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Assessment of Three Methods for Detection and Quantification of Nitrite-Oxidizing Bacteria and *Nitrobacter* in Freshwater Sediments (MPN-PCR, MPN-Griess, Immunofluorescence)

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Abstract

Nitrification in freshwater, a key process in the nitrogen cycle, is now well known to take place predominantly on suspended particles and in sediment. *Nitrobacter* is the most commonly isolated nitrite oxidizing bacteria from water environments. Three methods for counting nitrite oxidizing communities (especially *Nitrobacter*) in sediment were investigated: MPN-Griess, fluorescent antibodies (immunofluorescence), and a more recent molecular method coupling specific DNA amplification by PCR and statistical MPN quantification. After preliminary adjustments of the MPN-PCR technique, the detection level and the yield of each method were determined by inoculating a sediment with a pure *Nitrobacter* culture. The best recovery yield was obtained with the immunofluorescence technique (21.3%) and the lowest detection level was reached with the MPN-Griess method (10^3 *Nitrobacter*/g dry weight sediment). The MPN-PCR method resulted in the lowest recovery yields and needs further adaptation to become a reliable and precise tool for investigations of nitrifying bacteria in sediment.

Introduction

The improvement of freshwater quality requires identifying and limiting nitrogen pollution sources (wastewater, agriculture runoff) and calls for better knowledge of the biological processes and bacterial assemblages involved in the nitrogen cycle. Nitrogen, in the form of ammonia, nitrite, or nitrate, could act as an environmental stress in aquatic environments, depending on its concentration: together with phosphorus, it contributes to the eutrophication of lakes and rivers; oxygen is consumed during the nitrification process; it has toxicity for aquatic life (especially for fishes) in its free ammonia or nitrite forms, which are also a potential danger to drinking-water quality. Removal of the nitrogen forms from aquatic environments requires the coupling of two transformation stages: first, a nitrification stage that transforms NH_4^+ to NO_2^-, then NO_3^-, and second, the denitrification stage that transforms nitrates to gaseous nitrogen.

Nitrification, one of the key steps of this cycle, is mainly

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associated with suspended particles or bed sediment [1, 2, 7, 13]. Some parameters control nitrification whatever the environment may be: substrate concentrations, temperature, light, pH, inhibitory compounds, heterotrophic bacteria, etc. These parameters could change the relationship between nitrification rate and the nitrifying populations.

Sediment presents some specificities: an oxygen gradient that is very limited with depth, a diffusion gradient of NH_4^+ also linked to sediment depth, the effect of a rise in water level on nitrogen fluxes and on sediment remobilization. All of these modify nitrifying activity and the involved bacterial communities. Many studies in soil have shown the close connections in this process between population and activity and the necessity of linking together the studies on nitrifying communities and the studies on their activity in order to understand the dynamics of nitrification.

Especially in soils, the different strains of nitrite-oxidizing bacteria could have different specific activity under the same environmental conditions [17]: this is the case for *Nitrobacter,* which is frequently found in soils and freshwater and which is considered to be the main genus in nitrite oxidation. This implies great difficulty in precisely defining a relationship between bacterial number and activity. Thus, in order to understand the role and the distribution of *Nitrobacter* populations in aquatic environments (water, sediments, biofilms, etc.), it is at first necessary to have reliable detection and quantification methods.

Unlike studies in soil, in sediment the presence of nitrite-oxidizing bacteria is often inferred from chemical profiles in water or from activity measurements [2, 14, 37], but is seldom directly determined. Since, in aquatic environments, nitrifying activity and nitrifying populations are mainly localized on suspended particles or in sediments, it is necessary to develop methods to detect *Nitrobacter* populations or nitrifying communities in these matrices.

Techniques for such an enumeration are already more or less commonly used in soil, but not in sediment: the Most Probably Number technique with Griess reagent (MPN-Griess) which counts active nitrifying organisms [4, 47] or the fluorescent antibody staining technique (immunofluorescence) [12, 38] (Josserand, thesis, 1983). Recently, advances in molecular biology have allowed the development of detection techniques for *Nitrobacter* strains without a cultivation step [26, 35]. The coupling of PCR with the MPN method (MPN-PCR) has allowed detection and counting of *Nitrobacter* in soils [10].

These three techniques (MPN-Griess, immunofluorescence, and MPN-PCR) described here correspond to three different study levels of nitrifying communities: the global autotrophic nitrite-oxidizing community (active fraction) for the MPN-Griess technique, the species and even the strain (serotype) for immunofluorescence, and the genus for PCR (as used in this study, but of course it could be used at other taxonomic levels).

This work is the first assessment of these three methods in aquatic environments, especially in freshwater sediment. The main goal was to assess their limits, advantages, and drawbacks for the detection and the quantification of genus *Nitrobacter* or of nitrite-oxidizing communities, with special attention to the PCR method, in freshwater sediment.

Materials and Methods

Sediments

Two different sediments (called Neyrieux and Chalaronne) have been sampled in two small rivers located 50 km northeast of Lyon. The first one is sandy, with a poor organic content, and the second one is clayey and organic.

Bacteria

The species *Nitrobacter hamburgensis,* strain X_{14} (kindly provided by the laboratory of Microbial Ecology, University Lyon 1) was used for inoculation of sediment. This serotype is quite usually found in both terrestrial and aquatic environments [25]. The mixotrophic growth medium was from Bock et al. [6], with $NaNO_2$ 2 gL^{-1}, bactopeptone 1.5 gL^{-1}, yeast extract 1.5 gL^{-1}, and sodium pyruvate 0.55 gL^{-1}.

Sediment Inoculation

An exponential pure culture of *Nitrobacter hamburgensis* X_{14} was centrifuged at 22,000 × *g* for 30 min and the cell pellet was washed three times with sterile phosphate buffer (2 mM, pH 7.3). Inoculum densities were adjusted optically at 580 nm. Then a more precise estimation was obtained by DAPI staining. Sediment samples were inoculated in a volume of 400 μl per gram of dry sediment. Inoculation levels varied from 10^3 to 7×10^7 cells/g of dry weight sediment (dws), depending on the experiments.

Total Bacterial Counts (DAPI)

Total bacterial counts were made by direct microscopic observations on 0.2 μm Nuclepore black filters. Samples were stained with 4,6-diamidino-2-phenylindole (DAPI) [31] and sample treatment was adapted from Rebillard and Torre [32], allowing a precision of 10% [44].

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Nitrite-Oxidizer Counts (MPN-Griess)

The MPN-Griess technique was adapted from Schmidt and Belser [36]. Autotrophic and mixotrophic growth media, modified from Bock et al. [6] by adjusting $NaNO_2$ concentration to 5.0 mM, were used.

All incubations were carried out in 24-well microplates (Falcon), with each tenfold dilution in triplicate, and lasted 15 weeks. Observations were done every 2 weeks from the fifth week to the end of incubation: NO_2^- spot tests by the Griess–Illosvay method were carried out on aliquots from each well. MPN was calculated using a MacGrady table [30].

Nitrobacter Serotype X_{14} Counts (IF)

The immunofluorescence technique [12] was used to enumerate some well-defined strains of the genus *Nitrobacter*. The detailed protocol has been precisely described elsewhere [16].

Briefly, bacteria were desorbed from sediment samples: 9 ml of a 2 mM sterile phosphate buffer (1.6 mM K_2HPO_4, 0.4 mM KH_2PO_4, pH 7.2) were added to 1 g of fresh sediment, strongly mixed for 10 min in a Whirlimixer, then settled for 5 min. Overlying water was then sampled and diluted (serial dilutions 1:10). The bacterial suspension (5 ml) was fixed on a 0.2 μm Nuclepore black filter and counterstained with rhodamine–gelatine (0.7 ml). Rabbit antibodies (produced by Valbex, Centre of Bioexperimentation, Lyon, France), nonfluorescent and specific for *Nitrobacter* strain X_{14} (optimal dilution 1/320), were fixed on cell membrane. The free antibody excess was removed by washing out with 50 ml of a sterile NaCl solution (9 g/L), then 50 ml of sterile water. A goat serum anti-IgG, labeled with fluorescein isothiocyanate (FITC) (Sanofi-Pasteur Production), at the dilution 1:100, was then added (0.5 ml) and the excess was washed out with sterile water.

Enumerations were performed at 1000× magnification using a Nikon Labophot epifluorescence immersion microscope fitted with an HBO-100W mercury lamp, taking into account morphological criteria of *Nitrobacter* cells and the green fluorescence of FITC.

Nitrobacter Genus Counts (MPN-PCR)

The MPN-PCR technique was applied on the same samples, after specific pretreatment of sediment (bacterial extraction and DNA extraction) [22, 39].

DNA Extraction and Purification

Desorption step: One gram of fresh sediment was mixed with 9 ml of 0.1 M phosphate buffer (pH 4.5) and 0.6 g polyvinylpolypyrrolidone (PVPP) in a Waring blender for 3 cycles of 1 min of mixing and 1 min of cooling on ice. Sixty μl of a 20% sodium dodecyl sulfate (SDS) solution were added and the sample was centrifuged (10 min, 900 × *g*, 10°C). The supernatant (containing released cells) was removed and kept apart, and the pellet (containing coarse particles and nonreleased cells) was treated again twice as previously described, excepted that PVPP and SDS were omitted.

Concentration step: The supernatants were pooled and bacteria concentrated by centrifugation (30 min, 10,000 × *g*, 10°C). The bacterial pellet was washed two times with a deflocculating solution (0.1% sodium hexametaphosphate, 0.1% sodium pyrophosphate). The last washing of bacterial pellets was performed with 4.5 ml of extracting buffer (0.33 mM Tris-HCl, 0.001 M EDTA, pH 8.0).

Lysis step: Lysis of bacteria was carried out with lysozyme (final concentration 5 mg/ml) in 750 μl of extracting buffer, and incubated 2 h at 37°C. The solution was then heated to 60°C for 10 min, with SDS (final concentration 1%), and cooled in ice for 2 h. After a first centrifugation (20 min, 12,000 × *g*, 4°C), the supernatant was saved and the pellet washed a second time with 300 μl extracting buffer. The second supernatant was pooled with the first one to constitute the crude DNA solution.

Purification step: The DNA was then precipitated with isopropanol (1/2 v/v) and 3 M sodium acetate (pH 5.2) (1/10 v/v). The DNA pellet was washed with 100 μl ethanol (70%). The DNA pellet was then dried (15 min under vacuum) and recovered with 50 μl extracting buffer and incubated for 5 min at 37°C.

DNA was purified on an Elutip-d column (Schleicher & Schuell), following the manufacturer's recommendations. After purification, DNA was precipitated with isopropanol and finally washed with 70% ethanol.

DNA Amplification

Purified DNA serial dilutions (1:10) were carried out in triplicate and treated for the amplification of a 16S rDNA specific sequence of *Nitrobacter,* using a "booster" PCR. Oligonucleotide primers were: 5′ TTTTTGAGATTTGCTAG 3′ (FGPS1269′) and 5′ CTAAAACTCAAAGGAATTGA 3′ (FGPS872) (EuroGentec, Belgium).

Our PCR protocol was adapted from Degrange and Bardin [10]. In 0.5 ml Eppendorf tubes, 1 μl of each purified DNA dilution was added to 5 μl amplification buffer 10× (Tris-HCl 200 mM, pH 8.4, KCl 500 mM) (Gibco BRL); 1.5 μl $MgCl_2$ 50 mM (Gibco BRL); 2.5 μl 1% W-1 (Gibco BRL); 5 μl of a mixture of dATP, dTTP, dCTP, and dGTP each at 200 μM (Ultrapure dNTP Set—Pharmacia); 5 μl of each primer at 5×10^{-3} μM; 0.5 μl of Taq Polymerase (i.e., 2.5 U); and high-purity H_2O qsp 50 μl. The amplification step was carried out on a CETUS thermal cycler (Perkin Elmer 9600) with the following PCR cycles: predenaturation (3 min, 95°C); 10 [denaturation (45 s, 95°C)—annealing (30 s, 50°C)—extension (1 min, 72°C)] cycles; and a final extension (3 min, 72°C). Then, 2.5 μl of each 10 mM primer was added to each Eppendorf tube, and 60 denaturation–annealing–extension cycles and a final extension were run.

Samples of DNA extracted from sediment and PCR-amplified DNAs were checked by horizontal gel electrophoresis in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0) with 0.8 and 2% agarose (wt/vol), respectively, and then incubated in a 0.4 mg/l ethidium bromide staining solution and photographed under a 312 nm UV light source with Ilford FP4 films.

The number of *Nitrobacter* was determined from a MacGrady table [30].

Detection Level Determination and Yield Calculation

The yield of the pretreatments for MPN-PCR was calculated with both Neyrieux and Chalaronne sediments. After each step, total

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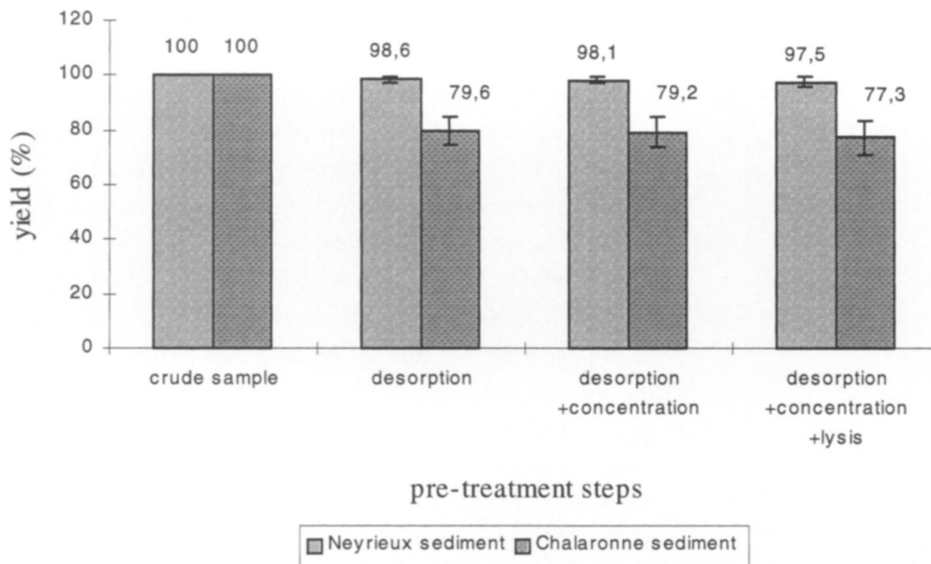

Fig. 1. Cumulative yields for different pretreatment steps (total bacterial counts by DAPI staining). Mean values for triplicates. Error bars are standard deviations.

bacteria were counted (by DAPI staining) and compared to the previous step counting. For the lysis step, the number of lysed bacteria was inferred from the number of nonlysed bacteria.

Quantification assays of DNA lost during purification on an Elutip-d were performed with calf thymus DNA (Sigma) which was directly eluted through the column. DNA measurements were carried out by fluorimetry, using DAPI staining [18] on a CytoFluor 2300.

The global yield (number of detected bacteria versus number of inoculated bacteria) and the detection levels for MPN-PCR, MPN-Griess, and IF were measured with the Neyrieux sediment inoculated with *Nitrobacter* strain X_{14}, at different adjusted concentrations (10^3 to 5×10^6 *Nitrobacter*/g dry weight sediment) and a noninoculated reference. No sterilization of sediment was performed to prevent destruction of the structure and possible competition with heterotrophic bacteria, and to avoid modifications of the PCR inhibitory compounds.

The three methods were tested in triplicate for each inoculation level.

Results

MPN-PCR: Yields of the Pretreatment Steps

The desorption yield of bacterial populations was about 99% for the Neyrieux sediment (sandy sediment) and 80% for the Chalaronne sediment (silty) (Fig. 1). For the two sediments, 99% of the desorbed bacteria were pelleted by centrifugation. The coupling lysozyme and SDS was effective, as more than 97% of the pelleted bacteria disappeared and their DNA was released. Taken as a whole, at the end of the three steps of sediment pretreatment (desorption, concentration, and lysis), DNA from 97% and 77% of total bacteria was released in Neyrieux and Chalaronne sediments, respectively (Fig. 1).

The small DNA concentrations in our sediment made reliable measurements of precipitation and purification yields difficult to obtain. Quantification assays of DNA lost during purification on an Elutip-d were performed with calf thymus DNA. These experiments showed that DNA losses were more pronounced and recovery variability was higher when DNA was diluted in freshwater (Saône river) than it was in extracting buffer (Table 1).

MPN-PCR: Global Yield and Detection Level

PCR products were systematically obtained from highly inoculated sediment (7.13×10^7 cells/g dry weight). An example is given in Fig. 2. Table 2 shows that the detection of *Nitrobacter* occurred in some samples only in slightly inoculated sediments (just one time out of six tests). Detection threshold is thus very high. Furthermore, the recovery rate (0.7%) and quantification reproducibility are very low.

MPN-Griess

The detection threshold by this technique is at least 10^3 *Nitrobacter* cells/g dry weight (the lowest inoculation level in our study), but when the concentration in the inoculum is low, incubation time allowing detection is long (up to 7 weeks, at 10^3 cells/g) (Fig. 3).

Taken as a whole, the yield *Nitrobacter* detected/ *Nitrobacter* inoculated depends on the length of incubation and on the concentration of the inoculum: it is inversely correlated with the inoculated number of *Nitrobacter* cells in

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Table 1. Purification rates of calf thymus DNA with an Elutip-d column

DNA solvent	DNA before purification (ng/ml)	DNA after purification (ng/ml)	DNA recovery (%)	
			Mean ± SD	Coefficient of variation
Extracting buffer	1380	574 639 626	44.4 ±2.5	5.6
Freshwater	889	414 253 298	36.2 ±9.4	26.0

SD: standard deviation.

the sediment (3.1 to 18.6%). The variability is high, whatever the inoculation level is (44.9 to 103.6%) (Table 2).

Immunofluorescence

The immunofluorescence method provides the highest recovery yields (11.5 to 21.3%) and the lowest coefficients of variation (<25%) (Table 2).

Discussion

The determination and comparison of the detection threshold and the yield of each method, and the calculation of the yield of each step for the MPN-PCR technique, are seldom undertaken in aquatic and soil studies.

The inoculation level of the sediment was a bit different for the three tested methods, depending on the theoretical threshold (especially IF) and on technical reasons (especially MPN-PCR: only one detection out of 6 assays at the inoculation levels 10^4 and 10^5 *Nitrobacter*/g dws, so lower inoculation levels were not justified).

Assessment of the MPN-PCR Method

The original aspect of this work, in regard to what has been done in soil, for example, is the determination of the yield of each step of the protocol used.

Bacteria desorption and DNA extraction: First assays in aquatic environments using the method described by Degrange and Bardin [10] (direct DNA extraction by physical treatment) without adaptation were unsuccessful (Montuelle and Bonnet, unpublished data). So we tried to define a pretreatment of crude samples by desorption of bacteria, before extracting DNA, to obtain a homogenized bacterial extract without particles whatever the sample may be (sediment, flocs from activated sludge, biofilms, etc.). This avoids disturbances due to the presence of particles during the DNA purification step and, from this point of view, allows a standardization of this process step (but of course, does not avoid disturbances linked to chemical compounds such as organic matter or humic acids).

The validity limits of methods for biomass characterization and biomass quantification based on *in situ* DNA analysis are generally not due to the analytical technique, but to the amount of contaminating compounds that could be accepted. Preliminary extraction of bacteria allows to partly avoid contamination troubles and substrata diversity. The DNA quantity that is recovered in this way is smaller than that obtained by direct lysis, but less contaminated [29].

Several bacterial extraction methods are used: shaking with or without chemicals (scattering compounds), ultrasonication, and Waring blender mixing. Comparison of these methods, with soil samples, has shown that the best extraction rate was obtained by Waring blender mixing and that this does not injure bacterial cells [21].

Our bacterial extraction technique, carried out with phosphate buffer, SDS, and PVPP at acid pH, was the technique chosen by Steffan et al. [39], who obtained, after three extraction cycles, an extraction rate of 32.2%, much lower than our results (98.6 and 79.6%). This could be due to a difference in sediment characteristics: Steffan's sediment had a high organic matter content with silt and clay (respectively 6.6, 28, and 44%) when compared to our sediments. A great number of bacteria are adsorbed on clay particles and are not desorbed by an extraction treatment, even after multiple blending–centrifugation cycles [3]. Our extraction rates were higher and were sufficient to carry out only three successive bacterial extractions. This number was also considered as well adapted when bacterial number was higher than 10^4 cells/g soil [15].

Lindahl and Bakken [21] showed that the representativeness of the extracted bacterial fraction is positively correlated with the extraction yield. However, even high rates could induce a quantitative and qualitative underestimation of bacterial assemblages because some bacteria are strongly bound to their substratum: for example, methanotrophic bacteria in peat or ammonium-oxidizing bacteria in clay soils. This underestimation is difficult to evaluate. *Nitrobacter* cells are known to be attached to sediment particles or suspended matter [7, 9, 13], but the strength of these attachments depends on multiple factors and is hard to estimate in advance.

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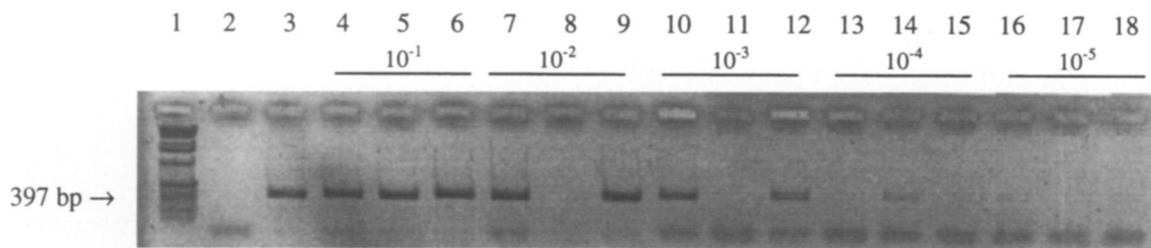

Fig. 2. Detection of *Nitrobacter* by MPN-PCR on electrophoresis gel. 1, 1-kb ladder; 2, negative control without DNA; 3, positive control with pure *N. hamburgensis* DNA; 4–18, DNA extracted from the highly inoculated sediment, serially diluted tenfold (from 10^{-1} to 10^{-5}), with three repeats for each dilution.

Taken as a whole, the concentration step of the desorbed bacterial fraction was very effective.

The treatment with lysozyme and SDS allows unselective disruption of bacterial cells. The lysis rate, expressed as DNA extraction, is important (>90%) and close to those obtained by Steffan et al. [39]. The choice of chemical lysis partly avoids problems caused by DNA strand breaks, which are more important when physical techniques such as ultrasonication are used, that release small DNA bits (about 500 bp) [15]. A subsequent analysis of DNA fragment size on electrophoresis gels showed that they were of different length ranging from 0.5 to 12 kb (data not shown).

DNA purification: Spectrophotometry is a widely used method for DNA quantification [15, 39, 43]. But the wavelength (260 nm) used for the DNA analysis is in the absorbency range of other molecules, such as phenolic or humic compounds [43]. Although this technique is well adapted to pure culture studies, more difficulty is encountered with environmental samples, where numerous compounds could interfere with DNA quantification. Furthermore, the evaluation of DNA purity level is not very exact because some compounds do not absorb at a precise wavelength, but at in a large zone: this is especially the case for humic compounds (abundant in sediments).

DNA measurement using DAPI labeling and fluorimetry is an interesting alternative, because fluorochrome binds specifically to DNA by inserting on the A–T bases. The interference risk is lower, but fluorescence intensity decreases if DNA is injured [18]. However, preliminary experiments are needed, particularly to define the best DAPI concentration (data not shown).

Our recovery yields measured by fluorimetry were lower than Tsai and Olson's results [45] in soil and sediment (60% recovery) with the same technique. These low rates could be explained by the fact that the DNA recovery rate after purification on Elutip-d columns decreases when very large DNA is treated [28] or when humic compounds interfere with DNA adsorption on the purification column [50]. This Elutip-d technique is one of the easiest to use for routine studies. The treatment diminishes the recovery rate, but is necessary to avoid inhibition of DNA polymerization during the next PCR step [27, 49].

Inhibiting compounds in the sediment (especially humic substances) which remain after DNA purification could also explain our low detection yield [42, 46, 49]. DNA may be injured during extraction and then not amplified by PCR [40]. Moreover, if chemical lysis releases an important amount of DNA, it may also cause interferences with PCR.

The global yield of *Nitrobacter* quantification in sediment with this PCR-MPN technique is low and the detection threshold is high.

The work of Malhautier et al. on biological filters also resulted in a low detection level and poor counting efficiencies, probably due to an important loss of DNA by adsorption and during the purification step on an Elutip-d column [24].

Besides a loss of biomass during the pretreatment steps (mainly due to purification and precipitation), it seems that the DNA amplification step is not optimized in spite of the use of a booster protocol. This protocol comprises two steps: first the primers are strongly diluted during 10 cycles to avoid the formation of dimeric primers; then they are concentrated during 60 resting cycles, when the annealing process is well engaged [34].

Although Degrange and Bardin [10] managed to detect low *Nitrobacter* cell densities in a soil sample by PCR (yield of more than 60% and a detection threshold of 10^2 *Nitrobacter*/g soil), obviously no method exists that is universally applicable to solid matrices such as soils or sediments. Our results show that DNA extraction is not responsible for failure, and more research should be directed toward the im-

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Table 2. Quantification of *Nitrobacter* (strain X_{14}) in Neyrieux inoculated sediment by three methods

Nitrobacter inoculation (*Nb*/g dws)	*Nitrobacter* detection			
	Number of positive results/number of assays	Mean (*Nb*/g dws) ± SD	Coefficient of variation (%)	yield[a] (%)
MPN-PCR[b]				
0 (reference)	0/3	0 ±0		
1.43×10^4	1/3	1.63×10^1 $\pm 2.83 \times 10^1$	173.2	0.1
1.09×10^5	0/3	0 ±0	0	0.0
7.13×10^7	3/3	4.88×10^5 $\pm 4.57 \times 10^5$	93.6	0.7
MPN-Griess[c]				
0 (reference)	0/3	0 ±0		
1.09×10^3	2/3	2.03×10^2 $\pm 1.76 \times 10^2$	86.7	18.6
1.07×10^4	3/3	1.17×10^3 $\pm 7.69 \times 10^2$	65.7	10.9
1.09×10^5	3/3	1.36×10^4 $\pm 6.11 \times 10^3$	44.9	12.5
1.08×10^6	3/3	8.31×10^4 $\pm 8.61 \times 10^4$	103.6	7.7
5.36×10^6	3/3	1.64×10^5 $\pm 1.30 \times 10^5$	79.3	3.1
Immunofluorescence[d]				
0 (reference)	0/3	0 ±0		
1.09×10^5	3/3	2.28×10^4 $\pm 2.5 \times 10^3$	11.0	20.9
1.08×10^6	3/3	1.24×10^5 $\pm 2.0 \times 10^4$	16.1	11.5
5.36×10^6	3/3	1.14×10^6 $\pm 2.82 \times 10^5$	24.7	21.3

[a] Yield is the quantity of detected *Nitrobacter* versus inoculated *Nitrobacter*.
[b] MPN-PCR quantification of *Nitrobacter* (strain X_{14}) in inoculated sediment.
[c] MPN-Griess quantification of *Nitrobacter* (strain X_{14}) in inoculated sediment (autotrophic growth medium $NaNO_2$ 5 mM; incubation time: 15 weeks).
[d] Immunofluorescence detection and quantification of *Nitrobacter* (strain X_{14}) in inoculated sediment.
SD: standard deviation.
dws: dry weight sediment.

provement of the DNA purification and amplification procedures.

MPN-Griess

Of the available methods for biomass estimation of nitrite-oxidizing bacterial communities, MPN-Griess is at the moment the most widely used, but it is well known that this technique underestimates the nitrifying community [8, 33]. For example, Belser and Mays [4] have estimated that MPN takes into account less than 5% of a community.

Our results confirm this fact: after a 15 weeks incubation time, the number of detected *Nitrobacter* is low in comparison to the inoculum bacterial density (3.1 to 18.6%), although we worked with inoculated populations, optimal conditions for MPN-Griess. Several causes could explain this low recovery yield: culture medium selectivity, presence of bacterial clusters, or nitrite concentration [8]. Another constraint on the MPN-Griess technique is the long incubation time necessary to obtain the highest nitrite-oxidizer number, under identical conditions. According to various authors this time ranges from several weeks to some months (with

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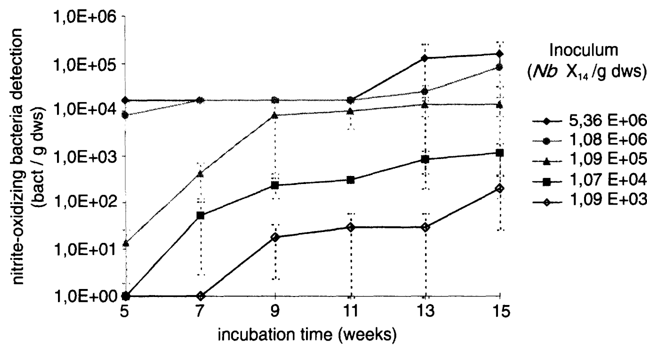

Fig. 3. Changes in the number of nitrite-oxidizing bacteria detected with the MPN-Griess method versus incubation time. Each point is a mean of triplicates. Error bars (dotted lines) are standard deviations.

the limit of liquid growth medium evaporation from the microplates). In our case, the increase between 9 and 15 weeks of incubation is slight, but the variability increases as well, and the error bars overlap each other after 15 weeks. Even if the nitrite oxidizing community is underestimated after 9 weeks of incubation, we have a better quantitative discrimination, which is interesting for *in situ* studies (for example, for comparing different sediments, or different study sites on the same river). So, we can consider that the last significant result is obtained after 9 incubation weeks.

As *Nitrobacter hamburgensis* grows better on a mixotrophic medium, other assays with a mixotrophic growth medium were carried out, but results after incubation were much the same as in autotrophic growth medium (data not shown). In this last study case, care must be taken with respect to the disappearance of nitrite: if the O_2 concentration is low in incubation microplates, nitrite and the nitrate produced could be denitrified by *Nitrobacter* [19]. In our microplates incubated with the mixotrophic media, the disappearance of NO_2^- was not systematically correlated with NO_3^- production.

We chose to establish three parallel incubations of the same sediment sample, and we obtained a great variability (Table 2), which led us to conclude that at least three counting assays are necessary when an enumeration is needed.

Immunofluorescence

After preliminary comparisons between immunofluorescence on Teflon-coated supercured microscopic slides [20, 25] and on Nuclepore filters [11, 23], we used filters, because of less variability of the results and better microscopic observation quality. But, depending on the quantity of bacteria and the quality of the sediment, it is sometimes impossible to work on filters (clogging), and so, even if it is less reliable, the slide method may be unavoidable.

Some authors frequently counted many more than 100 microscopic fields, yet saw much less than one cell per field [48]. To define more realistic experimental conditions (not too time-consuming), we considered that a significant enumeration was obtained by counting at least one bacteria per field and 100 fields, even if this could underestimate bacterial counts. The theoretical detection threshold is then 1.56×10^4 *Nitrobacter* cells/gram of dry sediment (for genus *Nitrobacter*, this number is not effective in all aquatic environments). But because sediment particles could clog the membrane filter, and because they are autofluorescent and hamper bacterial counting, at least a 1/10 dilution of sample and a small filtered volume are needed for a sufficient observation quality. The real enumeration level is therefore higher than the theoretical one: about 1 or 2×10^5 *Nitrobacter* cells/gdw of sediment. The recovery yields of inoculated *Nitrobacter* (from 11.5 to 21.3%; see Table 2) are close to the results of Josserand (thesis, 1983) and Degrange (thesis, 1996) in soil: 25 and 20%, respectively.

In situ, according to the studied serotype, the recovery yield may vary, since great variations in the recovery yield of three *Nitrobacter* strains (X_{14}, AG, LL) inoculated in sterile soil have been observed (with the X_{14} yield being the slightest) (Degrange, thesis, 1996). Moreover, lower efficiency of sediment-borne cell recovery could be possible, because of failure of the antiserum to react with encapsulated cells or with the bacteria covered by slime in biofilms [41].

The advantage of the immunofluorescence technique is its quickness and its high specificity for identification at the serotype level (very few cross reactions [12]) and relatively low coefficient of variation: 11 to 24.7% here, ad 10 to 30% in Laanbroek and Schotman [20]. Standard deviations are 5 times less than those of the MPN-Griess technique [5]. On the other hand, immunofluorescence does not allow quantification of the global *Nitrobacter* population because it is selective to a given serotype. Bonnet et al. [7], by combining the enumerations of five strains, have obtained 7 to 10% of the MPN-Griess numeration with activated sludge samples, and Montuelle et al. [25] have obtained 15% of the MPN-Griess result with six strains on treated wastewater samples. The immunofluorescence technique lacks exhaustiveness (even with a mixing of several serotypes) but is therefore powerful for studying strain competition or for assessing the influence of environment parameters on strains chosen as biological models of *Nitrobacter* sp.

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Each method used in our experiments has a specific observation level and gives different information on the nitrite-oxidizing community structure:

- Serotype for the immunofluorescence technique
- Genus level for the MPN-PCR technique
- Autotrophic nitrifying community for the MPN-Griess technique

Theoretically, the PCR method is the more appropriate one for our initial goal: exclusive *in situ* quantification of the nitrite-oxidizer genus *Nitrobacter*. But adaptations are still necessary to improve the method before considering it as a reliable enumeration tool in sedimentary environments: improvement of the extraction and purification yield and reduction of the detection level are imperative for application to aquatic environments.

Given the high detection threshold, an interesting application could be the detection and fast estimation of *Nitrobacter* quantity in water treatment plants (especially nitrifying systems), where quantities are greater than in sediment. Such detection assays, applying the protocol established in sediment on an activated sludge biomass, have been successful (Féray, unpublished data, 1996).

However, besides the advantage of the rapidity of the response (a few days as compared to a few months for MPN-Griess), PCR presents severe drawbacks compared to immunofluorescence or MPN-Griess. Thus, PCR still remains a technique for selective verification more than a routine tool for *in situ* sediment studies. MPN-Griess allows an increased number of treated samples. IF is useful to study the evolution of a particular serotype in different environments or submitted to different factors. Used in complement to each other and adapted to study aims, these techniques allow a better understanding of the relations between bacterial cell number and activity in freshwater systems.

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