

Comparison of Culture Methods for Isolation of Nontuberculous Mycobacteria from Surface Waters^{∇†}

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The environment is the likely source of most nontuberculous mycobacteria (NTM) involved in human infections, especially pulmonary, skin, and soft tissue infections. In order to measure the prevalence of NTM in different aquatic ecosystems, we tried to standardize the culture methods used for surface water testing since many procedures have been described previously. Cultivation of mycobacteria requires long-term incubation in rich media and inactivation of rapidly growing microorganisms whose growth impedes observation of mycobacterial colonies. Consequently, the two criteria used for evaluation of the methods examined were (i) the rate of inhibition of nontarget microorganisms and (ii) the efficiency of recovery of mycobacteria. We compared the competitive growth of *Mycobacterium chelonae* and *M. avium* with nontarget microorganisms on rich Middlebrook 7H11-mycobactin medium after treatment by several chemical decontamination methods that included acids, bases, detergent, or cetylpyridinium chloride (CPC) with and without an antibiotic cocktail, either PANTA (40 U/ml polymyxin, 4 µg/ml amphotericin B, 16 µg/ml nalidixic acid, 4 µg/ml trimethoprim, and 4 µg/ml azlocillin) or PANTAV (PANTA plus 10 µg/ml vancomycin). Our results showed that treatment for 30 min with CPC (final concentration, 0.05%) of water concentrated by centrifugation, followed by culture on a rich medium supplemented with PANTA, significantly decreased the growth of nontarget microorganisms (the concentrations were $6.2 \pm 0.4 \log_{10}$ CFU/liter on Middlebrook 7H11j medium and $4.2 \pm 0.2 \log_{10}$ CFU/liter on Middlebrook 7H11j medium containing PANTA [$P < 0.001$]), while the effect of this procedure on NTM was not as great (the concentrations of *M. chelonae* on the two media were $7.0 \pm 0.0 \log_{10}$ CFU/liter and $6.9 \pm 0.0 \log_{10}$ CFU/liter, respectively, and the concentrations of *M. avium* were $9.1 \pm 0.0 \log_{10}$ CFU/liter and $8.9 \pm 0.0 \log_{10}$ CFU/liter, respectively). We propose that this standardized culture procedure could be used for detection of NTM in aquatic samples.

It is generally accepted that environmental exposure, particularly exposure through water, is the main source of most human infections caused by nontuberculous mycobacteria (NTM). The incidence of waterborne NTM skin and soft tissue infections in immunocompetent patients is increasing (31), as is the incidence of pulmonary infections that occur due to aerosol inhalation (15, 31). Ingestion or inhalation of contaminated water (while swimming, for instance) could also be a source of NTM infections in children (31). Because NTM are emerging pathogens for humans and domestic animals, it is important to identify their environmental sources and reservoirs and to measure their proliferation and persistence in freshwater ecosystems. A robust and standardized method for environmental detection of NTM is necessary to do this.

NTM are ubiquitous and can be isolated from a variety of

aquatic ecosystems, including natural water, wastewater, drinking water, recreational water, and industrial water (16, 51). Even hospital water has been reported to be contaminated by NTM (31). More precisely, aquatic plants, amoebae, and aquatic vertebrates and invertebrates could be considered NTM reservoirs in aquatic ecosystems in natural environments and in drinking water distribution systems or buildings and homes (19, 26, 37). Once present in a system, mycobacteria may proliferate and persist (4).

Typically, the methods usually used for detection of NTM are methods that are used for clinical microbiology and have not been adapted for environmental samples. Surface water samples are quite different from clinical samples, since they may contain low levels of NTM but typically contain highly diverse bacterial communities in which the concentrations of bacteria range from 10^4 to 10^7 cells per ml (54). This microbial diversity makes it likely that nontarget species will overgrow NTM in nutrient-rich medium. Several studies have been conducted to determine the optimum decontamination method for inhibiting the growth of nontarget bacteria in NTM assays, although most of the methods were developed for clinical samples (2, 8, 20, 42, 56). Moreover, no clear consensus for treatment of environmental samples has emerged from these studies. The combination of chemical decon-

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TABLE 1. Chemical decontamination procedures for isolation of mycobacteria

Method	Decontamination reagent (concn)	Decontamination time (min)	Neutralization reagent (concn)
Löwenstein	H ₂ SO ₄ (4%)	15	NaOH (4%)
Petroff	NaOH (4%)	20	HCl (3%)
Tacquet-Tison	SDS (3%)-NaOH (1%) preheated at 37°C	30	H ₃ PO ₄ (1%)
Kubica	NaOH (2%)-N-acetyl-L-cysteine (0.5%)-Na ₃ C ₆ H ₅ O ₇ (1.5%)	15	NaKPO ₄ , pH 6.8 (67 mM)
Oxalic acid	(COOH) ₂ (5%)	30	NaOH (4%)
Cetylpyridinium chloride	CPC (0.10%)	30	H ₂ O

tamination and addition of antibiotics to culture medium has not been studied previously for water surface samples.

The aim of this study was to develop and validate an improved method for detecting and counting NTM in surface water. To do this, we compared the results for recovery of mycobacteria from water samples and inactivation of nontarget microorganisms (fungi and bacteria other than mycobacteria) when various antibiotics and chemical decontaminants were used.

MATERIALS AND METHODS

Sampling of surface water and artificial inoculation. Surface water samples were collected in triplicate between July 2008 and March 2009 from the Seine River (France) at water supply catchment sites near the Orly drinking water plant (DWP), Ivry DWP, and Joinville DWP. At each site, a 1-liter sample was collected in a sterile glass bottle used for bacterial analyses (Schott Duran). Samples were cooled to 4°C and transported to the laboratory within 6 h, and they were processed immediately after they arrived at the laboratory. The solids in each water sample were concentrated by centrifugation (7,000 × g, 15 min, 4°C), and the pellet was resuspended in a small volume of discarded supernatant, which was different in different experiments, as described below. Resuspended pellets were stored at 4°C before treatment.

To examine the recovery of mycobacteria, pellets were resuspended in 9 ml of supernatant and autoclaved for 20 min at 120°C before addition of 1 ml of a suspension containing a mixture of the fast-growing organism *Mycobacterium chelonae* ATCC 35752 (target cell titer, 10⁵ CFU/ml) and the slowly growing organism *M. avium* ATCC 25291 (target cell titer, 10⁷ CFU/ml) to obtain a final volume of 10 ml. To prepare the inoculum, the reference strains were cultivated on Löwenstein-Jensen medium (Bio-Rad) and transferred to API AUX medium (bioMérieux). Using a McFarland standard (a McFarland density of 3.0 corresponded to 10⁸ CFU/ml), the two strains were diluted appropriately with sterilized water and mixed. Because we had no information about the rate of cell loss with each decontamination method and in order to count cells after decontamination treatments, we used a high density of NTM that did not reflect the actual density in contaminated water. Inoculated autoclaved samples were designated “artificial samples.” For all other experiments, centrifuged pellets from environmental samples were resuspended in 10 ml of supernatant, and the resulting samples are referred to below as “natural samples.” The final volume of these samples was the same of that of the artificial samples described above.

Supplementation of media and growth conditions. All samples were inoculated onto Middlebrook 7H11j solid medium. This medium was prepared by supplementing Middlebrook 7H10 medium with 10% (vol/vol) oleic acid-albumin-dextrose-catalase enrichment (Becton Dickinson), 0.5% (vol/vol) glycerol, and 2 µg/ml mycobactine J (Synbiotics Corporation). Middlebrook 7H11j-PANTA medium was prepared by adding 2.0% (vol/vol) reconstituted PANTA PLUS antibiotic supplement (Becton Dickinson) to obtain the following final concentrations of antibiotics in the culture medium: 40 U/ml of polymyxin, 4 µg/ml of amphotericin B, 16 µg/ml of nalidixic acid, 4 µg/ml of trimethoprim, and 4 µg/ml of azlocillin. Middlebrook 7H11j-PANTAV medium was prepared by adding 2.0% (vol/vol) reconstituted PANTA PLUS antibiotic supplement and 10 µg/ml of vancomycin (Sigma) to Middlebrook 7H11j medium.

Sterility (no contamination) and fertility (growth of *M. chelonae* ATCC 35752) on these three media were controlled by streaking sterilized water and cell suspension, respectively. In order to study the impact of antibiotics on NTM and nontarget microorganisms, artificial and natural samples from Orly, Ivry, and Joinville were inoculated onto Middlebrook 7H11j medium without antibiotics,

Middlebrook 7H11j-PANTA medium, and Middlebrook 7H11j-PANTAV medium.

Chemical decontamination procedures. Artificial and natural samples from the Orly site were independently treated using six different methods and inoculated onto Middlebrook 7H11j medium, Middlebrook 7H11j-PANTA medium, and Middlebrook 7H11j-PANTAV medium in order to study the impact of chemical decontamination methods on recovery of NTM and inhibition of nontarget microorganisms.

Chemical decontamination was performed by using the following steps: decontamination, agitation, centrifugation, and resuspension. Six chemical decontamination methods, designated the Löwenstein (5), Petroff (30), Tacquet-Tison (41), Kubica (23), oxalic acid (56), and cetylpyridinium chloride (CPC) (28) methods, were used in this study (Table 1). Equal volumes of decontamination reagents were added to 50-ml tubes containing 10 ml of a resuspended pellet, and then the samples were shaken at 20°C in a Kahn agitator for the required time. Neutralization was checked by adding 2 drops of litmus pH indicator, except for the Kubica and CPC methods, for which the volume of the neutralization reagent was 10 ml. The suspensions were then centrifuged (3,000 × g, 15 min, 4°C), and each pellet was resuspended in 2 ml of sterile distilled water before 10-fold dilution and inoculation onto Middlebrook 7H11j medium with or without antibiotics. A control treatment in which sterile water was used as the decontamination solution was included in order to determine the initial level of contamination.

Counting method and statistical analyses. After the chemical decontamination treatments, natural and artificial samples were diluted 10-fold in sterilized water, inoculated onto media, and incubated at 30°C in order to count nontarget microorganisms and the reference NTM. For natural water samples, colonies with different morphologies were counted on each plate and inoculated onto Löwenstein-Jensen medium that was incubated at 30°C for isolation. Mycobacteria and other acid-fast bacilli were detected with Ziehl-Neelsen acid-fast stain using a Quick-TB kit (Rutherford Appleton Laboratory). Acid-fast bacilli were then identified by partial sequencing of the 16S rRNA gene and the *hsp65* gene as described below. The nontarget microorganisms included non-acid-fast bacteria and acid-fast bacteria that did not belong to the mycobacteria. Statistical analyses of data were carried out using a generalized linear model (GLM) in the Statgraphics Plus 5.1 software (Manugistics Inc., Rockville, MD).

Identification of acid-fast bacilli recovered from freshwater samples. In order to identify the acid-fast bacilli isolated from natural samples, the 16S rRNA gene (3) and the *hsp65* gene (22) were partially sequenced. Acid-fast colonies grown on Löwenstein-Jensen medium were resuspended in 100 µl of 1× Tris-EDTA buffer (10 mM Tris [pH 7.6], 1 mM EDTA), and DNA was extracted by heat shock treatment (96°C for 15 min). After centrifugation (16,000 × g, 15 min, 4°C), the supernatant was used for PCR amplification. PCRs were carried out by using conditions described in a previous study (32) and using PCR primers and amplification cycles described previously (3, 22). Appropriate dilutions of amplicons were purified with a QIAquick PCR purification kit (Qiagen) and were marked separately in both senses with a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems). Products were purified with a DyeEx 2.0 spin kit (Qiagen), and sequencing was carried out with an ABI PRISM 3100 genetic analyzer used according to the manufacturer's recommendations (Applied Biosystems).

Consensus sequences were compared with sequences obtained from the GenBank (<http://www.ncbi.nlm.nih.gov/>), BIBI (<http://umr5558-sud-str1.univ-lyon1.fr/lebib/lebib.cgi>), and RIDOM (<http://www.ridom-rdna.de>) databases by performing phylogenetic analyses (data not shown).

Nucleotide sequence accession numbers. The consensus sequences of the 16S rRNA and *hsp65* genes of acid-fast bacilli have been deposited in the GenBank database under accession numbers FJ770974 to FJ770999.

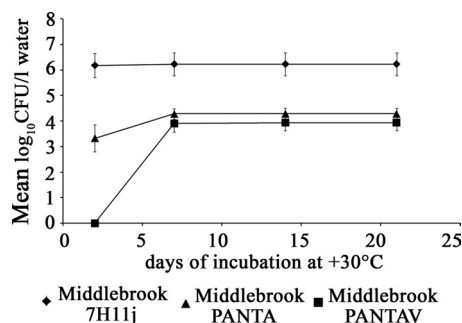


FIG. 1. Effect of addition of antibiotics to the medium on the numbers of growing nontarget microorganisms after different incubation times. Surface water samples were directly inoculated onto Middlebrook 7H11j medium with or without antibiotics. The error bars indicate standard deviations of the means.

RESULTS

Effect of addition of antibiotics to the culture medium. (i) Impact on overgrowth of nontarget microorganisms. The maximum densities of nontarget microorganisms were observed after 48 h of incubation on both Middlebrook 7H11j and Middlebrook 7H11j-PANTA media (Fig. 1). No nontarget microorganisms were detected on Middlebrook 7H11j-PANTAV medium after 48 h of incubation, and the maximum densities were observed after 7 days. After 14 and 21 days of incubation, filamentous fungal growth had started and interfered with counting of nontarget microorganisms even on Middlebrook 7H11j medium containing antibiotics (Fig. 1).

Addition of PANTA and addition of PANTAV to Middlebrook 7H11j medium resulted in significant 100-fold decreases in the densities of nontarget microorganisms (Fig. 1) compared to the results for Middlebrook 7H11j medium without antibiotics ($F = 120.19$, $df = 2$, $n = 108$, and $P < 0.001$, as determined by repeated-measures analysis by GLM and controlled by sampling area and incubation time). On average, after 21 days of incubation, the concentrations of nontarget microorganisms were $6.2 \pm 0.4 \log_{10}$ CFU/liter on Middlebrook 7H11j medium, $4.2 \pm 0.2 \log_{10}$ CFU/liter on Middlebrook 7H11j-PANTA medium, and $3.9 \pm 0.3 \log_{10}$ CFU/liter on Middlebrook 7H11j-PANTAV medium. There was no significant difference between the results for PANTA treatment and the results for PANTAV treatment ($F = 3.04$, $df = 1$, $n = 72$, and $P = 0.086$, as determined by repeated-measures analysis by GLM and controlled by sampling area and incubation time).

GLM analysis showed that the sampling area and the antibiotic treatment explained the observed variation in the density of nontarget microorganisms ($F = 20.82$, $df = 4$, $n = 108$, and $P < 0.001$, as determined by repeated-measures analysis by GLM and controlled by incubation time). Indeed, the densities of nontarget microorganisms on Middlebrook 7H11j medium after 7 days of incubation were $6.5 \pm 0.1 \log_{10}$ CFU/liter, $5.7 \pm 0.0 \log_{10}$ CFU/liter, and $6.3 \pm 0.4 \log_{10}$ CFU/liter for the Orly, Ivry, and Joinville catchment sites, respectively ($F = 20.98$, $df = 2$, $n = 108$, and $P < 0.001$, as determined by repeated-measures GLM and controlled by medium supplementation and incubation time).

(ii) Impact on recovery of NTM. GLM analysis showed that addition of PANTA and addition of PANTAV significantly de-

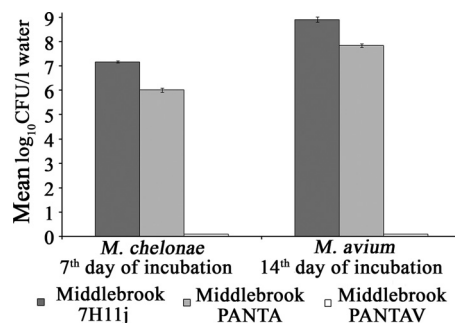


FIG. 2. Effect of addition of antibiotics to the medium on recovery of *M. chelonae* ATCC 35752 and *M. avium* ATCC 25291. The bars indicate the average numbers of mycobacteria recovered from artificial samples (9 experiments for each strain) inoculated onto Middlebrook 7H11j medium supplemented or not supplemented with antibiotics.

creased the densities of both strains examined ($F = 261.09$, $df = 2$, $n = 9$, and $P < 0.001$ for *M. chelonae*, as determined by GLM; $F = 52.99$, $df = 2$, $n = 9$, and $P < 0.001$ for *M. avium*, as determined by GLM). The densities of *M. chelonae* and *M. avium* were $7.17 \pm 0.04 \log_{10}$ CFU per liter and $8.91 \pm 0.10 \log_{10}$ CFU/liter, respectively, on Middlebrook 7H11j medium and $6.0 \pm 0.0 \log_{10}$ CFU/liter and $7.8 \pm 0.0 \log_{10}$ CFU/liter, respectively, on Middlebrook 7H11j-PANTA medium, whereas no growth was observed on Middlebrook 7H11j-PANTAV medium (Fig. 2).

Effect of chemical decontamination. (i) Impact on overgrowth of nontarget microorganisms. The effects of the differ-

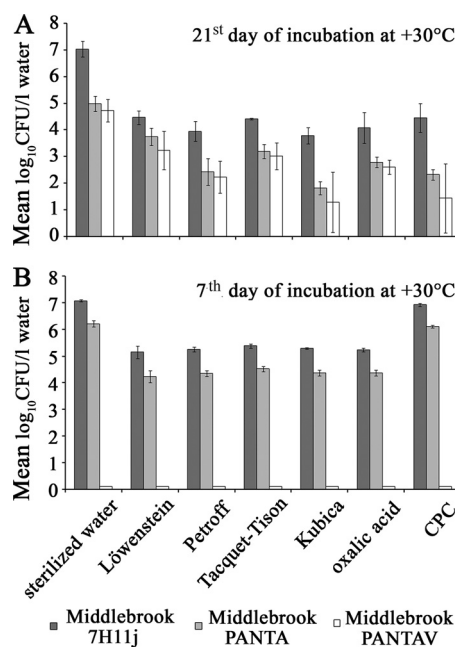


FIG. 3. Effect of chemical decontamination on (A) the numbers of nontarget microorganisms and (B) recovery of *M. chelonae* ATCC 35752. The average densities (numbers of CFU per liter) of nontarget microorganisms ($n = 378$) and of mycobacteria recovered from artificial samples ($n = 63$) isolated from Orly water samples inoculated onto Middlebrook 7H11j medium (with or without antibiotics) were determined after chemical decontamination. The error bars indicate standard deviations of the means.

ent chemical decontamination methods (Fig. 3A) were not significantly different ($F = 0.64$, $df = 5$, $n = 324$, and $P = 0.666$, as determined by repeated-measures GLM and controlled by medium supplementation and incubation time). The procedure used to count NTM CFU when there was no chemical decontamination treatment was hampered by the growth of non-acid-fast bacilli on Middlebrook 7H11j medium after 14 to 21 days of incubation and by the growth of fungi on Middlebrook 7H11j-PANTA and Middlebrook 7H11j-PANTAV media after 21 to 35 days. Compared to the results for the control treatment, every chemical decontamination treatment significantly decreased the density of the nontarget microorganisms ($F = 51.41$, $df = 6$, $n = 378$, and $P < 0.001$, as determined by repeated-measures GLM and controlled by medium supplementation and incubation time).

When antibiotics were not added, all chemical decontamination procedures resulted in an average count of $4.1 \pm 0.4 \log_{10}$ CFU/liter of nontarget microorganisms on Middlebrook 7H11j medium, which corresponded to a decrease of about 3 \log_{10} CFU/liter compared to the results for the control without a decontamination treatment on Middlebrook 7H11j medium (Fig. 3A). The combination of chemical decontamination and addition of antibiotics to the medium was more effective, because we observed a decrease of 4 to 4.5 \log_{10} CFU/liter in the level of nontarget microorganisms compared to the results for the control on Middlebrook 7H11j medium. All chemical decontamination procedures resulted in concentrations of $2.7 \pm 0.6 \log_{10}$ CFU/liter on Middlebrook 7H11j-PANTA medium and $2.4 \pm 0.7 \log_{10}$ CFU/liter on Middlebrook 7H11j-PANTAV medium (Fig. 3A).

(ii) Impact on NTM recovery. Compared to the results for the control treatment, all chemical decontamination methods (Löwenstein, Petroff, Tacquet-Tison, Kubica, oxalic acid, and CPC methods) significantly inhibited the growth of *M. chelonae* ($F = 276.66$, $df = 6$, $n = 63$, and $P < 0.001$, as determined by repeated-measures GLM and controlled by medium supplementation) and *M. avium* ($F = 59.11$, $df = 6$, $n = 63$, and $P < 0.001$, as determined repeated-measures GLM and controlled by medium supplementation). When the control treatment was removed from the GLM analysis, the impact of the CPC method was significantly less negative for *M. chelonae* ($F = 216.89$, $df = 5$, $n = 54$, and $P < 0.001$, as determined by GLM and controlled by medium supplementation) and *M. avium* ($F = 37.30$, $df = 5$, $n = 54$, and $P < 0.001$, as determined by GLM and controlled by medium supplementation) on Middlebrook 7H11j medium and Middlebrook 7H11j-PANTA medium. Indeed, after the control treatment $7.0 \pm 0.0 \log_{10}$ CFU/liter of *M. chelonae* and $9.1 \pm 0.0 \log_{10}$ CFU/liter of *M. avium* were recovered using Middlebrook 7H11j medium, whereas after the CPC treatment $6.9 \pm 0.0 \log_{10}$ CFU/liter of *M. chelonae* (Fig. 3B) and $8.9 \pm 0.0 \log_{10}$ CFU/liter of *M. avium* were recovered using this medium (data not shown). When vancomycin was added, there was no growth of either strain on Middlebrook 7H11j-PANTAV medium after all chemical decontamination treatments (Fig. 3B).

Isolation and identification of NTM from surface water. Thirteen acid-fast bacillus isolates were collected from the water samples and are described in Table S1 in the supplemental material. After phylogenetic analysis of the 16S rRNA and *hsp65* gene sequences (data not shown), 12 strains were

identified as mycobacteria, one strain was identified as *Rhodococcus* sp., and one strain was identified as *Nocardia* sp. (see Table S1 in the supplemental material). *M. chelonae* strains were regularly isolated from samples from the Orly catchment site on either Middlebrook 7H11j-PANTA medium or Middlebrook 7H11j-PANTAV medium (see Table S1 in the supplemental material), whereas no acid-fast bacilli were isolated from samples from the Ivry and Joinville catchment sites. However, the CFU quantities were less than 30 per plate, and thus the results did not allow reliable estimation of the densities of NTM at the Orly site (see Table S1 in the supplemental material).

Very diverse NTM were isolated from the Orly catchment site (8 different species). Rare species of mycobacteria (*M. psychrotolerans*, *M. setense*, *M. insubricum*, *M. porcinum*, *M. llatzerense*, *M. austroafricanum*, and *M. arupense*) were isolated sporadically from one of the triplicate samples on Middlebrook 7H11j, Middlebrook 7H11j-PANTA, or Middlebrook 7H11j-PANTAV medium after different kinds of chemical decontamination methods were used; *M. arupense*, *M. llatzerense*, *M. porcinum*, and *M. austroafricanum* were isolated after decontamination using the Tacquet-Tison method, *M. setense* and *M. psychrotolerans* were isolated after decontamination using the Löwenstein method, and *M. insubricum* was isolated after decontamination using the CPC method (see Table S1 in the supplemental material).

DISCUSSION

Our goal was to measure the effects of various methods known to inhibit the growth of nontarget microorganisms, while we also took into consideration the inhibitory effects of these methods on the growth of NTM. To our knowledge, this is the first time that addition of antibiotics to medium was combined with chemical decontamination (alone or in combination with addition of antibiotics during decontamination) and used to culture NTM from environmental water samples. We developed this bacteriological tool in order to use it for an extensive environmental analysis. Statistical analyses of our results demonstrated that the presence of PANTA significantly decreased the level of nontarget microorganisms, without being too aggressive against reference NTM. Nevertheless, the PANTA added did not remove all nontarget microorganisms from 1 liter of surface water. It does not seem advisable to decrease the sampling volume in order to avoid nontarget microorganisms, because decreasing the sampling volume would lead to a decrease in the lower limit of NTM detection. Chemical decontamination appeared to be necessary for highly sensitive detection of NTM. Decontamination with CPC appeared to be the best decontamination method since, on the one hand, it significantly decreased the level of nontarget microorganisms and, on the other hand, it was significantly less lethal for the NTM strains studied. Moreover, addition of PANTA and CPC decontamination were more efficient together than separately in terms of the objectives mentioned above. A statistical approach helped us determine the most appropriate method for NTM isolation, although the low numbers of NTM isolated from surface water did not allow accurate estimation of either the number or the diversity of NTM in the habitat.

Although filtration has been reported to be more effective than centrifugation for isolation of mycobacteria from drinking water (42), we used centrifugation for the Seine River samples due to the rapid clogging of filters by large quantities of suspended particles (20 to 92 mg/liter) (17). Different culture media have been used to isolate mycobacteria, but there has been no consensus (39). However, nutrient-rich Middlebrook 7H11 medium and a low incubation temperature ($\sim 32^{\circ}\text{C}$) seem to result in recovery of the widest range of *Mycobacterium* spp. (39). Reports have indicated that the sensitivity of Middlebrook 7H11 medium is improved by addition of mycobactine J (13, 40), and the specificity can be improved by addition of the PANTA antibiotic cocktail. The PANTA additive was initially used for detection of mycobacteria in clinical samples using BACTEC (Becton Dickinson) liquid medium detection systems (36). Two previous studies of *M. avium* subsp. *paratuberculosis* prevalence showed that PANTA used without a preliminary chemical decontamination step successfully suppressed the growth of background microorganisms associated with Cheddar cheese (13) and with raw milk cheeses (38). Additionally, inhibition of nontarget microorganisms in culture medium could be improved by addition of vancomycin at a final concentration of 8 $\mu\text{g/ml}$ (13), 10 $\mu\text{g/ml}$ (11), or 20 $\mu\text{g/ml}$ (1). In the present study, addition of the PANTA antibiotic cocktail appeared to significantly decrease the level of nontarget microorganisms, but it did not inhibit the growth of *M. chelonae* and *M. avium* so that they could not be quantified. Two previous studies of the prevalence of *M. avium* subsp. *paratuberculosis* in Cheddar cheese (13) and in raw milk cheeses (38) showed that addition of PANTA to Middlebrook 7H11j medium effectively removed nontarget microorganisms. However, we found that addition of PANTA was not sufficient to allow accurate enumeration of NTM in surface water. The concentrations of bacteria in raw milk cheeses usually range from 10^8 to 10^9 cells per ml depending on the ripening time (6), while freshwater usually contains between 10^4 and 10^7 cells per ml (54). Thus, it seems that the concentrations of nontarget microorganisms do not explain the finding that addition of PANTA was not sufficient to allow enumeration of NTM in surface water. In contrast, the higher diversity of microorganisms in surface water (21, 55) than in raw milk cheeses (34) could explain the finding that addition of PANTA was not sufficient to allow enumeration of NTM in surface water.

Addition of vancomycin to PANTA did not seem to be useful for counting NTM in surface water samples, because there was no significant difference between the effect of PANTA addition and the effect of PANTAV addition on removal of nontarget microorganisms. Moreover, on Middlebrook 7H11j-PANTAV medium, the growth of the *M. chelonae* and *M. avium* reference strains was significantly inhibited by vancomycin. Although these results cannot be extended to other NTM species that might be isolated from natural samples (some species may be less sensitive to vancomycin), vancomycin should not be added to media used for general NTM detection.

Our study showed that the CPC method was the most effective method studied for counting NTM in surface waters. This is in agreement with the results of Neumann et al., who showed that the CPC method was appropriate for decontamination of surface water (28). We found that CPC decontamination sig-

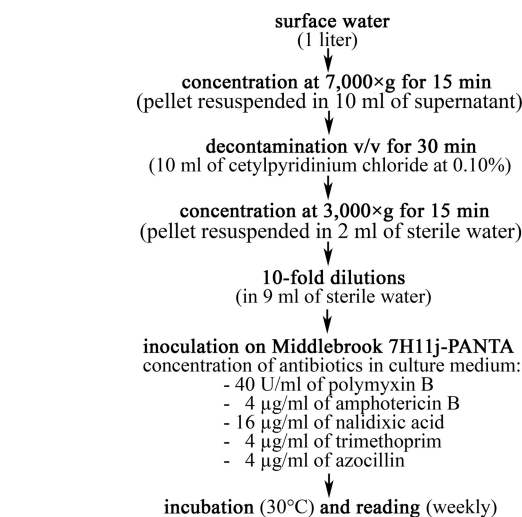


FIG. 4. Proposed microbial culture procedure for detection and measurement of mycobacteria in freshwater samples.

nificantly reduced the level of nontarget microorganisms, while it resulted in a smaller decrease in the density of NTM than the Lowenstein, Petroff, Tacquet-Tison, Kubica, and oxalic acid methods. Moreover, CPC can be used at different concentrations for decontamination of samples containing low (0.005% CPC) or high (0.05% CPC) levels of contaminating organisms (28). This method has been used mainly for samples with low levels of contamination, such as drinking water (49), water from distribution systems (29, 52), and tap water artificially inoculated with NTM (42). In our study, treatment with 0.05% CPC without antibiotics resulted in survival rates of 71.1% for *M. chelonae* and 70.0% for *M. avium*. In contrast, Thomson et al. reported that addition of 0.005% CPC to tap water samples spiked with *M. avium* and *M. intracellulare* (100 CFU/500 ml) resulted in a survival rate of only 3.6% for both strains (42). However, it is difficult to compare the results of these workers with our results, because the inocula (NTM densities and species), final concentrations of CPC (0.005% and 0.05%), and types of samples (surface water and tap water) were different.

Our study demonstrated for the first time that chemical decontamination and addition of PANTA to culture media are more effective together than separately for decreasing the concentrations of nontarget microorganisms. We thus recommend use of CPC decontamination (final concentration, 0.05%) combined with addition of PANTA to Middlebrook 7H11j medium for isolating and/or counting NTM in highly contaminated samples, such as surface waters (Fig. 4).

The diversity of NTM in the Orly water supply catchment was high, and the isolates included species such as *M. austroafricanum*, *M. porcinum*, and *M. chelonae*, as well as other more recently described species, such as *M. arupense*, *M. insubricum*, *M. llutzerense*, *M. psychrotolerans*, and *M. setense*. *M. chelonae* was the predominant NTM in the Seine River at the Orly sampling site. This species has been isolated previously from a water distribution system (51), tap water (12), treated surface water or groundwater (25), nail salon water (35), and public swimming pools (31). *M. chelonae* is an opportunistic pathogen that is able to cause pulmonary infections, cutaneous infections

after direct contact with contaminated water (35), or iatrogenic infections after surgery or subcutaneous injection (7, 33). Consequently, its presence in the water supply catchment may be a concern for water production in Paris.

Except for *M. psychrotolerans*, all of the strains isolated from the Orly water supply catchment were potential pathogens (10, 18, 24, 43, 44, 47, 48, 50, 53). These results show that water supply sources may contain very diverse NTM that could be potentially harmful to humans. In addition to NTM, the Seine River contained other harmful pathogens, including *Cryptosporidium* sp., *Giardia* sp., and *Salmonella* sp. (27, 46). Processes associated with potable water cycling and distribution or treatment and reuse of wastewater treatment plant rejects might be significant vehicles for NTM dissemination. Tap water has been suspected or identified as the source of infection in several cases (9, 14, 33, 45). Thus, determining the abundance of NTM in water resources might be an important public health issue. Our culture method might be useful for this purpose.

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