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Nucleic acid extraction from polluted estuarine water for detection of viruses and bacteria by PCR and RT-PCR analysis

Fabienne Petit[a*], Stéphanie Craquelin[a], Janine Guespin-Michel[a], Claudine Buffet-Janvresse[b]

[a]*Laboratoire de microbiologie du froid, UPRES 2123, IFRS 61 CNRS/Inserm, Faculté des sciences, 76821 Mont-Saint-Aignan, France*

[b]*Laboratoire de Virologie, IFRS 61 CNRS/Inserm, CHR, 76031 Rouen, France*

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Abstract — We describe an extraction protocol for genomic DNA and RNA of both viruses and bacteria from polluted estuary water. This procedure was adapted to the molecular study of microflora of estuarine water where bacteria and viruses are found free, forming low-density biofilms, or intimately associated with organo-mineral particles. The sensitivity of the method was determined with seeded samples for RT-PCR and PCR analysis of viruses (10 virions/mL), and bacteria (1 colony-forming unit mL). We report an example of molecular detection of both poliovirus and *Salmonella* in the Seine estuary (France) and an approach to studying their association with organo-mineral particles. © Elsevier, Paris

nucleic acid extraction / PCR and RT-PCR analysis / estuary water

1. Introduction

Recent advances in molecular biology have been of great value in the study of microbial populations in the environment. As only approximately 1% of these bacteria can be grown in pure cultures, other techniques can make an important contribution to ecological studies. Major technical advances in PCR analysis and the extraction of nucleic acids from environmental sources have made it possible to identify the species in a population without prior isolation of the bacteria, and to detect viruses without previous multiplication in cell cultures [2, 9, 13].

A research project was recently undertaken to investigate the ecology of an estuarian ecosystem (the river Seine estuary, France). It addresses issues including the dynamics of particles and the study of associated bacteria and viruses. The use of molecular methods is a novel approach, complementary to the classical ecological methods. The first step in all these molecular methods is DNA extraction from the studied environment. Numerous methods of DNA extraction allowing PCR analysis have been described [7]. None was entirely suitable for the aquatic environment of the Seine estuary, rich in both dissolved and particulate organic matter. Estuarine water is more heterogeneous than other environmental waters: whereas the concentration of suspended particulate matter is about 1 mg/L in groundwater and 10–50 mg/L in lakes and rivers, it is 0.1–10 g/L in estuarine water.

For ecological studies, the nucleic acid extraction protocol should yield DNA from virtually all the whole microorganisms in the sample. As 90% of planktonic bacteria are associated with suspended matter [1], several authors have suggested working only with sediments in lake or

* Correspondence and reprints
Tel. : 33 2 35 14 66 84; fax : 33 2 35 14 70 20; fabienne@univ-nover.fr

Abbreviations: CFU, colony forming unit; PCR, polymerase chain reaction; PFU, plaque-forming unit; PVP, polyvinylpolypyrrolidone; RT, reverse transcription; TCID, tissue culture infectious dose

estuary water samples [8, 22]. But the water of the Seine estuary and river contained both an autochtone population of planktonic or benthic microorganisms, and an allochtone population often originating from the effluent of treatment plants [4]. Only 24% to 68% of culturable fecal coliform bacteria may be associated with suspended matter [18]. We propose herein an extraction protocol for obtaining genomic nucleic acid from all these different populations. All PCR inhibitors, particularly humic acids and chemical pollutants, must be removed from the nucleic acid extract [21]. In estuarine water the concentration of humic compounds was three times higher than in marine waters and the Seine estuary is highly polluted with chemicals.

These difficulties have been overcome by developing a DNA extraction protocol suitable for estuarine water that also allows the simultaneous extraction of RNA from viruses. This protocol may be of general interest for studies of polluted aquatic environments. The sensitivity of this method was tested for RT-PCR and PCR analysis with samples seeded with known amounts of viral and bacterial markers. Finally, the procedure was used to detect both *Salmonella* and poliovirus in estuarine water.

2. Materials and methods

2.1. Sampling

Samples of water from the Seine estuary (France) were collected in sterile centrifugation bottles containing (0.1%) SDS, 1 mM Na_2EDTA pH 8.0 and 0.5% (v/v) guanidium isothiocyanate final concentration. All samples were transferred at below 4 °C, to our laboratory (journey of 1–8 h) where they were immediately processed or frozen at -20 °C until processing. This treatment should lyse most eukaryotic scavenging microorganisms and some of the most fragile Gram-negative bacteria and virus capsids.

Kilometric unit (Pk) is defined by the Financial Agency of the ‘Seine Normandie’ basin as 1000 at the mouth of the estuary and decreased upstream.

2.2. Bacteria and viruses

Escherichia coli HB101::Tn5 *tac1* was grown at 37 °C in LB medium containing 10 μg/mL kanamycin. Cells were counted by plating appropriate dilutions on LB agar medium. Attenuated poliovirus was obtained from an oral vaccine preparation (Mérieux). Polioviruses were titered on human MRC5 cells to determine the $TCID_{50}$ value (50% of the tissue culture infectious dose) [11]. Prior to nucleic acid extraction, water samples were inoculated with various amounts of both *E. coli* (10^0 to 10^6 CFU/mL) and poliovirus ($2 \times 10^0 - 2 \times 10^5$ PFU/mL).

2.3. Direct extraction of nucleic acids from estuary water

Seeded and unseeded samples were frozen at -80 °C overnight and freeze-dried. Nucleic acids were extracted by a direct lysis method as described by Picard et al. [12]. The nucleic acids were then precipitated with isopropanol.

2.4. Nucleic acid extraction from estuary water

Nucleic acids were extracted from estuary water samples as described in *figure 1*. Samples treated as described above were thawed at 4 °C and incubated at 70 °C for 30 min with gentle shaking. Organo-mineral particles were pelleted × 6000 *g*, 20 min) at 20 °C to prevent sodium dodecyl sulfate precipitation. Guanidium was added to the supernatant (subsequently referred to as crude supernatant), and the mixture incubated for 3 min at 95 °C to inactivate nucleases. Then proteins, SDS and humic compounds were removed by precipitation with 0.5 M potassium acetate [10, 16]. Nucleic acids were precipitated from the crude supernatant with isopropanol. The organo-mineral particles were washed twice in 40 mL of washing buffer (0.1% SDS (w/v), 2% polyvinylpyrrolidone (PVP) (w/v), 1 mM $Na_2P_2O_7$, 0.1 M K_2HPO_4/KH_2PO_4 pH 6.0) by vigorously vortexing for 5 min to dissociate sediment-bound bacteria, and then centrifuged at 1000 *g* for 10 min. The washed organo-mineral particles were discarded. The two supernatants

(washed supernatant) were pooled and centrifuged at 8000 *g* for 30 min. The resulting supernatant, the acellular washing supernatant, was incubated for 3 min at 95 °C and proteins were removed by potassium acetate treatment. Nucleic acids in the acellular washed supernatant were precipitated by isopropanol as described above. The cell pellet was treated with lysozyme (5 mg/mL), frozen and thawed (-80 °C to 95 °C) twice. Crude nucleic acids from the crude supernatant, the acellular washing supernatant and the cell pellet were finally extracted by phenol/ether extraction and isopropanol precipitation. Each fraction was suspended in 100 μL of sterile distilled water and then pooled.

The concentration and purity of the total nucleic acids preparation were determined by spectrophotometry, with absorbance measurements at 260 and 280 nm (Beckman DU 640). Nucleic acid extraction yields are expressed in micrograms of nucleic acids obtained per mL (of water sample).

2.5. Oligonucleotide primers

The primers for *E. coli* HB101::Tn5 *tac1* were GCCGCCGTGTTCCGGCTGTCA and CCGCCACACCCAGCCGGCCAC targeting the *aphI* kanamycin resistance gene on Tn5 *tac1* (546 bp). The primers for *Salmonella* PCR were those previously described by Smith et al. [17] for the *fliC* gene (889 bp), encoding phase 1 flagellin. Two synthetic oligonucleotides corresponding to the 5′ non-coding sequence of poliovirus type 1 were used as primers in the RT-PCR reaction [11, 23]. The sequences of these primers were: 5′-CAAGCACTTCTGTTTCCCCGC-3′ and 5′-ATTGTCACCATAAGCAGCCA-3′. The targeted sequence (434 bp) was conserved in most of the enteroviruses. All these primers were synthesized by Genset (Paris, France).

2.6. PCR amplification

DNA from estuary water (20 to 200 ng) were used for PCR in 100 μL of the following reaction mixture: 10 μL of 10x PCR buffer (10 mM Tris-HCl pH 8.3; 50 μM KCl), 200 μM of each dNTP (Boehringer), 2.5 U *Taq* polymerase (Boehringer), and 1 μM of each set of the appropriate primers. The $MgCl_2$ concentration was optimized: 2 mM for the *E. coli aphI* kanamycin resistance gene and 3 mM for the *fliC* gene in *Salmonella*. PCR amplification was performed in a Perkin Elmer thermocycler (geneAmp PCR System 2400) and consisted of the following steps: 5 min at 94 °C to denature the DNA, 30 cycles of 1 min for denaturation at 94 °C, annealing according to the primers used (*aphI* gene: 1 min at 65 °C; *fliC* gene: 1 min 30 at 55 °C), 2 min for extension at 72 °C (3 min for *Salmonella* PCR), and a final extension step of 5 min at 72 °C (7 min for *fliC* PCR).

Reverse transcription-polymerase chain reaction (RT-PCR) for amplification of poliovirus RNA was performed as described by Petitjean et al. [11]. The viral RNA was transcribed directly into cDNA by avian myeloblastosis virus reverse transcriptase (Boehringer). The single-stranded cDNA was then amplified enzymatically by the thermostable *Taq* DNA polymerase (Perkin Elmer Cetus) in 35 thermal cycles. Amplified DNA (10 μL) samples were analyzed by electrophoresis in a 2% agarose gel (FMC Bio Products, Rockland, Maine) in (Tris-acetate-EDTA buffer pH 7.5 (Sigma). The gel was stained with ethidium bromide and DNA detected by illumination with UV light.

3. Results

3.1. Extraction of total nucleic acids from estuary water

Total nucleic acid extraction from environmental water samples requires that loss of microorganisms be minimized during processing. We developed a protocol *(figure 1)* based on nucleic acid extraction from three different fractions of estuarine water, corresponding to viruses and lysed or entire cells, free or associated with sediment. The crude supernatant contained free viruses, and cells lysed by SDS (30 min, 70 °C) were mainly bacterioplankton [3]. Washing organo-mineral particles released viruses and cells intimately associated with

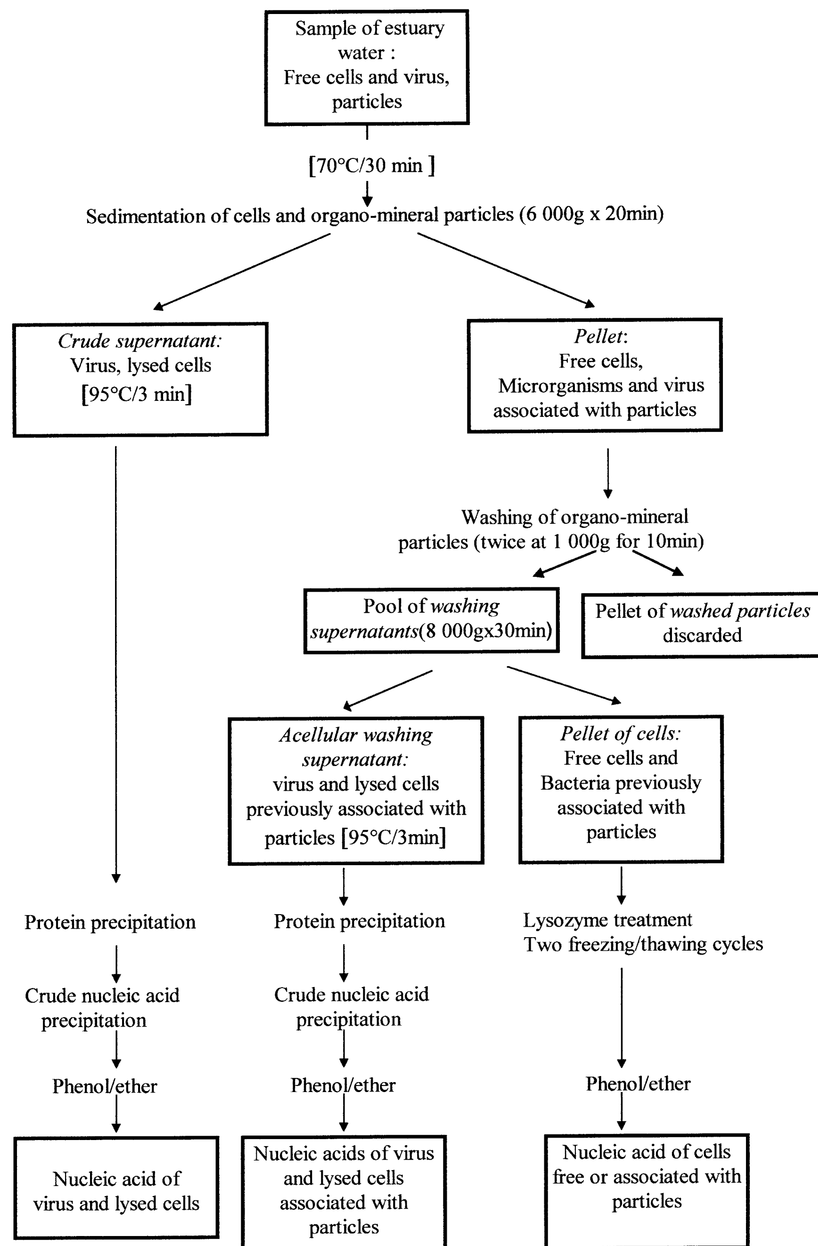

Figure 1. Nucleic acid extraction from estuary water.

organo-mineral particles and caused some bacteria to lyse. Intact bacteria, not lysed by SDS treatment, were pelleted and we sought to recover viruses and lysed cell content in an acellular washed supernatant. The amount of nucleic acid extracted from these three fractions depended on the water sample, and that obtained from the acellular washed supernatant could reach 31% of total nucleic acid extract *(table I)*. For comparison, a direct nucleic acid extraction procedure, without fractionation of the water sample *(table II)* yielded significantly

less total nucleic acid. The fractionation procedure we proposed improved the nucleic acid extraction from seeded deionized water by 25%, and from seeded estuarine water by 65%. This difference was probably due to poor release of microorganisms intimately associated with organo-mineral particles or to a greater nuclease activity. Nucleases were concentrated by freeze-drying (and were thus harder to inactivate) or because they were continuously released by cell lysis during the freeze-thaw cycles. In the same way, the higher nuclease activity in estuary water could also explain the lower yield obtained for estuary water than for deionized water with the direct extraction procedure.

Table I. Proportion of nucleic acid extracted from the various fractions of the estuary water sample. Nucleic acids were extracted from three fractions as described in *figure 1*: i, free virus and lysed cells; ii, virus and lysed cells associated with particles; and iii, cells free or associated with particles. The concentration of total nucleic acids was determined by measuring absorbance at 260 nm and 280 nm on a Beckman DU 640 spectophotometer and expressed in ng/mL of water sample, a, or percentage of total extract, b. Three water samples (Seine, France), containing 35 to 50 mg/mL of suspended particulate matter, were analysed (1, 2, and 3).

Estuarine water sample	*Fraction I*		*Fraction II*		*Fraction III*	
	a	b	a	b	a	b
1	13.4	42 %	9.9	31 %	8.8	37 %
2	78.1	64 %	5.3	0.4 %	38.5	32 %
3	75.0	55 %	21.8	16 %	38.5	29 %

Table II. Quantity of nucleic acid extracted from seeded water samples by direct extraction and sample fractionation methods. Total nucleic acid was extracted from seeded estuary and deionized water samples (10^6 CFU/mL). Nucleic acids were obtained with a direct lysis protocol or after fractionation of the water sample (see *figure 1* and Materials and methods). The concentration of total nucleic acid was determined as described in *table I*. Results were obtained from three independent experiments.

	Direct extraction	*Extraction from fractionated sample*
Deionized water	0.43 ± 0.20 µg/mL	0.66 ± 0.14 µg/mL
Estuary water	0.19 ± 0.04 µg/mL	0.76 ± 0.05 µg/mL

3.2. Sensitivity of the nucleic acid extraction methods

We assayed sensitivity with our method which detected *E.coli* and poliovirus, agents usually screened for sanitary control of environmental water. Nucleic acids were extracted from estuarine water samples containing various concentrations of marker bacteria (*E. coli* HB101::Tn5 *tac1*) and virus (attenuated poliovirus), as described in *figure 1*. PCR and RT-PCR analysis of bacterial DNA and viral RNA extracted in this way successfully detected as little as 10 virions/mL of poliovirus *(figure 2A)*, and 1 CFU/mL of *E. coli (figure 2B)*. Thus PVP treatment and precipitation by potassium acetate seemed to remove sufficient humic compounds and chemical pollutants present so as to allow PCR and RT-PCR analysis. However, *E.coli* DNA was better amplified in deionized water samples than in environmental water samples *(figure 2B)*. This suggested the residual presence of PCR inhibitors in nucleic acids preparations extracted from the water estuarine samples.

3.3. Detection of enterovirus and *Salmonella* in water from the Seine estuary

We tested whether the method could simultaneously detect enterovirus and *Salmonella* in estuary water. All samples were taken from the Seine (France) between Pk 847 and Pk 1000 (the mouth of the estuary). Nucleic acids were extracted from 500 mL of estuary water as described in *figure 1* and 200 ng of total nucleic acid was used as template for amplification of both enterovirus and *Salmonella* genes. The specificity of the amplified DNA was confirmed by hybridization or sequencing. An enterovirus RT-PCR product of 434 bp was detected in two sites of the Seine estuary *(figure 3A)*. RT-PCR analysis of the three different fractions independently indicated that the enteroviruses were both free and associated with organo-mineral particles in one sample, but only in the free state in the other *(figure 3B)*.

Salmonella screening was performed both by PCR analysis and the standard culture method. In samples from five sites along the Seine estuary, PCR amplified a DNA fragment of 889 bp, specific for the *Salmonella* genus*(figure 4A)*. Culture methods detected *Salmonella* in all these samples except sample # 4 (results not shown). Further PCR analysis of this sample detected

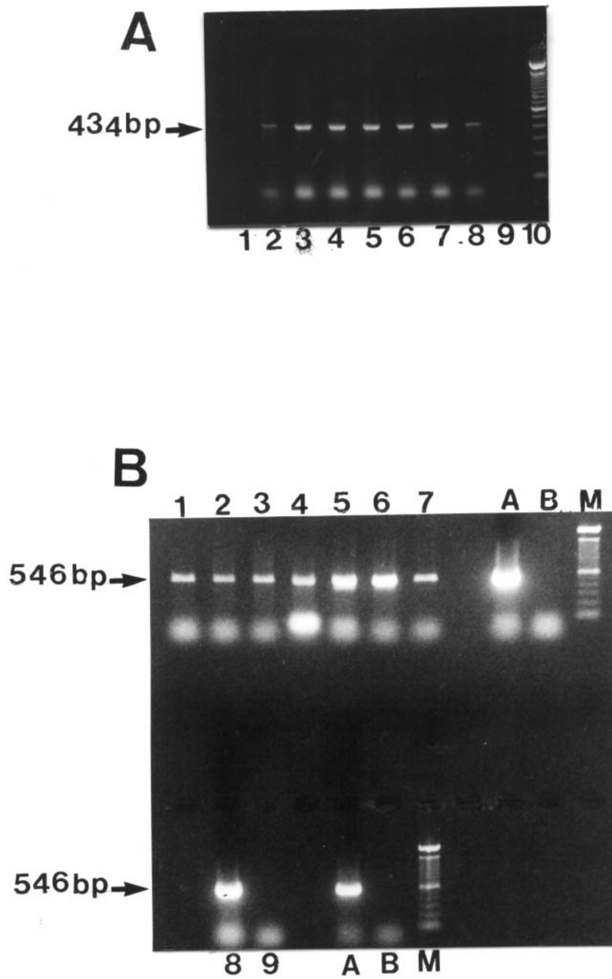

Figure 2. Sensitivity of the nucleic acid extraction method. Nucleic acids were extracted from estuary water samples (*figure 1*) seeded with various concentration of marker bacteria *E.coli* (HB 101::Tn*5 tac1*) and poliovirus. **A.** RT-PCR amplification of conserved sequence of enteroviruses (434 bp) from extract of estuary water sample inoculated with poliovirus: lane 1, negative control (unseeded estuary water); 2, positive control (RNA from poliovirus); 3, 10^6 virions/mL; 4, 10^5 virions/mL; 5, 10^4 virions/mL; 6, 10^3 virions/mL; 7, 10^2 virions/mL; 8, 10^1 virions/mL; 9, 10^0 virions/mL; and 10, 100-bp DNA size ladder. **B.** PCR amplification of *aphI* gene (546 bp) from extract of estuary water sample inoculated with *E. coli.* Lane 1, 10^0 CFU/mL; 2, 10^1 CFU/mL; 3, 10^2 CFU/mL; 4, 10^3 CFU/mL; 5, 10^4 CFU/mL; 6, 10^5 CFU/mL; 7, 10^6 CFU/mL; 8, control 10^6 UFC/mL in deionized water; 9, negative control (unseeded estuary water); A, positive control (DNA from *E.coli* HB 101::Tn5 *tac1*); B, negative PCR control (no DNA); and M, 100-bp DNA size ladder. Three different PCR experiments were performed with nucleic acids obtained from three independent extractions.

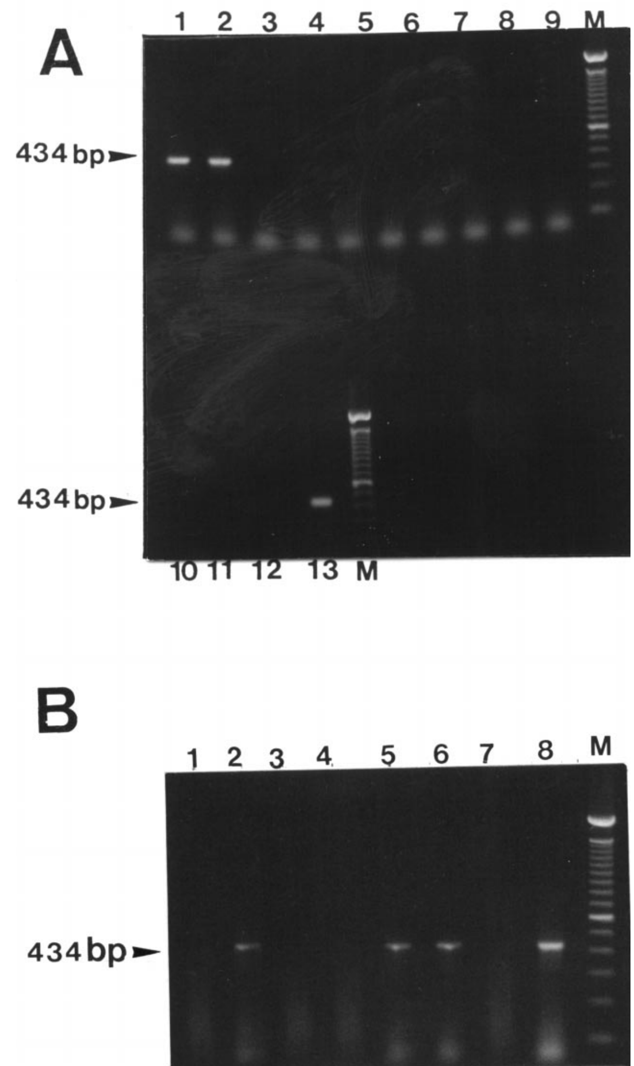

Figure 3. Detection of enterovirus in estuary water. Nucleic acids were extracted as described in *figure 1* from 11 samples collected from the Seine estuary (France) between Pk 847 and Pk 1000 in July 1995. Each extract was tested for enterovirus and *Salmonella*. **A.** RT-PCR amplification of conserved sequence of enteroviruses (434 bp) from various extracts of the estuary water samples. Lanes 1–11, estuary samples; 12, negative control (deionized water); 13, positive control (poliovirus RNA); and M, 100-bp DNA size ladder. **B.** RT-PCR amplification of conserved sequence of enteroviruses (434 bp) from the three different fractions from estuarine water of the two positive samples (corresponding to lanes 1–2, *figure 3A*). Lanes 1–2, acellular washing supernatant containing viruses and lysed cells previously associated with particles; 3–4, pellets of bacteria cells; 5–6, crude supernatant containing free viruses and lysed cells; 7, negative control (deionized water);. 8, positive control (poliovirus RNA); and M, 100-bp DNA size ladder. Lanes 1, 3, and 5, corresponding to positive sample of lane 1, (*figure 3A*) 2, 4, and 6, corresponding to positive sample of lane 2 (*figure 3A*).

Salmonella in the DNA extract corresponding to free cells or cells previously associated with particles. No amplification was observed with DNA extracted from crude supernatants, corresponding to the contents of lysed bacteria *(figure 4B)*. These results suggest that the bacteria were in a viable but nonculturable state and probably intimately associated with the biofilm surrounding organo-mineral particles.

4. Discussion

Several methods for DNA extraction from soil and sediments have been described but there is no widely used method suitable for environmental water, especially estuarine water [7, 14, 19]. For most studies of water samples, nucleic acids were only extracted from bacteria, isolated notably by centrifugation [6]. Planktonic bacteria from marine water have been isolated on 0.2-μm filters [3, 5] but this method is not suitable for estuarine water, which is too rich in suspended matter. Thus, some authors suggest DNA extraction from sediments of environmental water, but this would prevent the study of viruses and cells not associated with organo-mineral particles. Alternatively, direct extraction of nucleic acids is common for soil samples [10, 15]. But in this case, additional purification steps are required to eliminate PCR inhibitors, which decrease the yield of nucleic acid extraction [5, 14, 20, 21]. Furthermore, we show that such a procedure is less well suited to estuarine water, probably due to higher nuclease activity or chemical pollutant levels resulting from concentration by lyophilization.

We developed a standardized protocol for the extraction of total nucleic acids from estuarine water for subsequent PCR or RT-PCR detection of both viruses and bacteria. This method is adapted to the nature and organization of the microflora in estuarine water which commonly contains suspended matter colonized by microorganisms [8, 19]. An important aspect of the microbial population of estuarine water is the presence of bacteria and viruses, free or partially dissociated from particles forming low

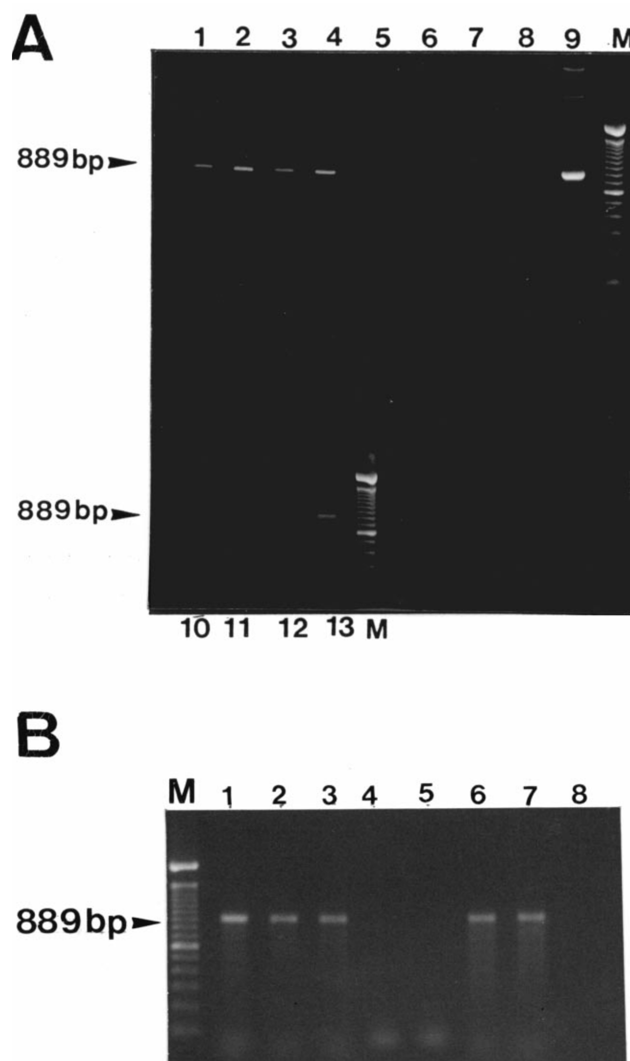

Figure 4. Detection of *Salmonella* in estuary water. Nucleic acids were extracted as described in *figure 1* from 11 samples collected from the Seine estuary (France) between Pk 847 and Pk 1000 in July 1995. Each extract was tested for enterovirus and *Salmonella*. **A.** PCR amplification of *Salmonella fliC* gene (889bp) from various extracts of estuary samples: lanes 1–11, estuary samples; 12, negative control (deionized water); 13, positive control (*Salmonella* DNA) ; and M, 100-bp DNA size ladder. **B.** PCR amplification of *Salmonella fliC* gene (889 bp) from sample where no *Salmonella* was detected by standard culture method (# 4, *figure 3A*). The three different fractions from estuarine water were PCR-analyzed; lanes 2–3, acellular washing supernatant containing viruses and lysed cells previously associated with particles; 4–5, crude supernatant containing free viruses and lysed cells; 6–7, pellets of bacteria cells; 8, negative control (deionized water); 1, positive control (*Salmonella* DNA); and M, 100-bp DNA size ladder.

density biofilms. We therefore extracted nucleic acids from three fractions of the water sample as follows: i) crude supernatant containing free viruses and lysed cells; ii) acellular washing supernatant containing viruses and lysed cells previously associated with particles; and iii) pellets of bacteria cells which has been free or associated with particles. Although initial sample centrifugations make this method slightly longer than direct lysis protocols (16 h), a greater amount of total nucleic acid was extracted, which allowed further PCR and RT-PCR analysis. Moreover, if the speed of the first centrifugation is decreased it is possible to separate particle-associated bacteria from free cells. The procedure we proposed herein could therefore also be used to compare these two distinct populations in estuary water samples.

The quality of nucleic acids thus extracted was sufficient for PCR and RT-PCR analysis. Inhibitors such as humic compounds and chemical pollutants were removed by PVP treatment to avoid the additional purification step suggested by some authors. [5, 14, 20, 21]. Nevertheless, purification of nucleic acid extracts with an Elutip-d column gave greater PCR efficiency, especially for samples from chemically polluted sites (data not shown), but there was a substantial reduction in the nucleic acid extraction yield.

Finally, this method seemed especially adapted for molecular epidemiology because it is possible to coextract genomic DNA and RNA of both viruses and bacteria. Moreover, for studying pathogenic bacteria, it was possible to avoid interference from DNA free in the estuarine water by analyzing only fractions corresponding to entire cells, whether associated or not with organo-mineral particles (i.e. fractions ii and iii described above).

DNA is rarely purified from organisms in estuarine water. Most studies address only sediments. The protocol proposed herein may allow the simultaneous study of bacteria and viruses, even RNA viruses, which make up the estuarine microbial population.

Résumé — Extraction des acides nucléiques d'eaux estuariennes polluées pour la détection de virus et de bactéries par PCR et RT-PCR. Nous proposons une méthode d'extraction des acides nucléiques adaptée à la recherche, par la technique de PCR, de bactéries et de virus présents dans les eaux d'un estuaire pollué. Les microorganismes présents dans un estuaire se trouvent soit à l'état libre dans les eaux, soit sous forme de biofilms de faible densité ou encore associés aux sédiments facilement décantables. Le protocole proposé permet d'extraire à la fois les ADN et les ARN génomiques de l'ensemble de ces virus et bactéries. Le seuil de détection de la technique, déterminé à partir d'échantillons d'eaux de la Seine supplémentés en microorganismes marqueurs, est de 1 UFC/mL pour les bactéries et de 10 virions/mL pour les virus. Une exploitation de cette technique est présentée pouvant aller de la détection moléculaire simultanée de *Salmonella* et de poliovirus dans les eaux de la Seine à l'étude de la localisation de ces microorganismes dans les sédiments. © Elsevier, Paris

extraction d'ADN / analyse par PCR et RT-PCR / eau d'estuaire

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