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Determination of the Viability of *Aeromonas hydrophila* in Different Types of Water by Flow Cytometry, and Comparison with Classical Methods

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The presence of *Aeromonas* spp. in water can represent a risk for human health. Therefore, it is important to know the physiological status of these bacteria and their survival in the environment. We studied the behavior of a strain of *Aeromonas hydrophila* in river water, spring water, brackish water, mineral water, and chlorinated drinking water, which had different physical and chemical characteristics. The bacterial content was evaluated by spectrophotometric and plate count techniques. Flow cytometric determination of viability was carried out using a dual-staining technique that enabled us to distinguish viable bacteria from damaged and membrane-compromised bacteria. The traditional methods showed that the bacterial content was variable and dependent on the type of water. The results obtained from the plate count analysis correlated with the absorbance data. In contrast, the flow cytometric analysis results did not correlate with the results obtained by traditional methods; in fact, this technique showed that there were viable cells even when the optical density was low or no longer detectable and there was no plate count value. According to our results, flow cytometry is a suitable method for assessing the viability of bacteria in water samples. Furthermore, it permits fast detection of bacteria that are in a viable but nonculturable state, which are not detectable by conventional methods.

During the last decade scientific interest has turned to members of the genus *Aeromonas* as human and animal pathogens. Many *Aeromonas* species have been associated with human diseases, including gastroenteritis, hemolytic-uremic syndrome, septicemia, etc. (21, 30, 48).

Aeromonas spp. are common aquatic microorganisms that occur in seawater, irrigation water, river water, brackish water, freshwater, groundwater, spring water, sewage-contaminated water, and activated sludge (4, 7, 9, 14, 15, 23, 24, 37, 43).

The wide distribution of aeromonads in different aquatic ecosystems underlines their capacity to adapt to environments with different trophic levels. Several studies have shown that the phenospecies *Aeromonas hydrophila* is prevalent in cleaner water, whereas *Aeromonas caviae* is prevalent in water with a high level of fecal pollution; the frequencies of the other phenospecies appear to be unrelated to environmental conditions (3, 15, 17, 23).

These bacteria have also been isolated from chlorinated and unchlorinated drinking water and from bottled mineral water, which shows that they are able to withstand long periods of nutrient limitation (6, 16, 19, 20, 29, 31, 46, 50). The survival of aeromonads in these ecosystems can be correlated with their capacity to produce and live within biofilms on the surfaces of pipes and bottles (26); on the other hand, it is known that *Aeromonas* spp. can cope with nutrient limitation conditions by entering into a starvation survival state (33).

The growing interest in these organisms addresses aquatic environments, because these environments represent an important reservoir of aeromonads. It is assumed that human infections arise from recreational activities, contaminated drinking water, or foods (23).

Therefore, due to the importance of water for survival and diffusion of the microorganisms, a study to evaluate the behavior of *A. hydrophila* in types of water with different physical and chemical characteristics was performed.

The bacterial content was evaluated by measuring absorbance and by using plate count agar; in addition, a flow cytometric analysis method was also used. Flow cytometry has become a method of choice for bacterial research. It has been used in basic (8, 13, 34, 39), clinical (2), and environmental (12, 47) studies. At present, the use of a flow cytometric technique in conjunction with a fluorescence technique provides a promising method for the detection and rapid enumeration of viable bacteria in environmental samples. Moreover, it provides information on the dynamics and physiological heterogeneity of bacterial populations (22).

MATERIALS AND METHODS

Bacteria. A strain of *A. hydrophila* that was previously isolated from irrigation water and was identified as described elsewhere (38) was used in this study. This strain was stored at −80°C until it was used.

Water microcosms. The water microcosms into which *A. hydrophila* was inoculated contained brackish water, river water, spring water, mineral water, and chlorinated drinking water. The samples of brackish water, river water, and

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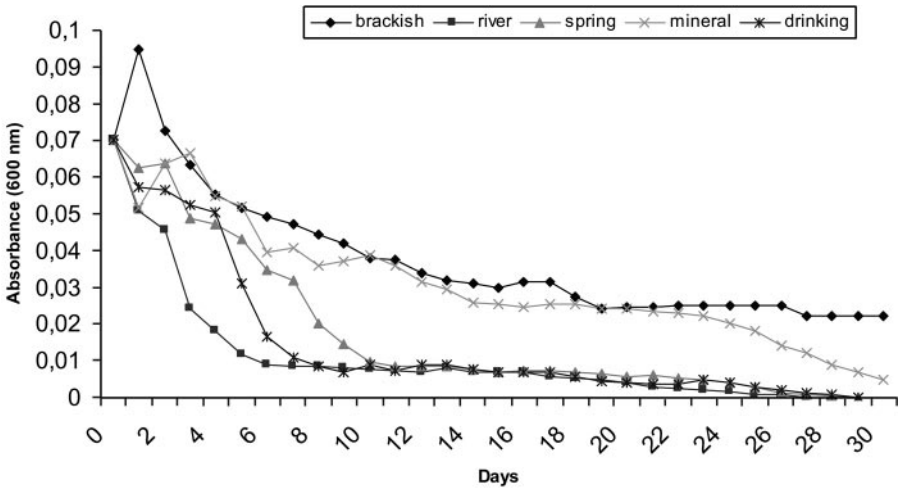

FIG. 1. Survival curves for *A. hydrophila* inoculated into different types of water as detected by measurement of absorbance.

spring water were collected with a "Mare-Lacus" sampler (International PBI, Milan, Italy); the drinking water was collected directly from the tap, and the mineral water was a commercial natural mineral water. Three liters of each type of water was filtered through a 0.22-μm membrane filter (Nalgene; International PBI) to remove autochthonous microbiota.

Inoculation of microcosms. The *A. hydrophila* strain was regenerated in 5 ml of tryptone soya broth (Oxoid, Milan, Italy) with 0.6% (wt/vol) yeast extract and 3% (wt/vol) Casamino Acids with overnight incubation at 28°C. One loopful of the culture was streaked on m-Aeromonas selective agar base (Havelaar, Biolife-Italiana, Milan, Italy) and incubated for 18 to 24 h at 28°C. Two or three colonies were inoculated into 40 ml of nutrient broth (Oxoid) and incubated at 28°C for 18 to 24 h with agitation at 150 rpm. The broth cultures were centrifuged at 3,000 rpm for 30 min, and each pellet was resuspended in 10 to 15 ml of the same water used for the experiments. The tests were performed in 1-liter screw-cap Pyrex brown glass bottles. These bottles were cleaned with a 10% solution of $K_2Cr_2O_7$ in concentrated H_2SO_4 and rinsed in hot tap water, in a 10% HNO_3 solution, and finally in distilled water. Then they were autoclaved at 121°C for 15 min. The *A. hydrophila* strain was inoculated into 800 ml of each type of water to obtain a bacterial concentration of about 5×10^6 CFU/ml, corresponding to an optical density at 600 nm of 0.07 (as determined with a UNICAM spectrophotometer) (45). The samples were incubated at room temperature (mainly at 22°C; range, 18°C to 25°C).

Control cultures were grown in nutrient broth and handled under the same conditions.

All the experiments were repeated three times, and the data were expressed as the mean for each sample.

Determination of the bacterial content. The bacterial content was evaluated after inoculation into the water samples and at regular intervals (1 day) over a 30-day period using turbidimetric determination and the plate count method. Plate counting was performed by streaking 1 ml of inoculated water in triplicate from decimal dilutions onto Havelaar agar (Biolife). The plates were incubated at 28°C for at least 24 h, and viable counts were expressed as CFU/ml.

Flow cytometry. The fluorescence intensity of stained bacteria was measured with a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with an argon laser set at 15 mV and tuned to an excitation wavelength of 488 nm. All data were analyzed statistically with the Cell Quest software. The fluorescent filters and detectors were all standard filters and detectors; green fluorescence was collected in the FL1 channel (525 nm), orange fluorescence was collected in the FL2 channel (585 nm), and red fluorescence was collected in the FL3 channel (>670 nm). All parameters were collected as logarithmic signals. Fluorescent microspheres (Becton Dickinson liquid counting beads) were added to each sample as an internal reference. The solutions used were routinely filtered through Millipore membrane filters (pore size, 0.22 μm; Millipore, Bedford, Mass.) before passage through the flow cytometer. Debris was excluded from analysis by scatter gating. For the flow cytometric analysis, 5×10^4 events were collected, and three independent experiments were carried out each time.

Viability staining with flow cytometry. Cell membrane integrity was assessed by using the nucleic acid double-staining procedure of Barbesti et al. (5) based on the permanent SYBR Green I (Molecular Probes) and impermanent propidium iodide (PI) (Molecular Probes) fluorescent probes. Both of these probes stain RNA and DNA (18).

Five milliliters of each sample was centrifuged at 3,000 rpm for 10 min and resuspended in 5 ml of filtered phosphate-buffered saline. One milliliter of the bacterial suspension was stained with 1:10,000 (vol/vol) SYBR Green I and a 10-μg/ml commercial PI solution (5) and incubated for 15 min in the dark at room temperature. Each cell was characterized by two fluorescence parameters that measured green fluorescence emission (FL1) and orange-red fluorescence emission (FL3).

The system permitted us to distinguish cells with intact membranes (live cells) (green), cells with damaged membranes (green plus orange-red), and cells with compromised membranes (orange-red; dead). Thus, the system was able to place the bacterial cells in three different cytometric clusters. The probes were tested using exponential-phase bacterial cells as a positive control and bacteria that were heat killed at 70°C as a negative control.

Statistical analysis. Analysis of variance applied to the regression model was used for statistical analysis for the correlation between the absorbance and CFU/ml. Cytometric data were analyzed using the Cell Quest software (Becton Dickinson); percentages of cells were determined by gating processing on acquired data files.

Data were acquired in triplicate for each test sample, and means, calculated with the Cell Quest software, were used for analysis of statistical significance by a Student paired *t* test.

Physical and chemical parameters. The physical and chemical parameters of the water samples that were determined before bacterial inoculation were conductivity at 20°C, total hardness, total alkalinity, pH, and the concentrations of chlorine, sulfates, phosphates, nitrates, ammonia, calcium, iron, silver, copper, magnesium, and total organic carbon (TOC). The concentrations of nitrates, ammonia, phosphates, sulfates, and TOC and the pH were also monitored at the end of the experiments in order to evaluate possible changes following bacterial growth. The tests were performed by APAT IRSA-CNR methods (1).

RESULTS

As shown in Fig. 1, the optical densities of the brackish water increased slightly (from 0.07 to 0.095) in the first 24 h and then gradually decreased to 0.022 in the last 4 days of the trial. A similar curve, but without an initial increase, was observed for the mineral water; in this case the optical density fell to 0.005 on day 30.

In the other types of water marked decreases in the optical density were observed in the first 6 to 10 days (from 0.07 to 0.008 to 0.009), and the optical density was 0 on day 27 for the river water and on day 29 for the spring water and the drinking water.

The survival curves, as determined by the standard plate

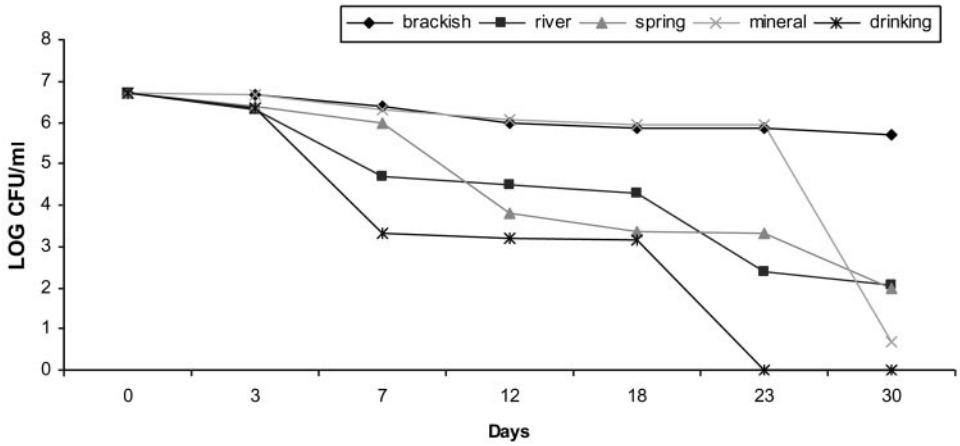

FIG. 2. Survival curves for *A. hydrophila* inoculated into different types of water as detected by plate counting.

count method, exhibited a similar behavior for the brackish water and mineral water until day 23; on day 30 the brackish water contained log 5.84 CFU/ml, and the mineral water contained log 0.69 CFU/ml. The lowest values were obtained for the drinking water; for this water no growth was found on day 23 or thereafter (Fig. 2).

There was a correlation between the absorbance values and the plate count values ($P < 0.001$; $R^2 = 0.096$ for river water; $R^2 = 0.097$ for brackish water; $R^2 = 0.099$ for the other types of water; $R^2 = 0.098$ for the control culture).

Cytofluorimetric analysis showed that the highest percentages of viable bacteria were generally found in drinking water, and the values ranged from 47 to 74% throughout the experiment. In contrast, the lowest values were observed in the mineral water, in which the percentages of viable cells were ~10% from day 12 onward. In the other types of water the values were still around 50% on day 30 (Fig. 3).

The percentages of damaged cells did not change much during the experiment; damaged cells were prevalent in the mineral water, and the percentages ranged from 9 to 30% during the experiment (Fig. 4 and 5).

In the control culture in nutrient broth, after increases in the optical density and CFU/ml on day 3 (1.72 and log 9.4 CFU/ml, respectively), there were gradual decreases, and the optical density and number of CFU/ml were 0.042 and log 8.4 CFU/ml at the end of the trial (Fig. 6). In contrast, cytofluorimetric analysis showed that there were low percentages of viable cells and high proportions of damaged cells beginning on day 3 (Fig. 3, 4, and 5).

In drinking water on day 23 of incubation, there were prominent cytometric clusters of live cells (74%), even though there was no plate growth during the same period (Fig. 2).

The initial analysis of physical and chemical parameters (Table 1) showed that the highest values for electrical conductivity, total hardness, and the concentrations of chlorine, sulfates, calcium, magnesium, and TOC were the values for the brackish water. At the end of the experiments (Table 2) the nitrate concentrations were lower in all types of water, while the concentrations of ammonia, phosphates, sulfates, and TOC were higher.

DISCUSSION

The aim of this study was to evaluate the capacity of a strain of *A. hydrophila* inoculated into different types of water to survive by using conventional methods of detection, such as absorbance and plate counting, and to compare these methods with the flow cytometric technique.

The data from the spectrophotometric analysis showed that the bacterial content was variable and depended on the type of water. In fact, when the bacterial strain was inoculated into brackish water, the optical densities were higher throughout

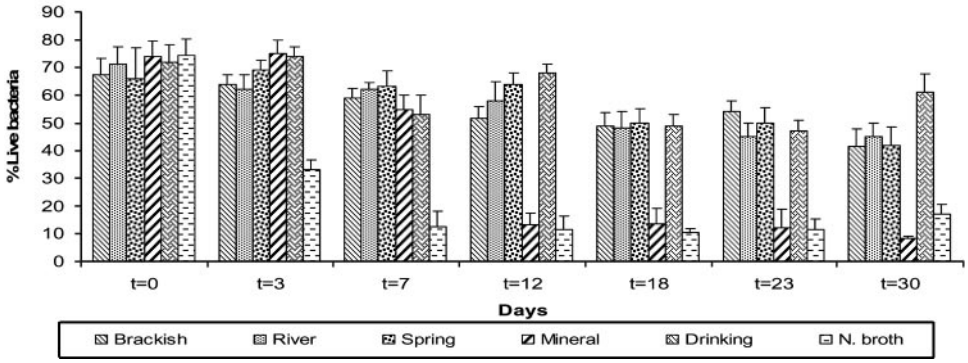

FIG. 3. Viable *A. hydrophila* evaluated by flow cytometry, as revealed in the FL1 channel. N. broth, nutrient broth.

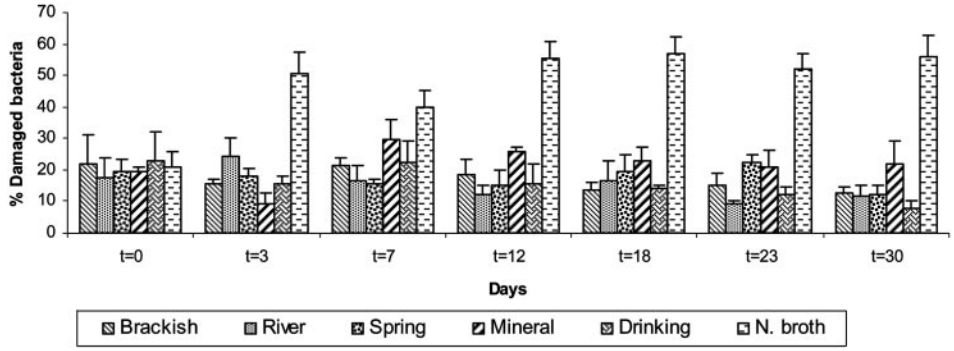

FIG. 4. Damaged *A. hydrophila* evaluated by flow cytometry, as revealed in the FL1 and FL3 channels. N. broth, nutrient broth.

the trial and were still 0.022 on day 30. In mineral water the bacteria behaved similarly; in contrast, in the river water, spring water, and drinking water the optical densities decreased after 8 to 10 days of incubation, and values could not be determined on the last days of the experiment. In some cases, including mineral water and drinking water, cytofluorimetric data were different from spectrophotometric data. In fact, from 12 day of incubation, the percentages of live bacteria in drinking water (~20%) were less than the values for the other types of water, while the optical densities were higher than those obtained for the other types of water (Fig. 1 and 3).

Estimating microbial growth by measurement of absorbance has the advantage of being rapid, nondestructive, inexpensive, and relatively easy to automate. However, absorbance is an indirect measure of bacterial behavior; it depends on the total number of cells present in the sample and does not allow workers to distinguish between live and dead cells. Furthermore, the sensitivity of this method appears to be correlated with microbiological culture density (10, 11).

When bacteria are monitored, it is very important to determine the viability of cells in addition to their abundance, since this is a critical issue in assessing water quality and preventing sanitation problems (41).

The plate count method is a widely used technique for determining the bacterial charge, but it supplies information related only to viability and growth capacity. In this study the results obtained from the plate count analysis statistically correlated with the absorbance data. However, the turbidimetric method for determining bacterial concentrations appeared to be less sensitive than the plate count method. In fact, in the river water and spring water a number of viable cells were

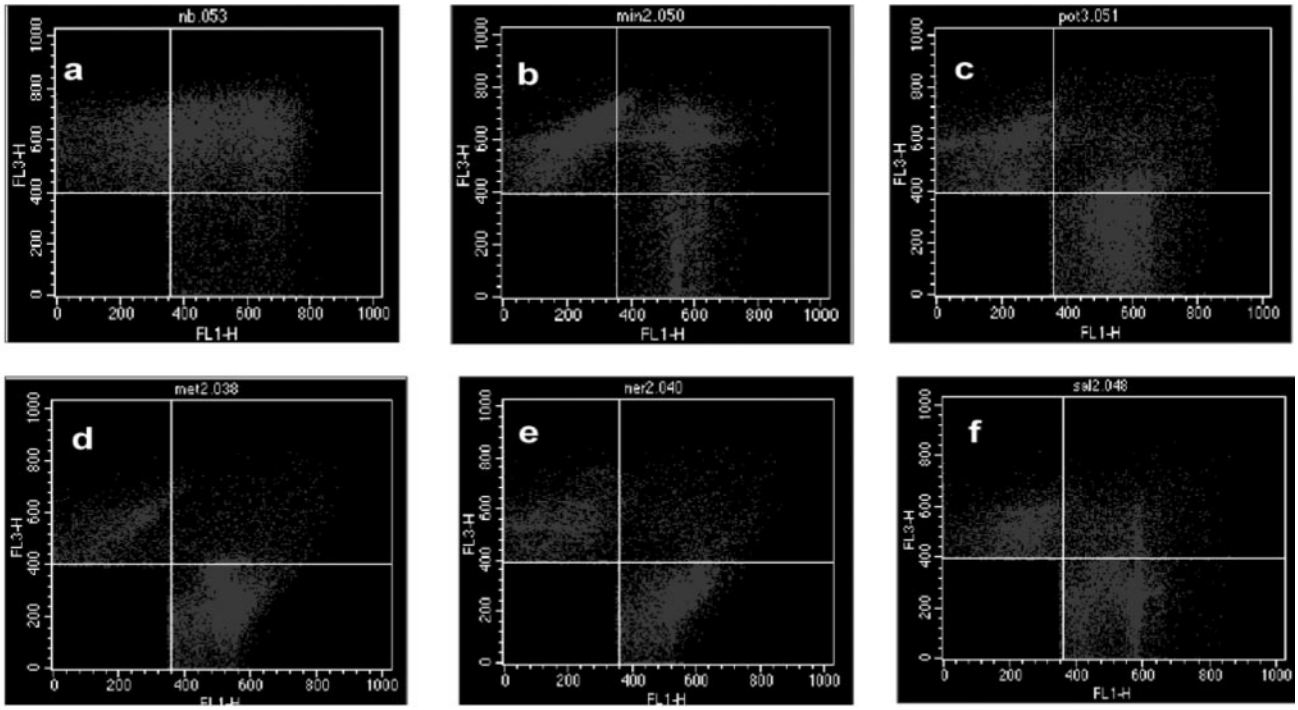

FIG. 5. Flow cytometric analysis of fractions of damaged and membrane-compromised cells in nutrient broth (a) and fractions of viable, damaged, and membrane-compromised cells in mineral water (b), drinking water (c), river water (d), spring water, (e) and brackish water (f).

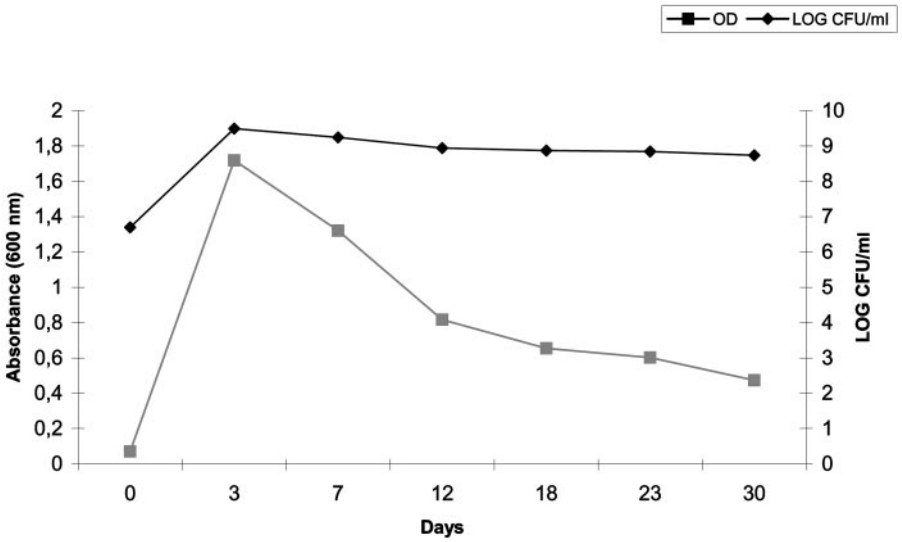

FIG. 6. Survival curves for *A. hydrophila* inoculated into nutrient broth as detected by plate counting and measurement of absorbance. OD, optical density.

detected when the optical density was no longer detectable. These findings agree with the results of other studies (11) in which the workers reported that the usefulness of turbidimetry was restricted to conditions under which high cell densities were reached. In such a case a more sensitive method, such as plate counting, might yield higher estimates. In any case, standard plate counting also has major disadvantages because it requires long incubation times and for many bacterial species there are not efficient growth media.

Recent investigations (35, 40) have demonstrated that many nondifferentiating bacteria, including *Aeromonas* spp., can respond to a number of environmental stresses by entrance into a viable but nonculturable state. Such forms cannot be detected by standard plating techniques, and their detection requires special resuscitation procedures (27, 28, 49). As a consequence, the routine plating method could underestimate the real bacterial presence, which could be a public health concern, especially if the cells maintain their virulence in the viable but nonculturable state (36).

New approaches have been developed to assess bacterial viability. Flow cytometry is a powerful tool for analyzing a cell population at the single-cell level, since it can be used both to identify and enumerate bacterial populations from environmental samples and to characterize functional properties of an individual cell (18, 42).

The flow cytometric nucleic acid double-staining protocol is based on simultaneous use of permeable fluorescent probes (SYBR Green dyes) (25) and an impermeable fluorescent probe (PI) and can distinguish viable, membrane-damaged, and membrane-compromised cells.

The efficiency of combined staining is magnified by the energy transfer from SYBR Green I to PI when both probes are bound to nucleic acids, as described by Barbesti et al. (5)

In this study flow cytometric analysis revealed viable *A. hydrophila* cells even when the optical densities were low or not detectable and there was no plate growth (e.g., drinking water) (Fig. 2).

For the control culture in nutrient broth, the optical densi-

TABLE 1. Physical and chemical parameters before the bacterial inoculum was added

Parameter	Brackish water	River water	Spring water	Mineral water	Drinking water
pH	7.81	7.83	8.01	7.5	7.82
Conductivity at 20°C (μS/cm)	41,400	575	263	400	512
Chlorine concn (mg/liter)	17,257	20.3	7.86	5.3	67.2
Sulfate concn (mg/liter)	1,969	51.4	4.68	1.93	36.6
Calcium concn (mg/liter)	384	78.3	51.5	71	61.9
Magnesium concn (mg/liter)	979	21.5	0.79	1	7.83
Total hardness (°F)	504	28.5	13.2		18.7
Nitrate concn (mg/liter)	2.18	7.03	5.21	2.4	2.59
Ammonia concn (mg/liter)	0.34	0.18	0.03	0	0.08
Iron concn (mg/liter)	0.106944	0.006	0.005		0.236
Phosphate concn (mg/liter)	0.11	0.58	<0.4		<0.4
Silver concn (mg/liter)	0.0001	0.0005	0.0001		0.0001
Copper concn (mg/liter)	0.0134	0.0016	0.004		0.003
Total alkalinity ($CaCO_3$ concn) (mg/liter)	140	236	152	216	160
TOC concn (mg/liter)	2.5	2.2	0.8	<0.5	0.7

TABLE 2. Physical and chemical parameters before the bacterial inoculum was added and at the end of the experiments

Parameter	Brackish water		River water		Spring water		Mineral water		Drinking water	
	Initial	End	Initial	End	Initial	End	Initial	End	Initial	End
pH	7.81	7	7.83	7	8.01	7	7.5	7	7.82	7
Sulfate concn (mg/liter)	1,969	2,186	51.4	53.2	4.68	5.64	1.93	2.07	36.6	45.9
Nitrate concn (mg/liter)	2.18	0.6	7.03	<0.300	5.21	<0.300	2.4	1.91	2.59	<0.0300
Ammonia concn (mg/liter)	0.34	3.5	0.18	4.25	0.03	5.75	0	3.7	0.08	6
Phosphate concn (mg/liter)	0.11	1.9	0.58	1.99	<0.4	2.11	0	1.84	<0.4	1.66
TOC concn (mg/liter)	2.5	20.6	2.2	8.2	0.8	1.1	0	3.9	0.7	1.5

ties were higher than those found in the water samples tested. At the same time cytofluorimetric analysis revealed the lowest viability and the highest percentage of damaged cells. In the control culture the live cell clusters tended to disappear, while the damaged clusters persisted. The percentages of live cells were high in all types of water, ranging from 50% to 80%, and the live cell clusters were well defined during all of the trials with the exception of the mineral water and control culture trials (Fig. 5). This behavior could have been due to excess nutrients that have inhibitory effects on microbial growth, especially when the microorganisms are oligotrophic (44).

The apparent discordance between the plate count data and the cytofluorimetric data requires further investigation. The analysis of subpopulations (sorting) performed by flow cytometry allows us to quantify and characterize cells with different physiological parameters, such as enzyme activity, respiration, membrane potential, intracellular pH, and membrane integrity; this could provide an explanation for the behavior of the bacteria when they are inoculated into media.

No significant correlation was found between survival and any single physical or chemical parameter for the brackish water, spring water, and drinking water; in any case, high percentages of viable cells usually corresponded to high values for at least one of the parameters tested. The mineral water was characterized by the absence of iron and phosphates, which are essential for bacterial growth (32). This fact, together with the low organic carbon concentration, could have been responsible for the reduced viability percentages that were found from day 12 onward. An interesting result is the increases in the concentrations of ammonia, phosphates, sulfates, and TOC observed at the end of the experiments. The increase in the ammonia content could have been due to anaerobic respiration, in which nitrates were utilized as an electron acceptor. On the other hand, a suffering state of the bacteria with dead cells and the resulting release of cellular components into the surrounding environment could explain the increases in the other parameters. Thus, the bacterial presence in bottled water could contribute to the changes in chemical parameters during storage.

Our data are preliminary, and further studies should be performed to better define the factors that can affect bacterial survival, such as storage temperature and interference by autochthonous flora.

According to our results, cytofluorimetric analysis had several advantages. SYBR Green I coupled with propidium iodide allowed us to assess the bacterial viability in water; the system provided rapid direct counting of high numbers of cells; and the system allowed us to directly analyze water samples regardless of cultivation, and therefore sample handling and possible contamination due to sample handling were reduced. Thus, the original characteristics of samples were likely to be analyzed.

Furthermore, the independence from cultivation makes flow cytometry an appealing technique for fast detection of the viability of pathogenic bacteria in the viable but nonculturable state, which can be present in the aquatic environment but not detectable by conventional methods.

In conclusion, flow cytometric analysis appears to be a valid tool for assessment of bacterial viability and for better microbiological characterization of water in order to control and prevent health risks.

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