison between 1 μL of a non-diluted environmental DNA sample and a range of known DNA concentrations (various dilutions of SuperLadder, Eurogentec) on agarose gel coloured with ethidium bromide. To obtain suitable PCR amplicons, 10- to 100-fold dilutions of crude DNA (1 μL of 1–10 ng/μL dilutions) were used as templates for subsequent PCR reactions. The DNA was visualized by UV transillumination (Gel Doc 200, BioRad). Digital images of the gels were obtained with a CCD camera controlled by the software Quantity one (BioRad).

2.5. Quantification of nitrobacter and nitrospira by cPCR

Fragments (397-bp) of 16S rDNA gene specific to the *Nitrobacter* genera were amplified with the FGPS 872 and FGPS 1269′ primers (Degrange and Bardin, 1995). 16S rDNA primers NSR1113f and NSR1264r (Dionisi et al., 2002) were used to target *Nitrospira* genera and amplify a 151-bp fragment.

Estimates of the *Nitrobacter* and *Nitrospira* 16S rDNA gene copy number were made by competitive PCR (cPCR) as described by Berthe et al. (1999) and Dionisi et al. (2002), respectively. The 16S rDNA gene copy number should be equivalent to the bacteria cell number because *Nitrobacter* and *Nitrospira* species typically possess only one 16S ribosomal DNA operon (Navarro et al., 1992; Dionisi et al., 2002). The cPCR for Seine River *Nitrobacter* has already been described by Berthe et al. (1999) and Cébron et al. (2003). Regarding cPCR on *Nitrospira*, we constructed a competitor (119-bp fragment) and a DNA target template (151-bp fragment) as described by Dionisi et al. (2002) by cloning both PCR fragments in pGEM-T (Promega). Two of our clones: NSR-Comp-3 and NSR-Elb-3 were used as competitor and target plasmid DNA template, respectively.

PCR amplifications were carried out in a total volume of 50 μL in 0.2 mL tubes using a DNA-microcycler (BioRad), according to the thermal profiles described in Table 1. The reaction mixtures were prepared in a 1X

Table 1
PCR thermal cycle profiles for *Nitrobacter* and *Nitrospira*

Primers	Target specificity	PCR conditions	Reference
FGPS 872/1269′	*Nitrobacter* 16SrDNA specific fragment	10 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C, followed by 7 min final extension at 72 °C	Degrange and Bardin (1995)
NSR 1113F/1264R	*Nitmspira* 16S rDNA specific fragment	10 min at 95 °C. followed by 40 cycles of 30 s at 94 °C, 30 s at 65 °C and 30 s at 72 °C, followed by 15 min final extension at 72 °C	Dionisi et al. (2002)

buffer (75 mM Tris, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 1.5 mM $MgCl_2$, 1 ng of DNA and 0.5 U of Hot Start *Taq* DNA polymerase (HotGoldtar, Eurogentec). Negative (sterile milliQ water) and positive control tubes were simultaneously performed for all PCR reactions to avoid any false positive or false negative results. Aliquots of amplification products were analyzed by gel electrophoresis on 1.7% (wt/vol) agarose gels (Eurogentec) for *Nitrobacter* PCR products and 2.5% (wt/vol) small fragments agarose gels (Eurogentec) for *Nitrospira* PCR products. DNA band intensities were estimated with image analysis software (Quantity One, Bio-Rad).

3. Results and discussion

3.1. Variations in inorganic nitrogen

In the upstream Orgeval river basin (104 km^2), located in an agricultural region of the Marne tributary (Fig. 1), ammonium (NH_4^+) and nitrite concentrations (NO_2^-) were low and variable (0.05–0.25 mg N-NH_4^+ L^{-1} and 0.005–0.1 mg N-NO_2^- L^{-1}, respectively, Table 2); however, these upstream river waters contain high quantities of nitrate (8.0–11.2 mg N-NO_3^- L^{-1}), presumably resulting from the fertilization of agricultural soils; these values are even higher than those found in the lower Seine after nitrification of the ammonium. Note that at the Loge station, a lower nitrate concentration was found (3.7 mg N-NO_3^- L^{-1}), perhaps due to riparian denitrification (Billen and Garnier, 1999; Sebilo, 2003) and because less fertilizer is applied to this forested sector.

During the water transit from the upstream basin to the confluence of the Marne and Seine Rivers (station 1, Fig. 1), the ammonia and nitrite concentrations remained low (0.1–0.3 mg N-NH_4^+ L^{-1}, 0.02–0.07 mg N-NO_2^- L^{-1}, Fig. 2), and nitrate concentrations were in the range of 2.8–4.4 mg N-NO_3^- L^{-1} (Fig. 2), relatively low values that may be due both to riparian denitrification and to dilution by water containing less nitrate. Benthic denitrification in the river bed cannot be excluded in the lower Marne, strongly affected by the Paris urbanization and by effluents creating favourable conditions (anoxic organic-matter-rich sediment), as observed in the lower Seine (Garnier et al., in press).

The impact of the Achères WWTP effluents is characterized by a sudden increase in ammonium concentrations: from 0.1–0.3 to 3.9–6.0 mg N-NH_4^+ L^{-1} (Fig. 2). The treated Achères WWTP domestic effluents contained concentrations of ammonium of up to 33 mg N-NH_4^+ L^{-1}, similar to those of the raw water without tertiary treatment (Table 2). Regarding nitrate, lower values in treated than in raw water (3–3.5 against 11–16 mg N-NO_3^- L^{-1}) indicated denitrification during the treatment by an activated sludge process. The nitrate concentrations in the raw water, much higher than those in domestic tap water, seem to confirm that nitrification occurs in the sewage network upstream of the WWTP (Brion and Billen, 2000; Garnier et al., 2002).

The nitrifying activity in the lower Seine River, from Paris to the estuary, was demonstrated by the inorganic nitrogen behaviour along the lower Seine, i.e. an increase in nitrate concentrations from 3 to 6 N-NO_3^- L^{-1} simultaneous with the complete nitrification of the ammonium discharged by the effluents (Fig. 2). Ammonium, brought by the Achères WWTP effluents, is firstly diluted in the Seine River, Achères effluents representing at the effluent outlet, about 20–30% of the Seine River water flow in summer period (25 $m^3 s^{-1}$ against 80–130 $m^3 s^{-1}$ depending on the hydrological year). Downstream, the Seine River nutrient content is then diluted by the Oise river tributary with an additional summer water flow of about 40–60 $m^3 s^{-1}$. Along the downstream transect to the upstream estuary, the ammonium is then totally oxidized within a sector of 200–250 km for the three sampling dates (July 2002, September 2002 and September 2003, Fig. 2), the Eure river tributary having a low effect (a summer water flow of 20–30 $m^3 s^{-1}$, compared to the one of the Seine at their confluences, i.e. 150–220 $m^3 s^{-1}$). As nitrite is an intermediate product of both nitrification and denitrification, there are strong variations in nitrite concentrations between Paris and the estuary. A similar general pattern in space was observed on the three sampling dates: after an increase in nitrite concentration (from 0.05–0.1 to 0.3–0.42 mg N-NO_2^- L^{-1}) within a distance of 50 km after the effluent discharge, a high concentration persisted down to the freshwater estuary where a rapid decrease occurred (September 2002 and 2003, Fig. 2). Nitrate concentrations decreased rapidly in the estuary, between stations 17 and 18, due to seawater dilution, contrary to the ammonium and nitrite concentrations which decreased in the freshwater estuary (Fig. 2).

Some divergence from the general pattern was also observed, including differences in the variation amplitudes and the spatial distribution, depending on the combination of the water residence time and the growth rate of the organisms, and the amount of ammonium brought by the effluents. In July 2002, nitrite concentrations remained high from station 6 (140 km) to station 16 (311 km) and then fell to low values at the last two stations. In September 2002, nitrite was produced farther downstream (station 8) and concentrations remained below 0.3 mg N-NO_2^- L^{-1}, as nitrite was consumed in the freshwater estuary. Nitrite production and consumption were amplified in the conditions of low water flow in September 2003.

Table 2
Ammonium, nitrite and nitrate concentrations and potential nitrite oxidizing activities (pNO-L and pNO-SPM) for samples from the upstream Orgeval River basin, the WWTPs (Achères -IN and -OUT for raw and treated water, and Rouen WWTP) the two major Seine river tributaries (Eure and Oise) and three Seine river stations (St. Maurice, Conflans and Duclair)

Stations from upstream to downstream		NH_4^+ (mg N L^{-1})	NO_2^- (mg N L^{-1})	NO_3^- (mg N L^{-1})	pNO-L (μmol N L^{-1} h^{-1})	pNO-SPM (μmol N g SPM^{-1} h^{-1})
Upstream Orgeval River basin	Soil loge	ND	ND	ND	*0.56*	0.03
	Soil rognon	ND	ND	ND	*1.02*	0.05
	Loge	0.05	0.005	3.7	*1.72*	0.09
	Rognon	0.16	0.07	9.15	*2.63*	0.13
	Avenelles	0.25	0.10	11.2	*3.51*	0.18
	Theil	0.15	0.03	8.0	*2.96*	0.15
St. Maurice st. 1		0.18 ± 0.08	0.05 ± 0.026	3.50 ± 0.06	0.046 ± 0.06	4.44 ± 4.26
Achères IN-	July 2002	24.6	ND	ND	*	*
	Sept 2002	29.7	1.36	16.04	*	*
	Sept 2003	29.7	0.84	11.16	*	*
Achères OUT-	July 2002	21.5	0.24	3.12	0.20	7.65
	Sept 2002	33	0.28	2.99	0.25	7.17
	Sept 2003	33.6	0.2	3.55	0.40	5.01
Conflans st. 3		4.92 ± 1.05	0.16 ± 0.04	3.99 ± 0.33	0.126 ± 0.109	11.01 ± 10.95
Oise		0.311	0.047	3.16	0.29	20.2
Eure		0.008	0.03	6.83	0.32	12.8
Rouen WWTP		ND	0	7.1	ND	ND
Duclair st. 14		0.37 ± 0.46	0.25 ± 0.10	6.18 ± 0.44	0.247 ± 0.083	4.99 ± 2.19

The pNO for upstream Orgeval River basin, expressed by liter, are shown in italic because it is not water samples but soil or sediment slurries.
*: Erroneous values due to inhibitor presence in samples; ND: non-determined values.

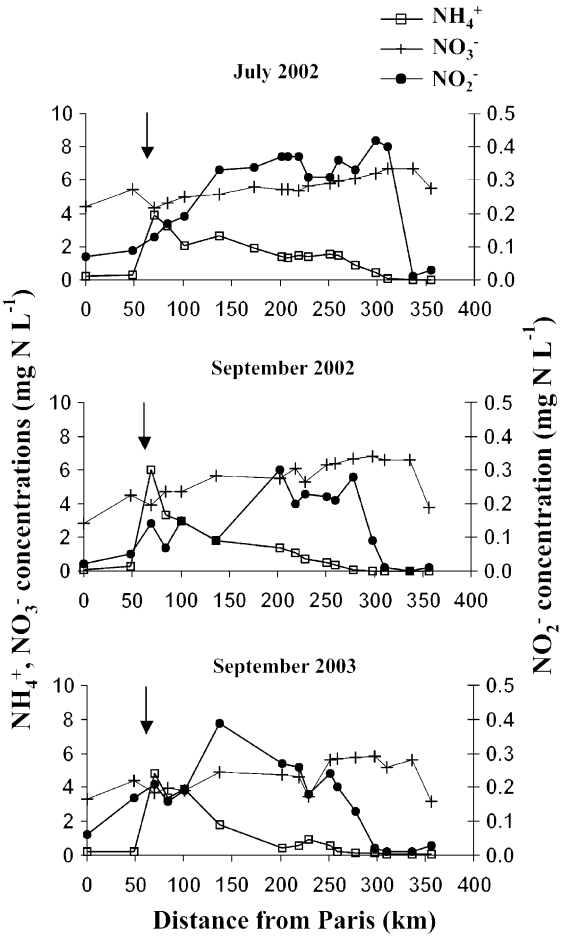

Fig. 2. Variations of the mineral nitrogen forms along the lower Seine River continuum for the sampling dates of July 2002, September 2002 and September 2003. The arrows represent the Achères WWTP discharge site. NH_4^+: ammonium in open square, NO_2^-: nitrite in black circle; NO_3^-: nitrate represented by crosses.

3.2. Potential nitrifying activities

pNO rates were calculated for the three sampling dates in the lower Seine River (Fig. 3), the head water samples, WWTP and tributaries samples (Table 2). The head water samples (Theil, Avenelles, Rognon and Loge) had comparatively high pNO activities when results are expressed by unit volume (Table 2). When data are expressed as SPM units (slurry incubations), the activities are lower by one to more than two orders of magnitude for head water samples than for the other samples (WWTP, tributaries and lower Seine River samples), and even lower than the values in the Seine upstream from the Achères WWTP effluent discharges (stations 1: 1–4 μmol N $gSPM^{-1}$ h^{-1}). Whereas the pNO activity of the raw WWTP water (-IN) is low and highly variable (Garnier et al., 2002), that of the treated water (-OUT) is high (5–7.65 μmol N $gSPM^{-1}$ h^{-1}). This result illustrates the role of the Achères effluents as a seeding source of nitrifying bacteria for the Seine River (Servais et al., 1999; Brion et al., 2000; Cébron et al., 2004; Garnier et al., 2002). Consequently, pNO and pNA (the pNAs measured with a ^{14}C method) increase in the Seine River downstream of the effluent output (Fig. 3). The high pNA in the WWTP effluents shows that the nitrifying bacteria could be active in the plant but optimal conditions would not be encountered for the nitrification to occur, because other groups of microorganisms (for example heterotrophic bacteria) can out compete the nitrifyers by consuming most of the oxygen faster. The hydraulic residence time in the Achères WWTP would also be too short to allow significant nitrification of ammonia to be detected in the plant.

In the lower Seine and freshwater estuary, the pNO and pNA patterns, i.e. a peak in the freshwater estuary, were similar on the three sampling dates, although the values in July 2002 were, approximately twice as high (Fig. 3). When pNO and pNA are expressed as SPM units, two maxima are observed, the first one just downstream of the effluent discharge (between stations 5 and 7, 100–170 km in July 2002, between stations 5 and 8, 100–200 km in September 2002 and immediately downstream of the WWTP input in September 2003), the second one in the upper estuary between stations 13 and 16 (260 and 310 km) (see also Brion et al., 2000; Cébron et al., 2003; Garnier et al., in press). In July 2002, the estuarine peak was the highest, while in September 2003 the maximum activities were observed immediately downstream of the effluent input, no clear pattern being observed in September 2002. These varied patterns, obtained by using volume or SPM units, show the propensity of nitrifying bacteria to attach themselves to particles. Nitrifying bacteria are probably brought to the receiving medium attached to aggregates and find favourable conditions for high activity in the estuary due to long water residence times and suitable summer temperatures (Brion et al., 2000). Similarly, the high nitrifying activities per SPM immediately downstream of the WWTP input in September 2003 may be explained by higher suspended matter concentrations during this dry summer, the proportion of the effluents discharged (containing SPM) increasing with the decreasing river flow.

3.3. Distribution of nitrite oxidizing bacterial populations: Nitrobacter and Nitrospira

Regarding the AOB, the major genus identified in the lower Seine River belonged to the lineage 6a represented by *Nitrosomonas oligotropha* and *ureae*-like bacteria belonging to the β subclass of the Proteobacteria

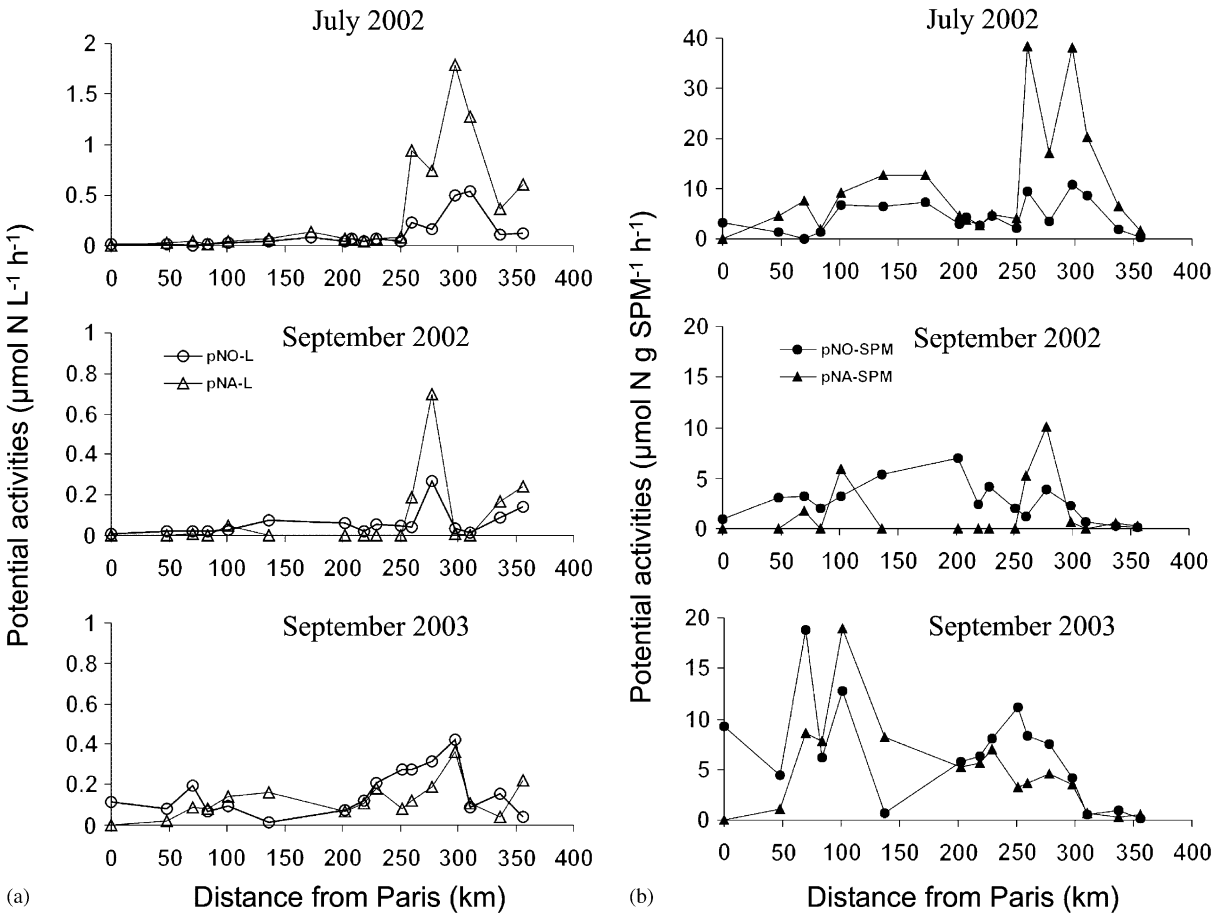

Fig. 3. Potential nitrite oxidizing rate (pNO) and potential nitrifying activity (pNA; ^{14}C method) along the lower Seine River continuum for the sampling dates of July 2002, September 2002 and September 2003. (a) pNA-L and pNO-L are expressed in $\mu mol\,N\,L^{-1}\,h^{-1}$; (b) pNA-SPM and pNO-SMP are expressed in $\mu mol\,N\,g\,SPM^{-1}\,h^{-1}$. SPM: suspended particulate matter.

(Cébron et al., 2003, 2004). These species, originating both from the upstream river and the WWTP effluents, can be considered as autochthonous bacteria, whose growth is enhanced by ammonia from the Achères WWTP, but also as allochthonous seeding bacteria subsisting and developing in the receiving medium as far as the estuary.

As opposed to the approach used for the bulk of AOB, targeting *amoA*-gene fragments (Rotthauwe et al., 1997), we targeted a fragment of the 16S rDNA gene, specific for both NOB genus detection (*Nitrobacter*: Degrange and Bardin, 1995; *Nitrospira*: Dionisi et al., 2002). These two NOB genera were specifically quantified by competitive PCR (Zacchar et al., 1993; Berthe et al., 1999; Dionisi et al., 2002). *Nitrobacter* and *Nitrospira* are the main representatives of the NOB community taking into account that (i) *Nitrobacter* is a genus known to dominate soil systems (Degrange and Bardin, 1995; Degrange et al., 1998; Grundmann and Normand, 2000), and consequently in hydrosystems through soil erosion and leaching and ii) the genus *Nitrospira*, is often found in wastewater (Dionisi et al., 2002; Regan et al., 2003).

The competitor DNA sequence is always chosen to be as close as possible to environmental target sequences with a small difference in unit length ($\pm 10\%$). The two DNA are co-amplified, to avoid any difference among the test tubes, temperature variations, etc.; if inhibitors are present in the environmental DNA, both competitor and environmental DNA amplification are impacted in the same way. Our competitor sequence had been cloned in a plasmid as described by Stephen et al. (1999) that avoid the difference in amplification efficiency between competitor and environmental DNA. An efficiency evaluation test of the cPCR method targetting *amoA* gene was rigorously realised by Bjerrum et al. (2002), further supporting the high sensitivity of the method. For example, Bjerrum et al. (2002) were able to detect

only 30 copies of amoA, that correspond to about 1000 cells g soil^{-1}.

In the upstream Orgeval River, as was done for the activity measurements, the NOB were quantified in suspended sediment (slurry) because we did not detect any nitrifying bacteria in the water samples. The *Nitrobacter* and *Nitrospira* 16S rDNA gene copy number increased from the smallest order 1 stream (Loge, Rognon) to higher order streams (2: Avenelles; 3: Le Theil) (Table 3). Within 8 km *Nitrobacter* increased 10 times, from 1.0–1.9 $\times 10^7$ to 1.7 $\times 10^8$ 16S rDNA gene copies.g SPM^{-1} but less than *Nitrospira* which increased by a factor of 20, from 4.1–4.4 $\times 10^6$ to 9.6 $\times 10^7$ 16S rDNA gene copies g SPM^{-1}. In all these samples *Nitrospira* were about 2–4 times less abundant than *Nitrobacter* and even less in soil samples where the abundance of *Nitrospira* was 200–500 times lower than that of *Nitrobacter*, supporting the general idea that soils are privileged habitats for *Nitrobacter* species and can be a seeding source of bacteria (Degrange et al., 1998; Grundmann and Normand, 2000). Furthermore, examining the results expressed in NOB number per total DNA quantity extracted from the samples, *Nitrospira* appeared to be weakly represented in the soil microflora (10–30 16S rDNA gene copies per ng of total DNA), while *Nitrobacter* show a strong presence (1.4–3.5.10^4 16S rDNA gene copies per ng of total DNA).

In the treated Achères WWTP effluents, both nitrite oxidizing bacterial genera had a high concentration (1.25–3.8 $\times 10^{10}$ *Nitrobacter* 16S rDNA gene copies g SPM^{-1} and 0.25–3.4 $\times 10^{10}$ *Nitrospira* 16S rDNA gene copies.g SPM^{-1}), revealing that the effluents of the Achères WWTP can be a seeding source of NOB, as they were shown to be a source of AOB (Cébron et al., 2004). These results differ from those found in recent literature, in which *Nitrospira*-like bacteria were the dominant nitrite oxidizers both in most full-scale WWTPs and in laboratory-scale reactors (Wagner et al., 1996; Juretschko et al., 1998; Schramm et al., 1998; Okabe et al., 1999). High serotype diversity and abundance of *Nitrobacter* (10^6–10^8 cells mL^{-1}) were also found in WWTP effluents (Montuelle et al., 1996). *Nitrobacter* genera able to grow heterotrophically can be favoured by WWTP organic matter-rich effluents, while the other known nitrite oxidizers, *Nitrospina*, *Nitrococcus* and *Nitrospira* would not (Ehrich et al., 1995). In the Seine River continuum, the proportion of *Nitrospira* increased immediately after the effluent outlet (Fig. 4 and 5), with *Nitrospira* spp. amounting to about 3–4 times the *Nitrobacter* spp. level down to the upstream

Table 3
Nitrobacter and *Nitrospira* 16S rDNA gene copy number for samples from the upstream Orgeval River basin, the WWTPs (Achères and Rouen), the two major Seine river tributaries (Eure and Oise) and three Seine river stations (St. Maurice, Conflans and Duclair) for comparison (see also Fig. 4a, b)

		Nitrobacte (16S rDNA gene copy number g SPM^{-1} or. g dry soil)	Nitrospira (16S rDNA gene copy number g SPM^{-1} or. g dry soil)	Nitrobacter (16S rDNA gene copy number ng DNA^{-1})	Nitrospira (16S rDNA gene copy number ng DNA^{-1})
Upstream Orgeval River basin	Soil loge	5.0×10^7	1.8×10^5	3.5×10^4	10
	Soil rognon	2.1×10^8	4.0×10^5	1.4×10^4	30
	Loge	1.0×10^7	4.1×10^6	4.2×10^3	6.5×10^2
	Rognon	1.9×10^7	4.4×10^6	2.5×10^3	5.7×10^2
	Avenelles	1.1×10^8	2.7×10^7	1.5×10^3	1×10^3
	Theil	1.7×10^8	9.6×10^7	9.8×10^3	5.6×10^3
St. Maurice st. 1		$3 \times 10^8 \pm 3.6 \times 10^8$	$1.4 \times 10^8 \pm 1.7 \times 10^8$	$1.2 \times 10^3 \pm 9.9 \times 10^2$	$1.3 \times 10^2 \pm 1.1 \times 10^2$
Achères IN-	July 2002	NDb	NDb	NDb	NDb
	Sept 2002	NDb	NDb	NDb	NDb
	Sept 2003	NDb	NDb	NDb	NDb
Achères OUT-	July 2002	2.5×10^{10}	3.4×10^{10}	6.5×10^3	8.9×10^3
	Sept 2002	3.8×10^{10}	2.5×10^9	8.7×10^3	5.8×10^3
	Sept 2003	1.25×10^{10}	6.4×10^9	1.1×10^4	5.8×10^3
Conflans st. 3		$6.7 \times 10^8 \pm 2.3 \times 10^8$	$8.3 \times 10^8 \pm 5.1 \times 10^8$	$3.7 \times 10^2 \pm 2.1 \times 10^2$	$5.5 \times 10^2 \pm 5.8 \times 10^2$
Eure		3.0×10^8	5.4×10^7	1.9×10^3	3.4×10^2
Oise		1.7×10^9	5.2×10^8	7.2×10^3	2.2×10^3
Rouen WWTP		—	—	7×10^3	2.1×10^3
Duclair st. 14		$4.1 \times 10^9 \pm 3.2 \times 10^9$	$2.1 \times 10^9 \pm 2.4 \times 10^9$	$2.4 \times 10^4 \pm 2.7 \times 10^4$	$7.8 \times 10^3 \pm 4.8 \times 10^3$

The values for the three Seine river stations are an average of the value from July 2002, September 2002 and september 2003 with the corresponding deviation values.
NDb: Not detected bacteria.

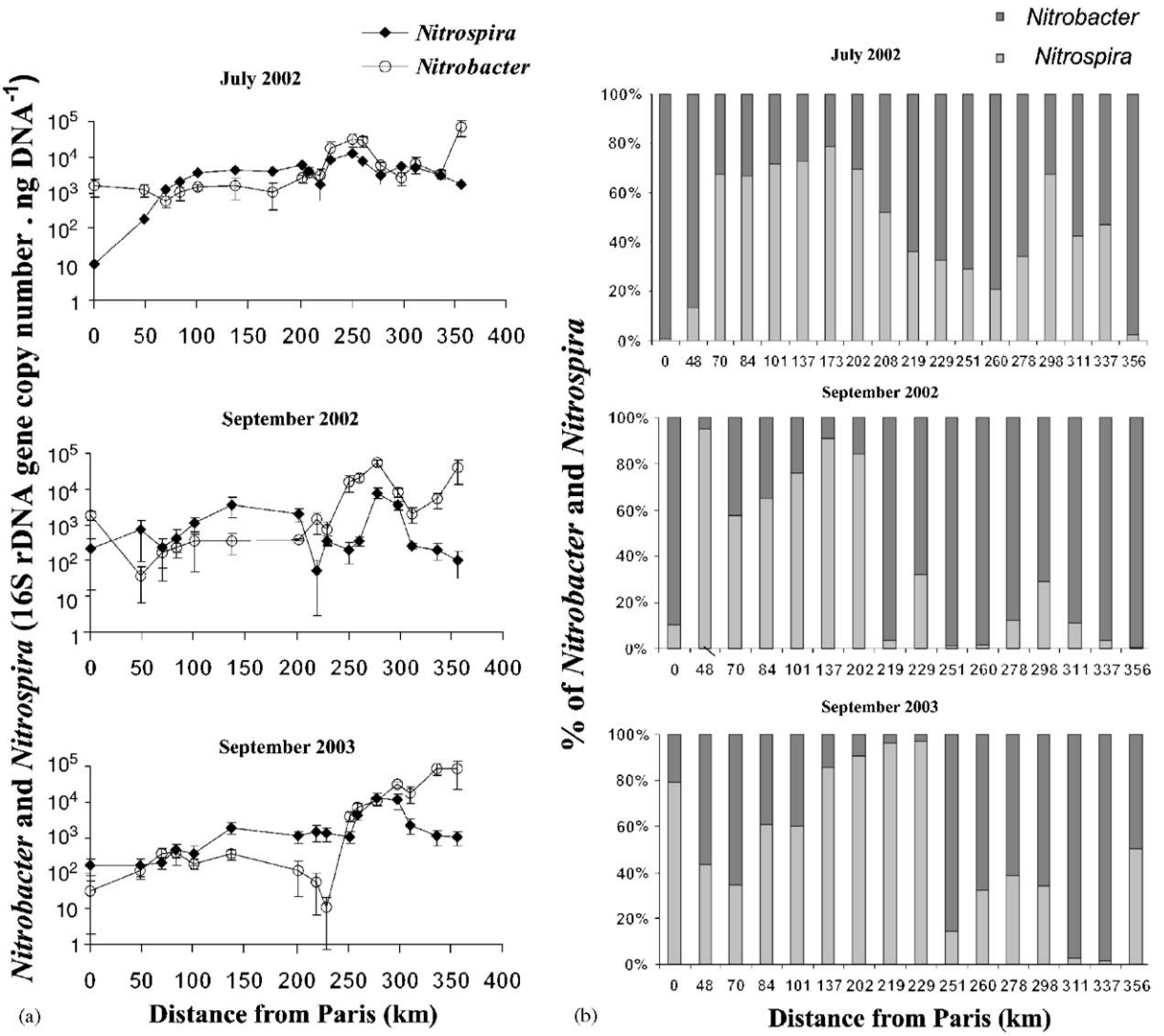

Fig. 4. Variations of the quantity of *Nitrobacter* and *Nitrospira* (16S rDNA gene copy number per DNA unit) along the lower Seine River continuum for the sampling dates of July 2002, September 2002 and September 2003. Results are expressed according to the quantity of DNA present in the PCR assays (a), and the variations of the proportion (%) of *Nitrobacter* and *Nitrospira* (b).

freshwater estuary (Fig. 4a and b). The predominance of *Nitrospira*-like species rather than *Nitrobacter* species responsible for oxidation of nitrite into nitrate have been reported from freshwater aquaria (Hovanec et al., 1998), nitrifying bioreactors and WWTPs (Burrell et al., 1998; Juretschko et al., 1998; Schramm et al., 1998, 1999; Daims et al., 2001) but not yet for natural, albeit human-impacted, hydrosystems. In the upper estuary, whereas the two genera reach their maximum concentrations, the dominance shifted: *Nitrobacter* spp. became more concentrated than *Nitrospira* (Fig. 4a and b). In addition, *Nitrobacter* must have been present in marine waters, since no dilution effect was observed in the lower saline estuary as opposed to *Nitrospira* concentrations which decreased in the salinity gradient (Fig. 4a). The same general pattern was observed on the three sampling dates (Fig. 4). Interestingly, the two NOB genera do not exclude each other; both were present at all stations, alternately dominating (Fig. 4b).

It is noteworthy that along the lower Seine River continuum, the NOB (number of gene copies per liter) were 10–100 times more concentrated than the AOB (Fig. 5, see also Cébron et al., 2004), a result in agreement with previous studies showing that the number of NOB might be 3–30 times higher than that of AOB in sediment (Smorczewski and Schmidt, 1991;

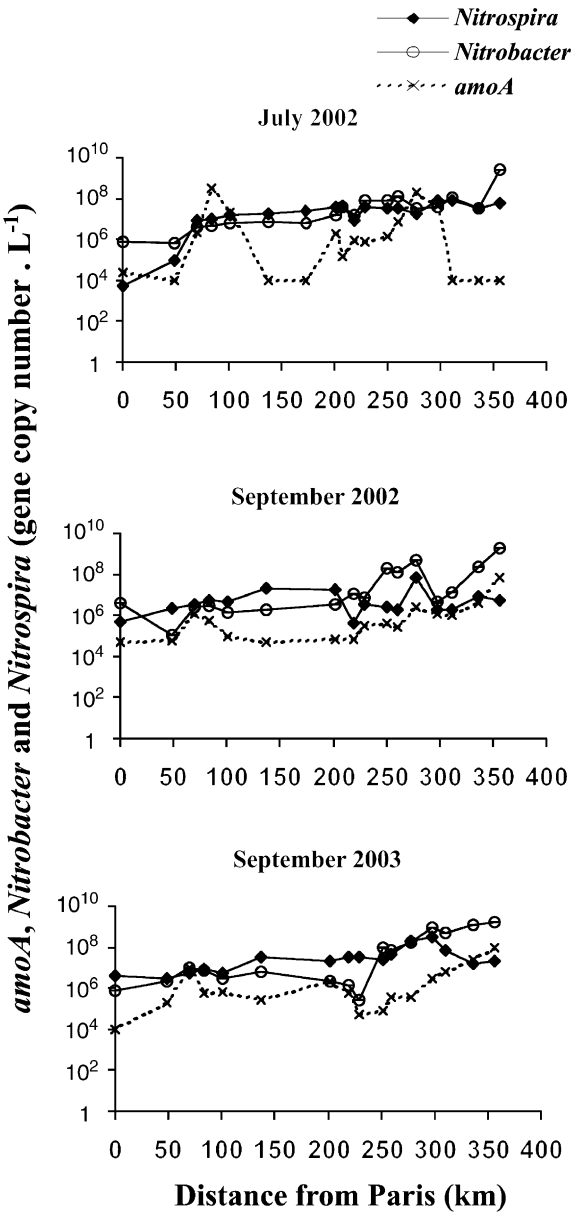

Fig. 5. Variations of 16SrDNA gene copy number of *Nitrobacter* and *Nitrospira* and *amoA* gene copy numbers per unit volume along the lower Seine River continuum for the sampling dates of July 2002, September 2002 and September 2003.

Altmann et al., 2003), nitrifying aggregates (Schramm et al., 1999) or biofilms (Gieseke et al., 2001). As NOB have weaker specific growth rates, more NOB than AOB are necessary to equilibrate the two steps of the nitrification process preventing a nitrite accumulation in the environment that may be toxic to aquatic life.

It has been suggested that *Nitrospira*-like nitrite oxidizers represent K-strategists with high substrate affinities and a low maximum growth rate adapted to low nitrite and oxygen concentrations. Conversely, *Nitrobacter* sp., as r-strategists, have low substrate affinity, high growth rate and develop large populations when nitrite and oxygen are present in high concentrations (Schramm et al., 1999; Gieseke et al., 2003). Consequently, a nitrite-weak environment may favour a *Nitrospira*-dominated NOB community, i.e. a Km of 0.01 mM nitrite (0.14 mg $N\text{-}NO_2^-.L^{-1}$), (Schramm et al., 1999). Such Km estimates are between 1 and 2 orders of magnitude lower than those reported for *Nitrobacter* strains (Keen and Prosser, 1987; Painter, 1970; Yoshioka et al., 1982). For example, *Nitrobacter* tolerated high nitrite concentrations in an experimental medium (>1 g $NaNO_2\,L^{-1}$, 14 mM) (Bock and Koops, 1992), with a preference for concentrations higher than 0.5 mM, whereas *Nitrospira* strains were inhibited in such conditions. Note however that *Nitrospira* and *Nitrobacter* grew simultaneously at nitrite concentrations of 0.2 g of $NaNO_2\,L^{-1}$ (3 mM), but *Nitrobacter* alone grew in cultures enriched with 2 g of $NaNO_2\,L^{-1}$ (28 mM) (Bartosch et al., 2002). In order to demonstrate a competitive advantage of *Nitrospira* vs. *Nitrobacter*, further physiological information on *Nitrospira*-like bacteria is needed. Moreover, organic carbon is able to support the growth of a majority of the *Nitrobacter* strains through a mixotrophic or even heterotrophic pathway, but their efficiency in using organic carbon as an energy substrate varies strongly (Bock et al., 1990); thus, *Nitrobacter* strains can have varying capacities in using organic carbon allowing them to coexist in a soil (Josserand and Cleyet-Marel, 1979; Both et al., 1992).

In the Seine River, nitrite concentrations were always below the inhibiting concentration level for *Nitrospira* (0.2–0.4 mgN L^{-1}, 0.014–0.028 mM) which may explain their steady state in the first 130 km downstream of the Achères effluents. Their rapid reactivity, possibly due to the sustained ribosome content during bacterial inactivity, might explain why *Nitrospira* dominated over *Nitrobacter* in this sector. Contrary to observations on cultures, *Nitrobacter* species appear to be well adapted to the rather low nitrite concentrations in the Seine headwaters and downstream estuary. Also, the large amount of *Nitrobacter* might be explained by their propensity to attach themselves to particles, i.e. to the soil matrix in the headwaters and to suspended solids in the estuary turbidity maximum where the silt from the mudflats is resuspended in time with the tidal cycles (Koops and Pommerening-Röser, 2001). Finally, *Nitrobacter* species present in the Seine River could have different physiological properties than the one isolated in pure culture, because of the cultivation-dependent method biais that often favour the isolation of microorganism capable of growing on nutrient rich medium.

Along the Seine River continuum, nutritional conditions (N and C substrates), but also hydro-sedimentary

characteristics (flow velocity, particle dynamics) vary widely, so that temporary limiting conditions would not prevent the co-existence of *Nitrobacter* and *Nitrospira* NOB.

In addition to a species-specific physiology, a study at the species level would be necessary to further investigate the shifts between *Nitrobacter* and *Nitrospira* genera along the Seine River continuum. Moreover, the two phylogenetically unrelated groups of NOB affiliated to the genera *Nitrobacter* and *Nitrospira* do not exclude the presence of each other from the Seine River system.

4. Conclusions

Two NOB genera, *Nitrobacter* and *Nitrospira*, coexist not only in the effluents of the Achères WWTP, but in the lower Seine River and its two principal tributaries (the Oise and the Eure), as well as in the sediment of the rivers of the upstream basin. However, the *Nitrobacter* genera predominated in particle-rich environments, i.e. sediment sampled in the upstream rivers and suspended material in the estuarine maximum turbidity. This study demonstrated that the abundance (number of gene copies per liter) of NOB is from 10 to 100 times higher than that of the ammonia-oxidizing bacteria.

The input of *Nitrobacter* and *Nitrospira* by the Achères WWTP effluents leads to a prevalence of the *Nitrospira* genera along a 100 km stretch downstream of the Achères effluent discharge. A ''succession'' of NOB genera along the Seine River continuum was clearly evidenced.

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References

Aamand, J., Ahl, T., Spieck, E., 1996. Monoclonal antibodies recognizing nitrite oxidoreductase of *Nitrobacter hamburgensis*, *N. winogradskyi*, and *N. vulgaris*. Appl. Environ. Microbiol. 62, 2352–2355.

Altmann, D., Stief, P., Amann, R., De Beer, D., Schramm, A., 2003. In situ distribution and activity of nitrifying bacteria in freshwater sediment. Environ. Microbiol. 5, 798–803.

Bartosch, S., Wolgast, I., Spieck, E., Bock, E., 1999. Identification of nitrite-oxidizing bacteria with monoclonal antibodies recognizing the nitrite oxidoreductase. Appl. Environ. Microbiol. 65, 4126–4133.

Bartosch, S., Hartwig, C., Spieck, E., Bock, E., 2002. Immunological detection of *Nitrospira*-like bacteria in various soils. Microbial. Ecol. 43, 26–33.

Berthe, T., Garnier, J., Petit, F., 1999. Quantification de bactéries nitrifiantes du genre *Nitrobacter* en milieux aquatiques (l'estuaire de la Seine, France). C. R. Acad. Sci Paris Sci. Ecol. 322, 517–526.

Bianchi, M., Bonin, P., Feliatra, 1994. Bacterial nitrification and denitrification rates in the Rhône River plume (northwestern Mediterranean Sea). Mar. Ecol. Prog. Ser. 103, 197–202.

Billen, G., 1976. Evaluation of nitrifying activity in sediments by dark ^{14}C-bicarbonate incorporation. Water Res. 10, 51–57.

Billen, G., Garnier, J., 1999. Nitrogen transfers through the Seine drainage network: a budget based on the application of the Riverstrahler model. Hydrobiologia 410, 139–150.

Bjerrum, L., Kjaer, T., Ramsing, N.B., 2002. Enumerating ammonia-oxidizing bacteria in environmental samples using competitive PCR. J. Microbiol. Methods 51, 227–239.

Bock, E., Koops, H.-P., 1992. The genus *Nitrobacter* and related genera. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Shleifer, K.-H. (Eds.), The Prokaryotes, second Ed. Springer, New York, pp. 2302–2309.

Bock, E., Koops, H.-P., Möller, U.C., Rudert, M., 1990. A new facultatively nitrite-oxidizing bacterium, *Nitrobacter vulgaris* sp. nov. Arch. Microbiol. 153, 105–110.

Both, G.J., Gerards, S., Laanbroek, H.J., 1992. Temporal and spatial variation in the nitrite-oxidizing bacterial community of a grassland soil. FEMS Microbiol. Ecol. 101, 99–112.

Brion, N., Billen, G., 1998. Une réévaluation de la méthode d'incorporation de $H^{14}CO_3^-$ pour mesurer la nitrification autotrophe et son application pour estimer des biomasses de bactéries nitrifiantes. Rev. Sci. Eau. 11, 283–302.

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.

Brion, N., Billen, G., Guézennec, L., Ficht, A., 2000. Distribution of nitrifying activity in the Seine River (France) from Paris to the estuary. Estuaries 23, 669–682.

Burrell, P.C., Keller, J., Blackall, L.L., 1998. Microbiology of a nitrite-oxidizing bioreactor. Appl. Environ. Microbiol. 64, 1878–1883.

Cébron, A., Berthe, T., Garnier, J., 2003. Nitrification and nitrifying bacteria in the lower Seine River and estuary (France). Appl. Environ. Microbiol. 69, 7091–7100.

Cébron, A., Coci, M., Garnier, J., Laanbroek, H.J., 2004. DGGE analysis of the ammonia oxidizing bacterial community structure in the lower Seine River: impact of the Paris wastewater effluents. Appl. Environ. Microbiol. 70, 6726–6737.

Chartrain, M., Rizet, M., Faup, G., 1983. Isolation of *Nitrobacter* from sewage and drinking water. Water Res. 17, 831–832.

Cho, J.-C., Kim, S.J., 2000. Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol. 66, 956–965.

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.-H., Wagner, M., 2001. In situ characterisation of *Nitrospira*-like nitrite-oxidizing bacteria active in wastewater treatment plants. Appl. Environ. Microbiol. 67, 5273–5284.

Degrange, V., Bardin, R., 1995. Detection and counting of *Nitrobacter* populations in soil by PCR. Appl. Environ. Microbiol. 61, 2093–2098.

Degrange, V., Lensi, R., Bardin, R., 1998. Activity, size and structure of a *Nitrobacter* community as affected by organic carbon and nitrite in sterile soil. FEMS Microbiol. Ecol. 24, 173–180.

Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira spp.* from full-scale wastewater treatment plants by competitive PCR. Appl. Environ. Microbiol. 68, 245–253.

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.

Garnier, J., Servais, P., Billen, G., Akopian, M., Brion, N., 2001. The oxygen budget in the Seine estuary: balance between photosynthesis and degradation of organic matter. Estuaries 24, 964–977.

Garnier, J., Billen, G., Berthe, T., Martinez, A., Pinault, S., Desruelle, M., Cébron, A., 2002. Nitrification de l'ammonium dans la basse Seine: impact de l'anthropisation de Paris à l'estuaire. Rapport de Synthèse 1998-2001, PIREN-Seine programme, 20p. http://www.sisyphe.jussieu.fr/internet/piren/

Garnier, J., Cébron, A., Tallec, G., Billen, G., Sebilo, M., Martinez, A., in press. Nitrous oxide emission in the Seine River estuary (France): comparison with upstream sector of the Seine basin. Biogeochemistry, in press.

Gieseke, A., Purkhold, U., Wagner, M., Amann, R., Schramm, A., 2001. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Appl. Environ. Microbiol. 67, 1351–1362.

Gieseke, A., Bjerrum, L., Wagner, M., Amann, R., 2003. Structure and activity of multiple nitrifying bacterial populations co-existing in a biofilm. Environ. Microbiol. 5, 355–369.

Grundmann, G.L., Normand, P., 2000. Microscale diversity of the genus *Nitrobacter* in soil on the basis of analysis of genes encoding rRNA. Appl. Environ. Microbiol. 66, 4543–4546.

Hovanec, T.A., Taylor, L.T., Blakis, A., Delong, E.F., 1998. *Nitrospira*-like bacteria associated with nitrite oxidation in freshwater aquaria. Appl. Environ. Microbiol. 64, 258–264.

Josserand, A., Cleyet-Marel, J.C., 1979. Isolation from soils of *Nitrobacter* and evidence for novel serotypes using immunofluorescence. Microbial Ecol. 5, 197–205.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Röser, A., Koops, H.P., Wagner, M., 1998. Combined molecular and conventional analysis of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64, 3042–3051.

Keen, G.A., Prosser, J.I., 1987. Steady state and transient state growth of autotrophic nitrifying bacteria. Arch. Microbiol. 147, 73–79.

Koops, H.P., Pommerening-Röser, A., 2001. Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol. 37, 1–9.

Maron, P.A., Cœur, C., Pink, C., Clays-Josserand, A., Lensi, R., Richaume, A., Potier, P., 2003. Use of polyclonal antibodies to detect and quantify the NOR protein of nitrite oxidizers in complex environments. J. Microbiol. Methods 53, 87–95.

Montuelle, B., Volat, B., Torio-Fernandez, M.M., Navarro, E., 1996. Changes in *Nitrobacter* serotypes biodiversity in a river: impact of a wastewater treatment plant discharge. Water Res. 30, 1057–1064.

Navarro, E., Fernandez, M.P., Grimont, F., Clays-Josserand, A., Bardin, R., 1992. Genomic heterogeneity of the genus *Nitrobacter*. Int. J. Syst. Bacteriol. 42, 554–560.

Okabe, S., Satoh, H., Watanabe, Y., 1999. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. 65, 3182–3191.

Painter, H.A., 1970. A review of literature on inorganic nitrogen metabolism in microorganisms. Water Res. 4, 393–450.

Regan, J.M., Harrington, G.W., Baribeau, H., DeLeon, R., Noguera, D.R., 2003. Diversity of nitrifying bacteria in full-scale chloraminated distribution systems. Water Res. 37, 197–205.

Rodier, J., 1984. In: Dunod (Ed.), L'analyse de l'eau (eaux naturelles, eaux résiduaires, eau de mer), seventh ed, Paris, 1364pp.

Rotthauwe, J.-H., Witzel, K.-P., Liesack, W., 1997. The ammonia mono-oxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63, 4704–4712.

Schramm, A., DeBeer, D., Wagner, M., Amann, R., 1998. Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Environ. Microbiol. 64, 3480–3485.

Schramm, A., DeBeer, D., VanDenHeuvel, J.C., Ottengraf, S., Amann, R., 1999. Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira* spp. along a macroscale gradient in a nitrifying bioreactor: quantification by in situ hybridization and the use of microsensors. Appl. Environ. Microbiol. 65, 3690–3696.

Sebilo, M., 2003. Utilisation du traçage isotopique naturel pour caractériser et quantifier les processus de nitrification et dénitrification à l'échelle du réseau hydrographique de la Seine. Thèse Doct., University Paris 6, 124pp

Servais, P., Garnier, J., Demarteau, N., Brion, N., Billen, G., 1999. Supply of organic matter and bacteria to aquatic ecosystems through waste water effluents. Water Res. 33, 3521–3531.

Slavyck, G., McIsaac, J.J., 1972. Comparison of two automated ammonium methods in a region of coastal upwelling. Deep-Sea Res. 19, 1–4.

Smorczewski, W.T., Schmidt, E.L., 1991. Numbers, activities and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, eutrophic lake sediment. Can. J. Microbiol. 37, 828–833.

Stephen, J.R., Chang, Y.J., Macnaughton, S.J., Kowalchuk, G.A., Leung, K.T., Flemming, C.A., White, D.C., 1999. Effect of toxic metals on indigenous soil b-subgroup Proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. Appl. Environ. Microbiol. 65, 95–101.

Wagner, M., Rath, G., Koops, H.-P., Flood, J., Amann, R., 1996. In situ analysis of nitrifying bacteria in sewage treatment plants. Water Sci. Technol. 34, 237–244.

Yoshioka, T., Terai, H., Saijo, Y., 1982. Growth kinetic studies of nitrifying bacteria by the immunofluorescent counting method. J. Gen. Appl. Microbiol. 28, 169–180.

Zacchar, V., Thomas, R.A., Goustin, A.S., 1993. Absolute quantification of target DNA: a simple competitive PCR for efficient analysis of multiple samples. Nucl. Acid Res. 21, 2017–2018.