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Influence of Water Chlorination on the Counting of Bacteria with DAPI (4′,6-Diamidino-2-Phenylindole)

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Counting bacteria in drinking water samples by the epifluorescence technique after 4′,6-diamidino-2-phenylindole (DAPI) staining is complicated by the fact that bacterial fluorescence varies with exposure of the cells to sodium hypochlorite. An *Escherichia coli* laboratory-grown suspension treated with sodium hypochlorite (5 or 15 mg of chlorine liter^{-1}) for 90 min was highly fluorescent after DAPI staining probably due to cell membrane permeation and better DAPI diffusion. At chlorine concentrations greater than 25 mg liter^{-1}, DAPI-stained bacteria had only a low fluorescence. Stronger chlorine doses altered the DNA structure, preventing the DAPI from complexing with the DNA. When calf thymus DNA was exposed to sodium hypochlorite (from 15 to 50 mg of chlorine liter^{-1} for 90 min), the DNA completely lost the ability to complex with DAPI. Exposure to monochloramine did not have a similar effect. Treatment of drinking water with sodium hypochlorite (about 0.5 mg of chlorine liter^{-1}) caused a significant increase in the percentage of poorly fluorescent bacteria, from 5% in unchlorinated waters (40 samples), to 35 to 39% in chlorinated waters (40 samples). The presence of the poorly fluorescent bacteria could explain the underestimation of the real number of bacteria after DAPI staining. Microscopic counting of both poorly and highly fluorescent bacteria is essential under these conditions to obtain the total number of bacteria. A similar effect of chlorination on acridine orange-stained bacteria was observed in treated drinking waters. The presence of the poorly fluorescent bacteria after DAPI staining could be interpreted as a sign of dead cells.

Epifluorescence direct counting is one of the best methods for the evaluation of bacterial counts in natural waters. Early studies were done with acridine orange [3,6-bis(dimethylamino)acridium chloride], but this fluorescent dye was replaced by DAPI (4′,6-diamidino-2-phenylindole), which has more stable fluorescence (26). DAPI is believed to be very specific for DNA and is thus used to count total (including nonviable and viable but nonculturable) bacteria in water (26).

DAPI is generally believed to bind to DNA preferentially at AT-rich regions within the minor groove of B-DNA in solution (5, 11, 16, 17). Footprinting experiments at low binding ratios indicate that DAPI prevents the cleavage of DNA at AT sequences of 3 to 4 bp, implying that DAPI binds to sites with the 3 or 4 nearest-neighbor AT base pairs (27). DAPI also binds to poly[d(G-C)$_2$], presumably in the major groove of the polynucleotide (14). Matsuzawa and Yoshikawa (20) suggest that DAPI molecules interact with DNA via the phosphate groups along the DNA strand. The DAPI bound to these sites seems to be governed by the pH of the medium. DAPI exists as a protonated form with a fluorescent emission maximum near 490 nm and as an unprotonated form with a fluorescent emission maximum at 440 nm (29). Hence, the maximum fluorescence of the DAPI is between 440 nm (blue) and 490 nm (blue-green), depending on whether the DAPI is bound inside or outside the minor groove of DNA (6, 15, 21).

The specificity of DAPI toward nucleic acids has been questioned recently by the finding that the dye interacts not only with DNA but also with extracted or synthetic biopolymers such as double-stranded RNA (11, 30), proteins (21), and phospholipids (9). A recent study has shown that bacteria without nucleoids (so-called ghosts) are stained by DAPI, suggesting that this dye is not specific for DNA (31). The peak of the emission spectrum of DAPI bound to proteins is shifted from 459 to 455 nm, and the quantum yield increases by a factor of 10 with respect to DAPI alone (21). There is also a small hypsochromic (blue) shift from 5 to 10 nm with the binding of DAPI to phospholipids (9). Consequently, the DAPI fluorescence spectrum is shifted when the dye is complexed with components other than DNA and as a function of the environmental conditions.

The intensity of the fluorescence of bacteria stained with DAPI also seems to vary. These variations in the spectrum of fluorescence, plus the decreases in the fluorescence quantum yield, could explain why DAPI staining underestimates the number of cells in seawater and sediments (12, 13, 23, 28). A recent study has shown that exposing bacteria to UV irradiation causes a decrease in the fluorescence of bacteria stained with DAPI (4). Paquin et al. (24) reported that the fluorescence of bacteria stained with DAPI in pure bacterial suspensions and in potable waters disinfected with chlorine varied greatly, from bright blue-fluorescing cells to poorly fluorescing bacteria. We have therefore examined this problem by using a strain of *Escherichia coli* in laboratory-grown suspensions, extracted calf thymus DNA solutions, and water samples.

The study was carried out to (i) quantify and explain the effects of two disinfectants (sodium hypochlorite and monochloramine) on the staining of bacteria with DAPI, (ii) measure the relationship between the decrease in fluorescence and the viability of the bacterial cells, and (iii) estimate the proportion of poorly fluorescent bacteria in treated potable water.

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MATERIALS AND METHODS

***E. coli* suspension.** *E. coli* (Institut Pasteur no. 54 127) was cultured at 37°C for 24 h on nutrient agar without glucose (6.0 g of peptone from trypsin-digested casein [Merck no. 7213] liter^{-1}, 3.0 g of extract of yeast powder [Merck no. 3753]

liter^{-1}, 18.0 g of agar [Merck no. 1614] liter^{-1}). Bacteria were suspended in a saline solution (2.25 g of sodium chloride [Merck no. 6400] liter^{-1}, 0.105 g of potassium chloride [Merck no. 4933] liter^{-1}, 0.12 g of calcium chloride [Sigma no. 3881] liter^{-1}, 0.05 g of calcium carbonate [Merck no. 6329] liter^{-1}, bacterium-free distilled water), and the concentration was adjusted to 10^8 cells ml^{-1}. The bacteria were starved for 24 h at 37°C in the saline solution and then collected by centrifugation at 10,000 × *g* for 20 min at 20°C. The bacterial pellet was washed with the same volume of sterile saline solution and resuspended in saline solution at 10^7 cells ml^{-1}.

Water samples. Four types of water samples were collected for bacterial analysis over a period of 6 months: raw Moselle river water (20 samples), nondisinfected drinking water from a small village (20 samples), drinking water taken after disinfection (residual chlorine, 0.3 to 0.83 mg liter^{-1}; mean 0.5 mg liter^{-1}) (20 samples), and chlorinated drinking water sampled at different points in a distribution network (residual chlorine, 0.02 to 0.25 mg liter^{-1}; mean, 0.05 mg liter^{-1}) (20 samples).

Sodium hypochlorite solution. A commercial solution of bleach (NaClO) was diluted to 2 g of chlorine liter^{-1} and adjusted to pH 8 with dilute HCl. The sodium hypochlorite concentration was determined by the DPD (*N*,*N*-diethyl-*p*-phenylenediamine) method (2). All the results are expressed in milligrams of chlorine as Cl_2 per liter.

Monochloramine solution. Monochloramine solutions were prepared immediately before use by mixing appropriate amounts of sodium hypochlorite and ammonium chloride. Pure monochloramine without free chlorine or other chloramines (di- or tri-) was obtained by conducting the reaction in phosphate buffer (pH 8.5) with a Cl_2/N molar ratio of 0.8 (3). The monochloramine concentration was determined by the DPD method (2). All the results are expressed in milligrams of chlorine as Cl_2 per liter.

Exposure of bacteria to chlorine or monochloramine. Sodium hypochlorite (2 g of chlorine liter^{-1}) or monochloramine (2 g of chlorine liter^{-1}) solution was added to *E. coli* suspensions (approximately 10^7 cells ml^{-1}) to obtain initial concentrations of 2, 5, 15, 25, and 50 mg of chlorine liter^{-1}. The suspensions containing sodium hypochlorite or monochloramine were incubated for 90 min at 20°C, and the residual chlorine was neutralized with sodium thiosulfate (final concentration, 0.02%).

Bacterial counting. Bacteria were counted by four methods (DAPI counting, acridine orange direct counting [AODC], heterotrophic plate counts [HPC], and CTC [5-cyano-2,3-ditolyl tetrazolium chloride] counting). The total number of bacteria was determined by DAPI staining. Aliquots of sample (pure or dilute, 9 ml) were placed in sterilized glass tubes containing 1 ml of DAPI (0.5 μg ml^{-1}) (Sigma no. D9542) and 1 ml of Triton X-100 (0.1%) (Prolabo no. 28817295) (all reagents were filter sterilized through cellulose nitrate filters; pore size, 0.2 μm). The tubes were mixed for 30 s and left to stand for 10 min. The samples were then filtered through black polycarbonate filters (DMF no. 111156; pore size, 0.2 μm). The filters were rinsed twice with 50 ml of bacterium-free distilled water, dried in hot air, hydrated with a drop of buffered glycerin (Diagnostics Pasteur no. 74921), and covered with a coverslip. The filters were examined under UV light (excitation filter BP330-385, barrier filter BA420) with an epifluorescence microscope (Olympus BX60) and an oil immersion objective (UPlan-FI 100× oil immersion universal objective). The highly fluorescent and poorly fluorescent bacteria in 30 fields were counted, and the results were expressed as cells per milliliter. The standard error is around 15% for each sample.

The method for AODC was the same as for DAPI, but the final concentration of acridine orange used (Merck no. 15931.0025) was 0.2 mg ml^{-1} per ml of sample, and the incubation time was 5 min.

For heterotrophic plate count bacteria, a 1-ml aliquot of sample or of its 10-fold dilution was mixed with melted, tempered nutrient agar without glucose and incubated for 24 h at 37 ± 2°C for *E. coli* cultures or 3 and 15 days at 20 ± 2°C for water samples. Colonies were counted and expressed as CFU per milliliter.

For direct counts of respiring bacteria by reduction of CTC, the samples were incubated with CTC (final concentration, 4 mM; Polysciences, Inc., no. 19292) in the dark at 37°C for 4 h. The samples were then filtered through black polycarbonate filters (DMF no. 111156). The filters were rinsed twice with 50 ml of bacterium-free distilled water, dried in hot air, hydrated with a drop of buffered glycerin (Diagnostics Pasteur no. 74921), and covered with a coverslip. The red-fluorescing CTC-formazan granules produced by respiring bacteria were examined under an epifluorescence microscope (objective, 100×) with a 450-nm filter for excitation and a 515-nm cutoff filter. The results are expressed as the number of CTC-positive cells per milliliter.

Fluorescence spectra of DNA. A 1 mg liter^{-1} solution of calf thymus DNA (Merck no. 24013) was prepared in phosphate buffer (pH 7.4) (22) and diluted to 1 μg ml^{-1} with bacterium-free distilled water just before the experiment. This DNA solution (1 ml) was incubated with 100 μl of DAPI (0.5 μg ml^{-1}) for 10 min. The fluorescence spectrum (400 to 800 nm) of this solution was obtained with a spectrofluorimeter (Hitachi model F-4500) and obtained at an excitation wavelength of 340 nm (1-cm^2 cuvette). The fluorescence intensity was expressed as arbitrary units of fluorescence.

Fluorescence spectra of *E. coli* suspensions. The emission spectra of *E. coli* suspensions stained with DAPI were obtained with a spectrofluorimeter (Hitachi model F-4500). *E. coli* suspensions (1 ml) were mixed with 100 μl of DAPI (0.5 μg ml^{-1}) for 10 min. The fluorescence spectrum (400 to 800 nm) of each suspension was recorded with an excitation wavelength of 340 nm (1-cm^2 cuvette). The fluorescence intensity was expressed as arbitrary units of fluorescence.

RESULTS

DAPI staining of *E. coli* suspensions. *E. coli* cells grown in the laboratory and incubated with sodium hypochlorite (0 to 50 mg of chlorine liter^{-1} because of the high chlorine demand of the suspension) for 90 min were stained with DAPI. The bacterial cells had very different intensities of fluorescence. Some bacteria fluoresced strongly (deep blue), while others showed weak fluorescence (pale blue bacteria, slightly green) (Fig. 1A and B). The DAPI concentration (0.5 to 3 μg ml^{-1}) and the DAPI-bacterium contact time (8 to 14 min) did not greatly influence the staining of the bacterial cells (results not shown). Hence, the poorly fluorescent bacteria were the cells that bound DAPI less efficiently or differently. The proportion of highly fluorescent bacteria in the *E. coli* suspension increased with the initial concentrations of sodium hypochlorite added (Fig. 2) and then dropped to zero at chlorine concentrations higher than 15 mg liter^{-1}. Parallel tests with monochloramine showed no great variation in the fraction of poorly fluorescent bacteria (mean, 35%), even at concentrations as high as 50 mg of chlorine liter^{-1} (Fig. 3).

There are at least two possible explanations for the reduction in DAPI fluorescence of chlorine-exposed bacteria. First, there may be less DAPI bound to the bacteria, resulting in reduced signal intensity (from highly to poorly fluorescent). Second, there may be a shift in the fluorescence spectrum to higher wavelengths (blue to green). The bacterium-bound DAPI fluoresced between 400 and 500 nm, with a peak at around 460 nm, regardless of the bacterial population examined (Fig. 4). Conversely, the intensities of the maximum emissions of the suspension, measured with a spectrofluorimeter, varied considerably and corroborated the microscopic findings of strong emission by lightly chlorinated suspensions and low emission by heavily chlorinated suspensions. The poorly fluorescent bacteria appeared slightly green under the microscope because of the great reduction in the intensity of fluorescence in the blue region (450 to 490 nm), whereas the intensity of fluorescence in the green region (490 to 540 nm) varied little. As a result, the fluorescence spectrum of heavily chlorinated bacteria (curves e and f in Fig. 4) was remarkably close to that of free DAPI in water (curve a in Fig. 4).

The increase in the fluorescence of bacteria exposed to lower chlorine doses that was observed microscopically and quantified by the spectrofluorimeter may have been due to permeation of the membrane, resulting in alteration of the bacterial cell envelopes. The bacteria treated with 5 or 15 mg of chlorine liter^{-1} appeared to be porous and slightly swollen (Fig. 1C), suggesting that this allowed more DAPI to enter and form a greater number of DNA bonds.

Fluorescence of DNA solutions. The possibility that high doses of sodium hypochlorite acted on bacterial DNA to prevent DAPI binding was examined by using calf thymus DNA solutions treated with various concentrations of sodium hypochlorite or monochloramine. The residual oxidant was neutralized, and the DNA was stained with DAPI. The fluorescence of the samples treated with sodium hypochlorite was altered. Both the intensity (Fig. 5) and the maximum emission wavelength (Table 1) changed. The fluorescence intensity decreased by 83% at the lowest chlorine concentration (5 mg liter^{-1}). The DAPI-stained DNA fluorescence intensity was comparable to that of DAPI in aqueous solution from 15 mg of chlorine liter^{-1} upward, and the maximum emission wavelength, which

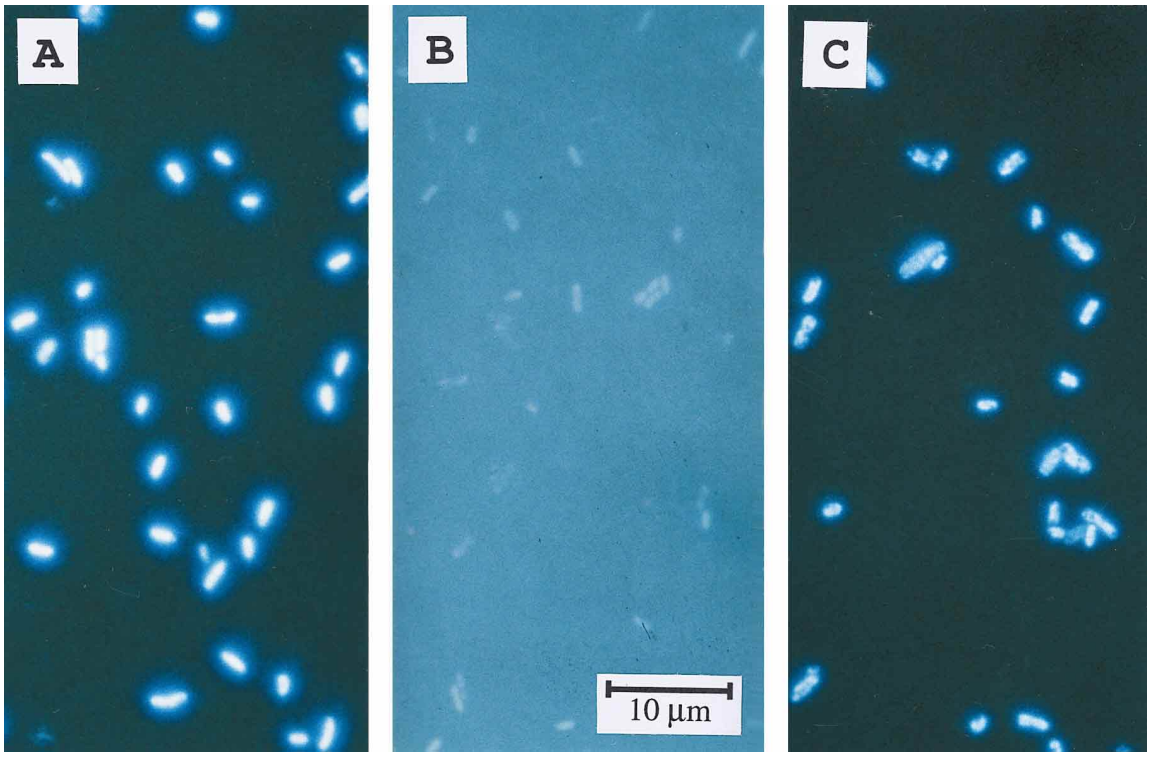

FIG. 1. DAPI-stained *E. coli* observed by epifluorescence microscopy. (A) Initial suspension, highly fluorescent bacteria. (B) After chlorination (25 mg of chlorine liter^{-1} for 90 min), poorly fluorescent bacteria. (C) After chlorination (15 mg of chlorine liter^{-1} for 90 min), bacteria appeared fluorescent but porous and slightly swollen.

was 456 nm when the DAPI was complexed with the DNA, was close to the maximum wavelength of noncomplexed DAPI in water (480 nm) from 25 mg of chlorine liter^{-1} upward. The DAPI fluorochrome therefore did not complex with DNA when it was exposed to more than 5 mg of chlorine liter^{-1}. The intensity of fluorescence and the maximum emission wavelength of DNA incubated with monochloramine did not alter with the monochloramine concentration (Fig. 5). These results confirmed that DNA was altered by sodium hypochlorite but

TABLE 1. Intensity and wavelengths (λ_{max}) at maximum fluorescence of DAPI in water or in DNA solutions[a]

DAPI solution in different systems	Fluorescence intensity of DAPI (a.u.)[b]	λ_{max} (nm)
Bacterium-free distilled water	125	482
NaClO (50 mg of chlorine liter^{-1}) neutralized[c]	160	484
DNA (1 µg ml^{-1}) in bacterium-free distilled water	4,541	457
DNA (1 µg ml^{-1}) in NaClO (50 mg of chlorine liter^{-1})-neutralized solution[c]	4,640	456
DNA treated with 5 mg of chlorine liter of NaClO^{-1}	757	455
DNA treated with 15 mg of chlorine liter of NaClO^{-1}	156	465
DNA treated with 25 mg of chlorine liter of NaClO^{-1}	165	484
DNA treated with 50 mg of chlorine liter of NaClO^{-1}	160	480
DNA treated with 5 mg of chlorine liter of monochloramine^{-1}	4,590	456
DNA treated with 15 mg of chlorine liter of monochloramine^{-1}	4,321	456
DNA treated with 25 mg of chlorine liter of monochloramine^{-1}	4,235	456
DNA treated with 50 mg of chlorine liter of monochloramine^{-1}	4,252	457

[a] When treated for 90 min with sodium hypochlorite or monochloramine, the solutions were neutralized with an excess of sodium thiosulfate.
[b] a.u., arbitrary units.
[c] Neutralized solution with an excess of sodium thiosulfate.

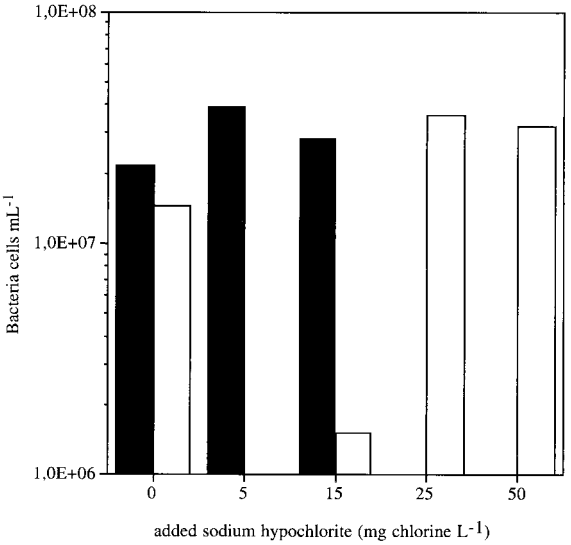

FIG. 2. Number of DAPI-stained highly fluorescent bacteria (black bars) and poorly fluorescent bacteria (white bars) as a function of the concentration of chlorine (milligrams of chlorine per liter) added to an *E. coli* laboratory-grown suspension. (The total number of cells remained constant.)

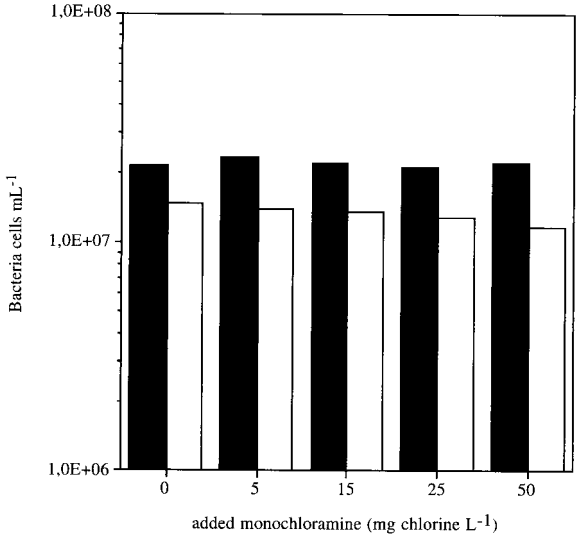

FIG. 3. Number of DAPI-stained highly fluorescent bacteria (black bars) and poorly fluorescent bacteria (white bars) as a function of the concentration of monochloramine (milligrams of chlorine per liter) added to an *E. coli* suspension. (The total number of cells remained constant.)

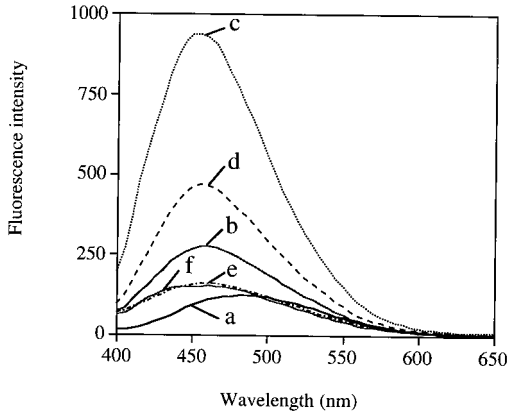

FIG. 4. Fluorescence emission spectra of an *E. coli* suspension (3×10^7 cells ml^{-1}) stained with DAPI. The excitation wavelength for the measurement of fluorescence spectra was 340 nm. (a) DAPI (0.05 μg ml^{-1}); (b) DAPI plus *E. coli*; (c) DAPI plus *E. coli* chlorinated with 5 mg of chlorine $liter^{-1}$ for 90 min; (d) DAPI plus *E. coli* chlorinated with 15 mg of chlorine $liter^{-1}$ for 90 min; (e) DAPI plus *E. coli* chlorinated with 25 mg of chlorine $liter^{-1}$ for 90 min; (f) DAPI plus *E. coli* chlorinated with 50 mg of chlorine $liter^{-1}$ for 90 min.

that monochloramine had no effect on the binding of DAPI to bacterial DNA.

Viability of chlorinated *E. coli* suspensions. The appearance of a high proportion of poorly fluorescent bacteria in chlorination tests suggests that there may be a link between the reduction in the intensity of fluorescence of the chlorinated bacteria and the viability of the bacterial suspension. The viability of hypochlorite-treated *E. coli* was therefore estimated by counting the number of bacteria culturable on agar and the number of bacteria with respiratory activity (CTC-positive bacteria) and comparing these with the numbers of highly fluorescent and poorly fluorescent bacteria. The nonchlorinated *E. coli* suspension contained a mean of 2.2×10^7 bacteria per ml, of which 64% were culturable on agar and 96% had an active respiratory chain (Table 2). This untreated *E. coli* suspension stained with DAPI contained 70% highly fluorescent and 30% poorly fluorescent bacteria.

Bacteria treated with sodium hypochlorite (2 mg of chlorine $liter^{-1}$) were no longer agar culturable, but 40% of them still had an active respiratory chain. At higher concentrations of sodium hypochlorite (5 mg of chlorine $liter^{-1}$), CTC-positive bacteria could not be detected. Bacteria treated with 5 or 10 mg of chlorine $liter^{-1}$ were 100% fluorescent, although no viable bacteria could be detected after exposure to these concentrations. Chlorination at over 10 mg $liter^{-1}$ produced 100% poorly fluorescent bacteria. Thus, the culturability and respiratory activity of *E. coli* treated with chlorine were lost before the appearance of poorly fluorescent bacteria.

Bacterial analysis of different water samples. The water samples analyzed contained 10^4 to 10^6 bacterial cells per ml, of which less than 1% were culturable (Table 3). The proportion of DAPI-stained poorly fluorescent bacteria was high in chlorinated waters (33 to 39%), and low (less than 5%) in nonchlorinated waters. The number of bacteria counted in the nonchlorinated waters after acridine orange staining was generally slightly greater (30 of 40 samples) than the number obtained after DAPI staining (highly fluorescent plus poorly fluorescent bacteria). This is in keeping with the findings of Porter and Feig (26). However, the total number of DAPI-stained bacteria (highly fluorescent plus poorly fluorescent bacteria) in chlorinated water was almost always greater (34 of 40 samples) than the total number of acridine orange-stained bacteria. This suggests that chlorine exposure significantly in-

TABLE 2. Number of cells in a suspension of *E. coli* chlorinated with different concentrations of chlorine (contact time, 90 min) counted by different methods

NaClO concn (mg of Cl_2 $liter^{-1}$)	No. of cells (10^7 ml^{-1}) counted by:			
	HPC, 24 h	CTC	DAPI (fluorescent cells)	DAPI (total cells)
0	1.45	2.19	1.60	2.27
2	—[a]	0.89	1.90	2.23
5	—	—	2.01	2.01
10	—	—	2.30	2.30
15	—	—	—	2.10
25	—	—	—	2.26
50	—	—	—	2.40

[a] —, not detectable.

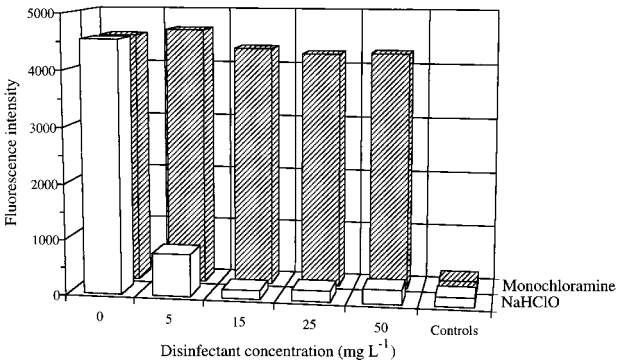

FIG. 5. Fluorescence intensity (measured at the maximum of fluorescence) of a DNA solution (1 μg $liter^{-1}$) stained with DAPI before and after chlorination/chloramination (with a contact time of 90 min followed by neutralization) and DAPI solution in water (controls without disinfectant).

TABLE 3. Number of bacteria in water samples determined by different methods

Water type	Mean no. of bacteria ml^{-1} counted by:					
	HPC		AODC (total cells)	DAPI[a]		
	3 days	15 days		Highly fluorescent cells	Poorly fluorescent cells	Total cells
Raw surface water	8.2×10^3	1.5×10^4	1.8×10^6	1.5×10^6 (99.5%)	7.8×10^3 (0.5%)	1.5×10^6
Nonchlorinated drinking water	8	3.0×10^1	6.5×10^4	6.1×10^4 (95.3%)	3.0×10^3 (4.7%)	6.4×10^4
Chlorinated drinking water (residual chlorine, 0.5 mg $liter^{-1}$)	1	4.9×10^2	3.4×10^5	3.2×10^5 (60.4%)	2.1×10^5 (39.6%)	5.3×10^5
Chlorinated drinking water (residual chlorine, 0.05 mg $liter^{-1}$)	1.0×10^2	2.2×10^3	2.6×10^5	2.4×10^5 (66.7%)	1.2×10^5 (33.3%)	3.6×10^5

[a] The values in parentheses are the percentages of highly or poorly fluorescent bacteria compared to the total number of cells stained with DAPI ($n = 20$ for each type of water).

terferes with the counting of bacteria by acridine orange staining.

DISCUSSION

The staining of bacterial cells with DAPI implies the efficient diffusion of the fluorochrome through the cell envelope (19) and the integrity of the DNA that allows the binding of DAPI at specific sites (15, 16). Any change in the structure of a bacterium therefore causes a change in the DAPI fluorescence.

The DAPI staining of bacterial cells varied with the nature and concentration of the disinfectant used. The intensity of fluorescence of *E. coli* treated with sodium hypochlorite (5 to 15 mg of chlorine $liter^{-1}$ because of the strong chlorine demand of our system) increased, probably due to increased membrane permeability, allowing better diffusion of the DAPI to the DNA. Several groups have reported that sodium hypochlorite improves the permeation of the bacterial membrane (10, 19) without causing lysis (1, 7).

DAPI staining was, however, reduced by altering the structure of the bacterial DNA by high-chlorine treatment of *E. coli* suspensions or by using chlorinated drinking waters. Sodium hypochlorite or chlorine dioxide treatment also leads to the appearance of poorly fluorescent bacteria in strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (24). Our study shows that this reduction in the fluorescence of chlorinated DAPI-stained bacteria can be due to the inability of the DAPI to complex with the DNA modified by the reaction with the chlorine. A substitution reaction between sodium hypochlorite and the hydrogen of the amine group of the cytosine (25) could interfere with the binding of DAPI to the DNA via a hydrogen bond (16). The poor complexing of the DAPI with the DNA could also be due to physical damage to the DNA (strand separation and breaks) as a result of the chlorine treatment (10).

This change in the staining by a fluorochrome is not due solely to oxidants. Aging of the bacteria and exposure to UV (4) also lead to significant changes in the bacterial structure and to lower fluorescence of DAPI-stained bacteria. Our observations suggest that DAPI is not the only fluorescent dye to be affected by cellular changes. Acridine orange, for example, binds less efficiently to cell structures exposed to chlorine. The difficulty in acridine orange staining is not surprising, since the dye must bind to specific sites in order to stain DNA or RNA (8), and these sites can also be affected by chlorination in the same way as DAPI.

The reduction in the fluorescence of DAPI-stained bacteria makes counting them more difficult and leads to the risk of undercounting the number of bacterial cells. This phenomenon has been reported on several occasions with seawater and river estuary water (12, 13, 23, 28) and with disinfected water (18). The DAPI no longer complexes to the chlorine-treated DNA, and the chlorinated bacteria have low fluorescence. This low residual fluorescence of highly chlorinated bacteria (50 mg of chlorine $liter^{-1}$) could stem from the fluorescence of DAPI bound to membrane structures on account of a nonspecific DAPI staining of the DNA, as suggested by studies on different synthetic biopolymers (9, 21). The change in the DNA structure and the nonspecific DAPI binding undoubtedly explain the staining of bacteria termed ghosts by Zweifel and Hagström (31).

Cell alterations caused by oxidative stresses (sodium hypochlorite, chlorine dioxide, UV), which lead to a reduction in the fluorescence of DAPI-stained bacteria, generally occur after the loss of viability (measured by culturability and respiratory activity) of the bacteria. Thus, low fluorescence could be interpreted as a sign of dead cells when the stress is caused by sodium hypochlorite or UV. However, death was not the determining factor in the loss of DAPI fluorescence, because bacteria killed by high doses of monochloramine were still fluorescent after DAPI staining.

In conclusion, the fluorescence of DAPI-stained bacteria depends on the extent to which the bacterial membrane and DNA structure are altered. This brings into question the counting of the total number of bacteria or, at the very least, requires careful counting. Hence, the performance of the tool used for observation (microscope, image analyzer) is of fundamental importance in counting highly fluorescent and, especially, poorly fluorescent bacteria.

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