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Comparison of Methods for Isolation of Mycobacteria from Water

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Twelve methods for the isolation of mycobacteria were compared by applying them in parallel to 26 samples of surface water and 109 samples of treated water. Each method was defined by a particular combination of decontamination method, growth medium, and incubation temperature. For the decontamination of surface water, we used cetylpyridinium chloride (CPC) (30 min, 0.05%), as well as sample preincubation in tryptic soy broth (TSB) followed by decontamination with a cocktail of NaOH, cycloheximide, and malachite green. Treated water was decontaminated with 0.005 and 0.05% CPC (30 min). After enrichment by filtration, all samples were incubated on Löwenstein-Jensen medium (LJ), Ogawa egg yolk medium (OEY), and Ogawa whole-egg medium containing ofloxacin and ethambutol (OEOE) at temperatures of 30 and 37°C. The efficacy of each method was determined by calculating the positivity rate, negativity rate, contamination rate, mean number of mycobacterial colonies grown, and mean number of different mycobacterial strains isolated. The last value was determined by subjecting the isolates to PCR restriction analysis and mycolic acid thin-layer chromatography. Statistical analysis demonstrated that both the TSB method and 0.05% CPC were appropriate for the decontamination of surface water. Decontamination with 0.005% CPC was best for treated water. The results for incubation on LJ were at least equal to those for incubation on OEY and always superior to the results with OEOE. At an incubation temperature of 30°C, all methods achieved higher yields than at 37°C.

Mycobacteria other than the *Mycobacterium tuberculosis* complex and *M. leprae* are often referred to as atypical or nontuberculous mycobacteria. Some of them, such as members of the *M. avium* complex and *M. kansasii*, have been reported to cause infections and disease with increasing frequency, especially in immunocompromised patients (6, 13, 25, 26, 28), and are thought to be transmitted not from person to person but from the inanimate environment (10, 13, 17, 25, 26); hence, they have been designated potentially pathogenic environmental mycobacteria (25). Water apparently plays a major role, as it is the natural habitat as well as the source and vector of transmission of this group of organisms (3, 4, 13, 20, 23, 24), and further mycobacteriological studies of water may contribute to our knowledge about their ecology and epidemiology.

For the isolation of mycobacteria from water, many studies have employed minor modifications of methods developed for the diagnosis of tuberculosis, but these seem to be inadequate for environmental samples. Thus, only a small percentage of environmental mycobacteria will survive pretreatment with higher concentrations of sodium hydroxide (NaOH) (4, 9, 19), a decontamination procedure widely employed for sputum samples. Likewise, standard growth media such as Löwenstein-Jensen (LJ) medium have been developed for cultivation of members of the *M. tuberculosis* complex, but these media have a pH of 7.0, which lies well above the pH optimum of 5.4 to 6.5 found for many atypical mycobacteria (16). Moreover, incubation periods of 6 to 8 weeks are usually chosen for the primary isolation of *M. tuberculosis*, whereas for environmental mycobacteria the incubation periods may have to be prolonged for up to 6 months (8, 15).

Only a few authors have tried to compare different methods for the isolation of mycobacteria from environmental samples. Two studies compared decontamination methods and found formaldehyde (2) or cetylpyridinium chloride (CPC) (19) to be more suitable for the isolation of mycobacteria from water samples than NaOH and other substances. Portaels et al. (15) compared different decontamination methods and culture media for the isolation of mycobacteria from soil specimens collected in Louisiana. They obtained the best results by preincubation of the soil samples in tryptic soy broth (TSB), followed by decontamination with malachite green, cycloheximide, and NaOH and by inoculation of the samples onto Ogawa medium containing cycloheximide. Kamala et al. (9) also compared different methods for isolating mycobacteria from samples of soil and water taken in Madras in southern India. Those authors found that decontamination with 1% NaOH and 3% sodium lauryl sulfate was significantly superior to the use of higher concentrations of NaOH and other decontamination procedures and that inoculation of LJ medium gave higher mycobacterial yields than inoculation of Falkinham's selective medium, a minimal agar medium containing Tween 80 as the sole carbon source (5). The Madras study also suggested that a larger spectrum of mycobacterial strains is isolated with 37°C rather than 30°C used as the incubation temperature. In a mycobacteriological study of tap water samples from Berlin, Germany, Peters et al. (13) obtained the highest isolation rates and numbers of isolates by decontaminating with CPC rather than with a combination of NaOH and *N*-acetyl-L-cysteine and by incubating the concentrates on LJ medium rather than on Middlebrook 7H10 agar or in BACTEC broth.

There are certain limitations in the designs of the two studies of water samples mentioned above. In the Madras study (9), small numbers of water samples were tested in parallel (maximum number, 15). Furthermore, a great variety of water samples (from taps, wells, and water coolers) were processed by using the same methods, and the volumes processed are not exactly specified. In the Berlin study (13), 59 tap water samples were tested in parallel, but the results were not subjected to

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TABLE 1. Isolation methods evaluated

Isolation method	Decontamination	Medium	Incubation temp (°C)	Water[a]
A	CPC, 0.005%	LJ	30	TW
B	CPC, 0.005%	LJ	37	TW
C	CPC, 0.005%	OEY	30	TW
D	CPC, 0.005%	OEY	37	TW
E	CPC, 0.005%	OEOE	30	TW
F	CPC, 0.005%	OEOE	37	TW
G	CPC, 0.05%	LJ	30	TW, SW
H	CPC, 0.05%	LJ	37	TW, SW
I	CPC, 0.05%	OEY	30	TW, SW
K	CPC, 0.05%	OEY	37	TW, SW
L	CPC, 0.05%	OEOE	30	TW, SW
M	CPC, 0.05%	OEOE	37	TW, SW
N	TSB	LJ	30	SW
O	TSB	LJ	37	SW
P	TSB	OEY	30	SW
Q	TSB	OEY	37	SW
R	TSB	OEOE	30	SW
S	TSB	OEOE	37	SW

[a] Type of water for which the method was used. SW, surface water; TW, treated water.

statistical analysis. In addition, only one temperature (37°C) was chosen for incubation. In both studies, primary isolation was carried out by incubating the growth media for 8 weeks or, in the case of BACTEC broth used in the Berlin study, until a growth index of ≥100 was reached.

The objective of the present study was to further optimize methods for isolating environmental mycobacteria from water by the parallel application of 12 isolation methods. The study was conducted in such a way as to ensure, as the criteria of evaluation, exact quantitative figures for the numbers of mycobacterial CFU and the spectra of different mycobacterial strains isolated. It was found necessary to consider methods for the investigation of treated water and surface water independently from each other, since these types of water may differ considerably in the extents and spectra of microbial colonization.

MATERIALS AND METHODS

Samples. A total of 135 water samples were taken between July 1993 and August 1994 in three different regions of Germany (North Rhine-Westphalia, Saxony, and Berlin). The mean pH was 7.63 (standard deviation [SD], 0.42). With regard to the isolation methods, two groups of samples were distinguished. The first group consisted of 26 surface water samples from drinking water reservoirs (n = 4) or from rivers, brooks, and ponds (n = 22). The second group (referred to as the group of treated water samples) consisted of 109 samples of water either taken from different water works (three in North Rhine-Westphalia, two in Saxony, and one in Berlin) after having undergone incomplete treatment such as flocculation and filtration but before chlorination (n = 44) or taken from different peripheral domestic outlets (taps) after complete drinking water treatment (n = 65). Of the 65 tap water samples, 40 were taken from cold water distribution systems (temperature of water samples, 5 to 25°C; mean temperature, 15.1°C [SD, 4.5°C]), and 15 were taken from systems with heated water (sample temperature, 30 to 68°C, mean temperature, 47.8°C [SD, 9.0°C]). The samples were cooled to 4 to 8°C and processed within 1 week.

Isolation methods. All samples were subjected in parallel to 12 of the isolation methods listed in Table 1, with each method being characterized by a particular combination of decontamination technique, growth medium, and incubation temperature. Methods A through M were applied to treated water samples, of which 100 ml was processed for each isolation method, whereas methods G through S were applied to samples of surface water, of which 10 ml was processed for each isolation method, as they were expected to contain higher concentrations of mycobacteria and nonmycobacterial contaminants.

Decontamination methods. Two different decontamination methods were employed. The CPC method was carried out as described previously (19) by adding CPC to the samples to give a final concentration of 0.005 or 0.05% and shaking the mixture for 30 s. After an exposure time of 30 min, the samples were immediately filtered (cellulose acetate membrane filters [no. 11106-50-ACN; Sartorius, Göttingen, Germany]; diameter, 50 mm; pore size, 0.45 μm) and rinsed with 100 ml of sterile water to remove residual CPC. A strip 10 mm wide was then aseptically cut out from the center of the filter and placed on the medium.

The TSB method employed was a modification of the method described by Wolinsky and Rynearson (27) and Portaels et al. (15). Ten milliliters of the sample was mixed with the same volume of double-concentrated TSB (catalog no. 0370-01-1; Difco, Detroit, Mich.) and preincubated at 37°C for 5 h to allow spore-forming bacteria to germinate. If particles had sedimented during preincubation, the supernatant was transferred into a new sterile screw cap glass bottle. The following sterile aqueous solutions were then added to the mixture: 20 ml of malachite green oxalate (0.2%), 5 ml of cycloheximide (400 μg/ml), and 20 ml of NaOH (1 M [4.0%]). After exposure for 30 min, the mixture was acidified by carefully adding a 1 M (3.65%) solution of HCl until it lost its turbidity and the malachite green solution changed to yellow (pH 3). The mixture was then immediately filtered and further processed as described for the CPC method.

Growth media. For primary culture the following growth media were used: LJ medium containing glycerol (LJ) was purchased as slanted medium (catalog no. 913013; Biotest, Dreieich, Germany), Ogawa egg yolk medium (OEY) was prepared as slanted medium as described by Ogawa and Motomura (11), and Ogawa medium containing ofloxacin and ethambutol (OEOE) was prepared as Ogawa whole-egg slanted medium, as described by Tsukamura et al. (22), with addition of ofloxacin (final concentration, 2.5 mg/liter) and ethambutol (final concentration, 1 mg/liter) as described by Ichiyama et al. (7).

Incubation conditions. After inoculation, the media were incubated at 30 and 37°C for 12 weeks and checked for microbial growth at intervals of 2 weeks, taking care that the screw caps of the culture tubes were loose enough to allow a certain degree of gas exchange (judged by the gradual evaporation of the liquid collected on the bottom of the tubes) and tight enough to prevent visually observable desiccation of the media.

Isolation and characterization of mycobacterial strains. At the end of the 12-week incubation period, a representative number of the colonies grown were selected and characterized. The selection was done by picking at least one colony of each colony type grown in each tube. Different colony types were distinguished by their pigmentation, size, and surface structure. The colonies were checked for acid and alcohol fastness by the Ziehl-Neelsen technique, streaked out on Middlebrook 7H10 agar containing oleic acid-albumin-dextrose-catalase supplement (catalog no. 0627-17-4 and 0722-73-9; Difco), and again subcultured as pure cultures on the medium on which they had grown in primary culture. In cases where mycobacterial colonies were about to be overgrown by contaminants or other rapidly growing mycobacteria, colonies were picked before the incubation period was terminated.

All of the 586 mycobacterial strains isolated were tested for their pigmentation behavior and then characterized by mycolic acid thin-layer chromatography (TLC), as described previously (18). The procedure included alkaline hydrolysis of covalently bound cell wall lipids and esterification with iodomethane. The mycolic acid methyl esters were then developed on silica gel sheets with, as the solvent system, dichloromethane and a 95:5 mixture of petroleum ether and acetone.

In addition, all isolates were subjected to PCR restriction analysis (PRA), as described by Telenti et al. (21). For this purpose, a 439-bp fragment of the gene coding for the 65-kDa heat shock protein was amplified by PCR and digested with restriction endonucleases *Bst*EII and *Hae*III, yielding characteristic PRA restriction fragment patterns for different mycobacterial species. For some species (e.g., *M. gordonae* and *M. flavescens*) the procedure allows several subtypes to be distinguished.

Evaluation. Of the total number of samples tested by each isolation method, the positivity, negativity, and contamination rates were determined, which add up to 100% for each method. The positivity rate indicates the proportion of samples yielding mycobacterial colonies, the negativity rate indicates the proportion of samples not yielding any microbial growth, and the contamination rate indicates the proportion of samples from which no mycobacteria could be isolated during primary culture because of nonmycobacterial overgrowth (contamination) of the medium.

In addition, the total number of mycobacterial colonies grown and the number of different strains isolated were determined for each culture tube. Isolates were considered to be different if they had different PRA patterns. In rare cases in which no PRA patterns were obtained, the presence of different pigmentation behavior and mycolic acid TLC patterns was decisive. From these data, the mean concentration (CFU per liter) of mycobacteria found and the mean number of different mycobacterial strains isolated were determined for each method. For calculation of the mean values, culture tubes with and without mycobacterial growth were taken into consideration, excluding tubes that showed nonmycobacterial contamination or yielded confluent growth. All tubes in which no microbial growth was observed were included with a value of zero.

For statistical analysis of the results, McNemar's test was applied for comparing the positivity, negativity, and contamination rates of the isolation methods. This test was used to compare the positivity and the overall negativity rates (i.e., negativity plus contamination rates) of the methods in order to establish a rank list. The mycobacterial concentrations and numbers of different mycobacterial

TABLE 2. Performance of methods for the isolation of mycobacteria from treated water[a]

Isolation method	Rate (%)			n^b	Mean no. of:	
	Positivity	Negativity	Contamination		Mycobacterial CFU/liter	Different mycobacterial strains isolated from 100 ml
A	55.0	34.9	10.1	98	383	1.3
B	11.9	82.6	5.5	103	55	0.2
C	43.1	32.1	24.8	82	329	1.2
D	11.0	78.9	10.1	98	49	0.2
E	25.7	65.1	9.2	98	83	0.5
F	2.8	95.4	1.8	107	16	0.03
G	56.0	33.9	10.1	98	323	1.2
H	11.0	82.6	6.4	102	56	0.2
I	45.0	38.5	16.5	91	200	1.2
K	9.2	81.7	9.2	99	12	0.1
L	28.4	62.4	9.2	99	44	0.4
M	2.8	95.4	1.8	107	2	0.03

[a] For the evaluation, 109 samples were tested in parallel.
[b] n, number of samples tested to calculate the mean mycobacterial CFU per liter and the mean number of different mycobacterial strains, excluding samples in which contamination or confluent growth occurred.

strains were compared by using the Wilcoxon signed rank test. Both tests were performed with the Statistical Analysis Systems (SAS) program.

RESULTS

For each isolation method employed, Tables 2 and 3 give the positivity, negativity, and contamination rates, as well as the mean concentration of mycobacteria found and the mean number of different mycobacterial strains isolated.

In general, samples testing positive for mycobacterial growth by isolation methods with low positivity rates also tested positive by methods with high positivity rates. A significant exception was samples that tested positive by the isolation methods with an incubation temperature of 37°C. Of these samples, 18% were rated as contaminated by the corresponding method with an incubation temperature of 30°C. Similarly, 12 and 15% of the samples that tested positive with OEOE were rated as contaminated with LJ and OEY, respectively.

The data show that for any combination of decontamination method and growth medium, incubation at 30°C yielded higher positivity rates, higher mean numbers of mycobacterial CFU per liter, and higher mean numbers of different mycobacterial strains than incubation at 37°C. For any of the different combinations of decontamination method and growth medium, the differences between positivity rates at 30 and 37°C proved to be statistically significant ($P < 0.001$ for treated water and $P \leq 0.03$ for surface water by McNemar's test [data not shown]), with the only exceptions being the differences between methods N and O and methods R and S. Similar statistical results were obtained by analyzing the differences between the mycobacterial concentrations and the numbers of different mycobacterial strains at 30 and 37°C.

Since the statistical difference between the results at the two incubation temperatures was obvious, the results were further tested within four groups of methods characterized by the two types of samples collected (treated and surface water) and the two different incubation temperatures employed (30 and 37°C). According to the test results, Table 4 lists the methods with the highest performance within the four groups of isolation methods. Statistically, these methods were not different in terms of positivity rate, mean mycobacterial concentration, or mean number of different mycobacterial strains ($P > 0.05$; data not shown). For each of these criteria, the methods are ranked according to the data given in Tables 2 and 3. Table 4 also summarizes the performance of the methods by all three criteria in terms of their overall performance.

The mycolic acid TLC patterns of all 586 isolates were char-

TABLE 3. Performance of methods for the isolation of mycobacteria from surface water[a]

Isolation method	Rate (%)			n^b	Mean no. of:	
	Positivity	Negativity	Contamination		Mycobacterial CFU/liter	Different mycobacterial strains isolated from 10 ml
G	50.0	15.5	34.6	17	932	1.65
H	19.2	69.2	11.5	23	100	0.3
I	30.8	19.2	50.0	13	660	0.85
K	7.7	34.6	57.7	11	90	0.2
L	38.5	46.2	15.4	21	173	0.5
M	7.7	88.5	3.8	25	145	0.1
N	50.0	30.8	19.2	21	1,461	1.6
O	30.8	53.8	15.4	23	165	0.5
P	50.0	23.1	26.9	17	1,048	1.5
Q	19.2	46.2	34.6	17	291	0.5
R	7.7	80.8	11.5	23	43	0.1
S	3.8	96.2	0.0	26	13	0.04

[a] For the evaluation, 26 samples were tested in parallel.
[b] n, number of samples tested to calculate the mean mycobacterial CFU per liter and the mean number of different mycobacterial strains, excluding samples in which contamination or confluent growth occurred.

TABLE 4. Test results[a]

Water type and incubation temp (°C)	Method(s) with:			
	Highest positivity rate	Highest mean mycobacterial CFU/liter	Highest mean no. of different mycobacterial strains isolated	Best overall performance
Treated water				
30	G, A	A, C	A, C, G, I	A
37	B, D, H, K	H, B, D	B, D, H, K	B, D, H
Surface water				
30	G, N, P, L, I	N, P, G	G, N, P, I	G, N, P
37	O, H, Q	Q, O, M, H, K	O, Q, H, K, S	O, Q, H

[a] Statistically equal methods with the best performance regarding positivity rate, mean number of mycobacterial CFU/liter, and mean number of different mycobacterial strains isolated, ranked according to the data given in Tables 2 and 3.

TABLE 6. Mycobacterial species isolated from 26 samples of surface water

Isolation method	Species isolated (no. of samples from which species was isolated)
G	*M. chelonae* (2), *M. gordonae* (6), *M. kansasii* (1), *M. mucogenicum* (4), *M. nonchromogenicum* (1), *M. peregrinum* (3)
H	*M. gordonae* (2), *M. nonchromogenicum* (1)
I	*M. chelonae* (3), *M. fortuitum* (2), *M. gordonae* (8), *M. mucogenicum* (2), *M. peregrinum* (1)
K	*M. chelonae* (1)
L	*M. chelonae* (2)
M	
N	*M. fortuitum* (1), *M. gordonae* (10), *M. hiberniae* (1)
O	*M. gordonae* (2), *M. hiberniae* (1), *M. peregrinum* (1)
P	*M. gordonae* (4), *M. peregrinum* (1), *M. fortuitum* (2),
Q	*M. gordonae* (2)
R	
S	

acteristic of members of the genus *Mycobacterium*. Of these isolates, 287 strains (49%) were identified at the species level. The remaining isolates yielded PRA patterns which so far cannot be attributed to certain mycobacterial species. For each isolation method, Tables 5 and 6 list the strains identified and the numbers of samples in which these species were found.

DISCUSSION

In the present study we compared methods for the isolation of mycobacteria from water which, based on the literature available, were expected to be most appropriate for this purpose. In addition to the positivity rates and numbers of different mycobacterial strains isolated, we used the numbers of mycobacterial colonies grown (CFU per liter) as a third criterion to assess the overall performance of the isolation methods. This criterion was added since sample concentration by membrane filtration and primary isolation by direct incubation of the filters on solid media provide good quantitative results, which cannot be obtained by using other methods such as centrifugation and liquid media. The mean numbers of CFU per liter and the mean numbers of different mycobacterial strains (Tables 2 and 3) were calculated from data sets of different sizes, excluding contaminated samples. Samples rated as contaminated by one isolation method usually yielded high numbers of mycobacterial colonies by other methods. In methods with high contamination rates, the mean counts therefore probably underestimate the real values, thus stressing the inferiority of the method.

If ranked according to performance by each of the three criteria (Table 4), the isolation methods do not always occupy the same ranks, but there is good agreement between the results; i.e., the high positivity rate of a method usually coincides with high numbers of different mycobacterial strains isolated and high numbers of mycobacterial colonies grown.

The TSB method was originally developed for decontamination of soil samples heavily colonized by fungi and spore-forming bacteria (15), and decontamination of surface water with CPC (0.05% for 30 min) was not expected to perform almost as well as the TSB method (differences in performance were statistically not significant in most cases). Thus, apparently bacterial spores and fungi within surface water samples were sufficiently inactivated by low CPC concentrations. Table 3 indicates that the performance of these methods for the decontamination of surface water might be improved by increasing the concentration of and/or time of exposure to CPC or, with the TSB method, by decreasing the concentration of NaOH: with the CPC method the contamination rate (34.6% with LJ at 30°C) was higher than the negativity rate (15.5% with LJ at 30°C), whereas with the TSB method the contamination rate was lower than the negativity rate (19.2 and 30.8%, respectively, with LJ at 30°C). As the load of bacterial spores and fungi within treated water is generally much lower than that in surface water, there will not be much point in using the TSB method for decontamination of treated water. Here, the CPC method seems to be the method of choice; in other studies this method also proved to result in higher mycobacterial yields than decontamination methods using NaOH without sample preincubation (13, 19). The differences in the performance of the two CPC concentrations compared for the decontamination of treated water were insubstantial, and in most cases the performances did not differ statistically. Apparently, CPC concentrations have to be changed by a factor of 100 rather than by a factor of 10 (as in this study) in order to achieve marked effects on the positivity and contamination rates or on the numbers of mycobacteria cultivated. More significant effects might be achieved by a prolongation of the time of exposure to CPC. Still, in view of the best overall

TABLE 5. Mycobacterial species isolated from 109 samples of treated water

Isolation method	Species isolated (no. of samples from which species was isolated)
A	*M. avium* (1), *M. chelonae* (11), *M. fortuitum* (1), *M. gordonae* (25), *M. mucogenicum* (10), *M. peregrinum* (2)
B	*M. chelonae* (1), *M. gordonae* (4), *M. kansasii* (2), *M. peregrinum* (1), *M. xenopi* (1)
C	*M. chelonae* (4), *M. fortuitum* (1), *M. gordonae* (24), *M. kansasii* (1), *M. mucogenicum* (9), *M. peregrinum* (1)
D	*M. avium* (1), *M. gordonae* (2), *M. kansasii* (2), *M. xenopi* (1)
E	*M. chelonae* (8), *M. intracellulare* (1)
F	*M. chelonae* (2)
G	*M. avium* (2), *M. chelonae* (14), *M. gordonae* (31), *M. intracellulare* (1), *M. kansasii* (4), *M. mucogenicum* (15), *M. peregrinum* (2)
H	*M. gordonae* (4), *M. intracellulare* (1), *M. kansasii* (1)
I	*M. chelonae* (5), *M. fortuitum* (1), *M. gordonae* (20), *M. kansasii* (1), *M. mucogenicum* (10)
K	*M. avium* (1), *M. chelonae* (1), *M. gordonae* (1), *M. kansasii* (1), *M. peregrinum* (1)
L	*M. chelonae* (11), *M. gordonae* (1), *M. intracellulare* (1)
M	*M. chelonae* (1)

performance (Table 4), the lower concentration of CPC (0.005%) seems to be favorable for decontaminating samples of treated water. It is difficult to give clear statements on the influence of the decontamination procedure on the spectrum of mycobacterial species isolated. Table 5 does not suggest any significant effect of the CPC concentration on the spectrum of mycobacterial isolates. However, the data given in Table 6 suggest that decontamination with CPC and TSB does not yield the same spectrum of mycobacterial isolates: *M. hiberniae* was not isolated with the CPC method, whereas *M. chelonae*, *M. nonchromogenicum*, and *M. mucogenicum* were not isolated with the TSB method.

Three media (LJ, OEY, and OEOE) were included in this study. Of these, LJ gave the best results in the Madras (9) and Berlin (13) studies compared with Falkinham's selective medium as well as with Middlebrook 7H10 agar and BACTEC broth, respectively. We included OEY because of its good performance (highest positivity rates) when methods for the isolation of mycobacteria from soil samples were compared by Portaels et al. (15). OEY, with its pH of 6.0, is said to meet the pH requirements of most atypical mycobacteria better than LJ, with its pH of 7.0 (16), and our finding that LJ had equal (if not superior) performance was not anticipated. However, there is a marked difference between the mycobacterial spectra in soil and water (17), and mycobacteriological data obtained from the investigation of soil specimens from Louisiana may not automatically be valid for European water samples with neutral to slightly alkaline pHs. OEOE was included as a medium for the selective isolation of members of the *M. avium* complex and *M. scrofulaceum*. Its contents of ofloxacin and ethambutol are said to suppress most rapidly growing mycobacteria and mycobacteria belonging to the *M. terrae* complex, which often overgrow or suppress other slow growers (7). Tables 5 and 6 show that the growth of the rapid grower *M. chelonae* was not markedly suppressed on OEOE. Interestingly, none of the *M. avium* strains were isolated on OEOE, but two of four samples testing positive for *M. intracellulare* were examined with this medium. OEOE was not expected to perform better than the other two media tested. However, Tables 2 and 3 show that the positivity rates are reduced less by the use of OEOE (reduction by approximately 40 to 80% in comparison to the other two media) than the mycobacterial concentrations and numbers of different mycobacterial strains (reduction by approximately 60 to 90%). The reduction in all three performance criteria was most distinct when the TSB decontamination method was combined with the use of OEOE in isolation methods R and S (reduction by 80 to 97% in comparison to the other two media). In addition to the inhibitory effect on mycobacterial growth, the antimicrobial agents present in OEOE resulted in a marked reduction of contamination rates (Tables 2 and 3).

All methods using an incubation temperature of 30°C resulted in higher positivity rates, higher mean numbers of mycobacterial colonies grown, and higher mean numbers of different mycobacterial strains isolated than did methods using 37°C. On the other hand, incubation at 30°C was usually also associated with higher contamination rates than incubation at 37°C. These findings do not fully confirm the data reported by Kamala et al. (9), who found a greater variety of mycobacterial strains at 37°C than at 30°C. It remains unclear whether this discrepancy reflects the difference in mycobacterial strains occurring in the environments of southern India and central Europe or whether it is due to the different techniques of differentiation (traditional methods used by Kamala et al. versus molecular methods used in this study). However, our results correspond to the findings of other authors (1, 14) that incubation of environmental samples at 30°C results in higher yields of mycobacterial isolates than incubation at 37°C. Furthermore, the data given in Table 5 confirm the findings of Portaels (14) that mesophilic mycobacterial species, such as members of the *M. avium* complex with a growth optimum of approximately 37°C, may well be isolated at 30°C if the time of incubation is long enough. However, 30°C will not support the growth of *M. xenopi.*

In summary, our findings indicate that the best results (highest mycobacterial yields and highest number of different mycobacterial strains) can be achieved by employing the following isolation methods. Surface water can be decontaminated either by the TSB method or by CPC at a concentration of 0.05% (for 30 min). For treated water the favorable decontamination method seems to be 0.005% CPC (30 min), although this concentration is not crucial, as explained above. The incubation temperature with the best results is 30°C. Concerning the culture media for treated water, the overall performance of LJ was superior to that of OEY. For surface water we can also recommend LJ and, alternatively, OEY, which performed equally. As expected, the use of OEOE results in a significant decrease in the variety of mycobacterial strains isolated.

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