THE MICROBIOLOGICAL QUALITY OF WATER

Edited by

DAVID W. SUTCLIFFE

Published by the Freshwater Biological Association

Invited papers from a specialised conference held in London
on 12-13 December 1995
by the
Freshwater Biological Association,
The Ferry House, Far Sawrey, Ambleside, Cumbria LA22 0LP
and
International Water Supply Association,
1 Queen Anne's Gate, London SW1H 9BT, UK

ISBN 0-900386-57-6

Persistence of viable but non-culturable bacteria during the production and distribution of drinking water

P. CERVANTES[1], V. MENNECART[1], C. ROBERT[1],
M. R. DE ROUBIN[2] AND J. C. JORET[3]
ANJOU RECHERCHE, Laboratoire Central/Centre de Recherche Compagnie Gentrale des Eaux
[1]Département Recherche (GRAAL), 109-111 rue des Côtes, 78600 Maisons-Laffitte, France
[2]Département Contrôle, Chemin de la Digue, 78600 Maisons-Laffitte, France
[3]Laboratoire Central/Centre de Recherche, Chemin de la Digue, 78600 Maisons-Laffitte, France

The direct measurement of *in situ* respiring bacteria using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) shows that, especially for Gram-negative bacteria, large numbers of viable but non-culturable (VBNC) bacteria are present in finished water from a conventional water treatment plant, and the regrowth of bacteria along distribution networks can be seen rapidly by using this very sensitive technique. The level of bacterial inactivation with chlorine is much less important than has been previously supposed (based on experiments with non-injured laboratory strains of bacteria and classical culture techniques).

Threshold values of VBNC bacteria leaving water treatment plants or regrowing along distribution systems have to be determined for better control of coliform regrowth and health-risks associated with the consumption of drinking water.

Introduction

The methods used for evaluating the bacteriological quality of potable water are often based on the cultivation of planktonic bacteria in a sample (e.g. coliform counts and heterotrophic plate counts). However, culture methods not only require incubation periods of several days, but they are often insensitive and unable to detect the injured bacteria (Morita 1985; Brock 1987; Wayne *et al.* 1987; Yu & McFeters 1994). This may lead to an overestimation of the efficiency of water treatment steps, particularly disinfection steps, and to an underevaluation of the numbers of bacteria present in finished water and able to regrow along the distribution system. Consequently, more rapid, sensitive and convenient monitoring methods are needed for the quantitative assessment of viable but non-culturable (VBNC) microorganisms.

During this study, direct epifluorescent microscopic quantification of respiring (i.e. viable) bacteria was performed on raw river water, partially treated water (sand-filtered, ozonated and biologically activated carbon (GAC)-filtered), finished water (post-chlorinated) and samples from the distribution system at an industrial scale.

Additionally, we report some results of a bench-scale study aimed at evaluating the effect of chlorine on the inactivation of indigenous VBNC bacteria from GAC-filtered waters.

Material and methods

Enumeration of bacteria with DAPI and CTC

(a) DAPI and CTC. The DNA-binding fluorochrome, 4-6-diamidino-2-phenylindole (DAPI), was purchased from Sigma Chemical Co. (St Louis, Missouri, USA). The redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was purchased from Polysciences Inc. (Warrington, Pennsylvania, USA).

(b) Total counts of bacteria, using DAPI. A 250 µg ml^{-1} DAPI stock solution was prepared in tris-HCl buffer (125 mM, pH 7.2), stored at 4°C for up to 2 weeks and filter-sterilized before use. Water samples were incubated with DAPI (final concentration 2.5 µg ml^{-1}) for 20 min at 20°C. Samples (1 to 30 ml) were then filtered through polycarbonate black membranes (pore size 0.2 µm, diameter 25 mm; Schumacher, Germany) which had been premoistened with sterile deionized water.

Microscopic preparations were observed under the x100 oil immersion fluorescence objective of a Nikon microscope equipped with a Xenon lamp, a 330-380 nm excitation filter and a 420 nm cutoff filter. Twenty to fifty visual fields were randomly selected for the enumeration of bacteria.

(c) Viable but non-culturable bacteria (VBNC). Water samples (10 to 100 ml) were filtered through 0.2 µm porosity black polycarbonate filters (Nuclepore). The indigenous viable bacteria were incubated for 2 h at 20°C with 2 mM (final concentration) of CTC prefiltered through 0.2 µm porosity membranes. The stained bacteria were enumerated by microscopic observation with an epifluorescent illumination system (Nikon microscope equipped with a Xenon lamp, a 510-560 nm excitation filter and a 590 nm cutoff filter).

Strains of bacteria

The pure strains tested were purchased from the collection of the Institut Pasteur (CIP, Paris, France) or isolated from water. Bacteria were cultivated for 16 h at 37°C on plate-count agar (PCA) and resuspended in sterile water prior to the staining experiments.

Water samples

(a) Drinking water treatment plant. Water samples were taken at different steps of a full-scale drinking water treatment plant located at Méry sur Oise, France (270,000 m^3 day^{-1}): raw river water; rapid (flow velocity 5 m h^{-1}) sand-filtered water (after coagulation-flocculation-sedimentation); ozonated water (residual ozone *ca.* 0.2 mg l^{-1} after 10 min contact time); GAC-filtered water (flow velocity 5 m h^{-1}); post-chlorinated water (applied chlorine dose *ca.* 2 mg l^{-1}, contact time 2-4 h).

(b) Distribution system. Three points (DS1, DS2 and DS3) were sampled along the full-scale distribution system at Méry sur Oise, with estimated mean detention times of 4 h, 10-12 h, and 13-15 h, respectively.

Enumeration of culturable bacteria

Culturable bacteria from different water samples and suspensions of bacterial strains were enumerated by the pour-plate technique using PCA (3 days incubation at 20°C) and R_2A medium described by Reasoner & Geldreich (1985) (11 days incubation at 20°C).

Chlorination experiments

As part of an extensive study aimed at re-evaluating the contact times needed for inactivating indigenous bacteria from GAC-filtered water with chlorine, different laboratory experiments were performed at 20°C as follows: samples of GAC-filtered water taken at the Méry sur Oise treatment plant were respectively treated with an initial dosage of free chlorine at 0.25 mg l^{-1}, 1.44 to 1.76 mg l^{-1}, and 6.6 mg l^{-1}. Samples of treated water were taken after 5, 10, 15, 30, 60 and 120 min contact time and tested for residual chlorine (colorimetric method using N-N diethyl phenylene 1-4 diamine) or bacterial counts (CTC technique) after immediate neutralization of residual disinfectant with a sodium thiosulphate solution.

Contact-time values expressed in mg min^{-1} l^{-1} were calculated by mathematical integration of the concentration of residual free-chlorine in water *versus* time.

Results and discussion

Optimum conditions for staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)

The results published by Rodriguez *et al.* (1992), Schaule *et al.* (1993) and Yu & McFeters (1994) suggested a possible dose–response relationship between the CTC concentration/incubation time and the numbers of respiring bacteria in the water samples. Thus, preliminary experiments were carried out to determine the optimum conditions for staining respiring bacteria in our water samples. Final CTC concentrations of 1, 2 and 5 mM were used to stain *Escherichia coli* cultured for a full 16 hours and river water samples cultured for 2 to 6 h. Typical results are shown in Plate 1 (see page 105) and Figures 1 and 2. The enumeration of total bacteria (after DAPI staining) and culturable bacteria (on R_2A medium) was performed on the same samples. The following observations are noteworthy.

The coloured crystals of reduced CTC were deposited intracellularly, allowing easy microscopic visualization and enumeration, as shown in Plate 1 (p. 105).

Significantly higher counts were obtained when samples were incubated with 2 mM CTC for 2 hours. This result was observed with both the *E. coli* pure strain (Fig. 1) and the river water samples (typical data in Fig. 2).

The proportion of respiring bacteria detected with CTC, expressed as the percentage of the total count obtained after DAPI staining, was *ca.* 80% for the *E. coli* strain (Fig. 1) and 60 to 70% for the river water sample (Fig. 2).

Approximately 80 to 100% of culturable bacteria from river water samples were detected by staining with CTC (Fig. 3).

Efficacy of CTC staining for different strains of bacteria

Under the experimental conditions previously determined, several strains were tested in order to determine their ability to reduce CTC (Table 1). The proportion of respiring bacteria, expressed as the percentage of total bacteria stained with DAPI, varied from 30 to 86% for the Gram-negative strains, whereas the percentage of CTC-stained bacteria remained below 10% when Gram-positive strains were tested.

The results of this experiment suggest that bacterial counts after CTC staining strongly depend on the nature of the bacteria present in the sample; the efficiency of the technique appears to be satisfactory for the Gram-negative strains but poor for the Gram-positive bacteria.

Table 1. *Efficiency of CTC reduction by different strains of bacteria.*
*Water isolates.

Bacterial strains	Efficacy of CTC staining (%)
Escherichia coli 52.167	86
Enterobacter sp. 55.49	93
*Acinetobacter calco aceticus**	31
Staphylococcus 53.154	0.2
*Streptococcus faecalis**	0.6
Bacillus sp. 52.62	10

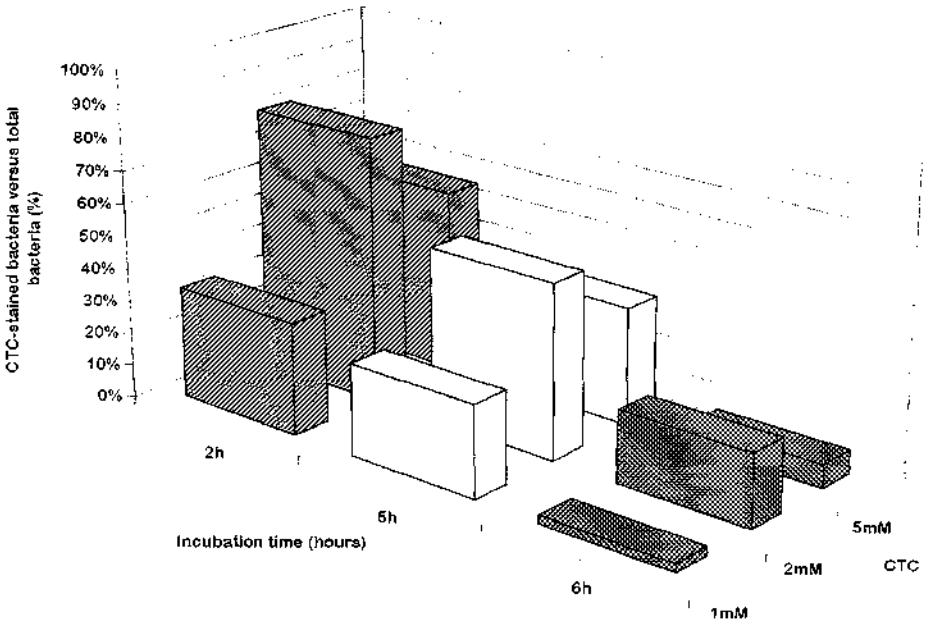

Figure 1. The proportions (%) of CTC-stained *Escherichia coli*, plotted against incubation time (hours at 20°C) and concentrations of CTC (mM).

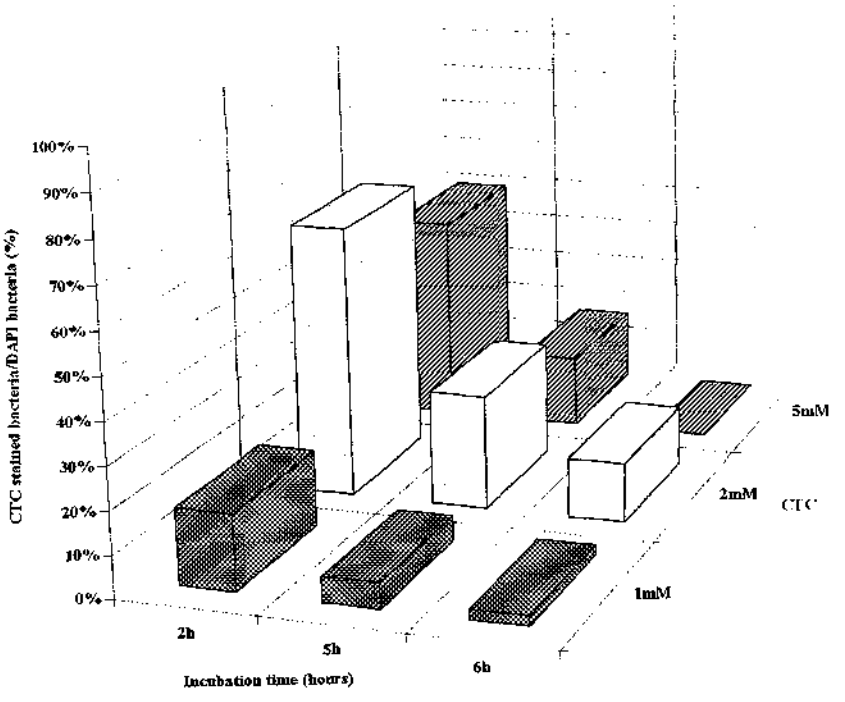

Figure 2. The proportions (%) of CTC-stained bacteria and DAPI-stained bacteria, plotted against incubation time (hours at 20°C) and concentrations of CTC (mM).

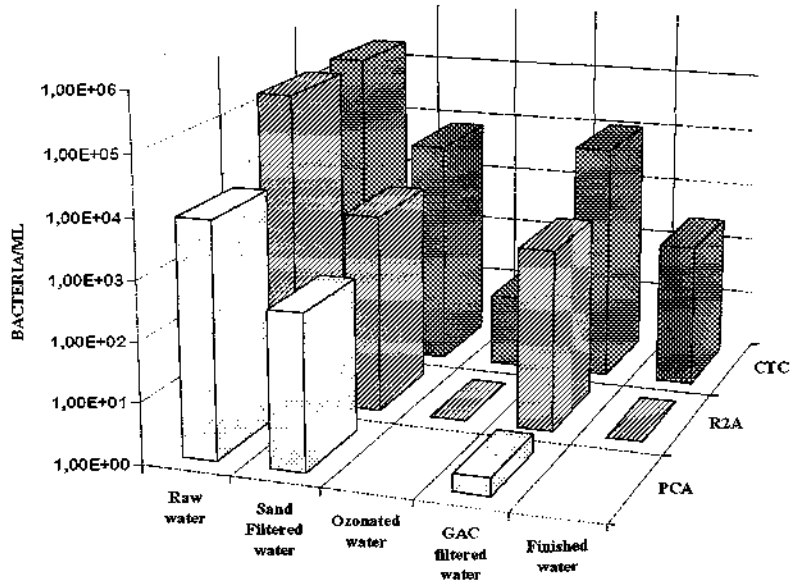

Figure 3. Efficiency of the drinking water treatment plant at Méry sur Oise for removal and inactivation of culturable (PCA, R_2A) bacteria and *in situ* respiring CTC-stained bacteria. The ordinate is scaled from 0 to 10^6.

Table 2. *Some characteristics of the water samples studied in full-scale experiments at the Méry sur Oise treatment plant.*

*Free-chlorine doses applied: 2 mg l^{-1} at start and 0.4 mg l^{-1} after partial dechlorination.
DOC = dissolved organic carbon; BDOC = biodegradable organic carbon.
ND = not determined.

Origin	DOC (mg l^{-1})	BDOC (mg l^{-1})	pH	Residual oxidant (mg l^{-1})	Total coliforms (Numbers 100 l^{-1})	Faecal coliforms (Numbers 100 l^{-1})
Water treatment plant						
River water	3.6	1.4	7.7–8.2	0	3000–6000	500–1000
Sand-filtered	2.3	0.5	7.5–7.6	0	15–80	15–70
Ozonated	2.2	0.8	7.5–7.7	0.4–0.5	0	0
GAC-filtered	1.8	0.5	7.5–7.7	0	0	0
Finished	1.8	0.5	7.5–7.7	2.0–0.4*	0	0
Distribution system						
DS1 to DS3	ND	ND	7.6–7.7	0.34–0.05	0	0

Characteristics of water samples taken from a full-scale treatment plant

Some characteristics of the samples taken from the treatment plant at Méry sur Oise are given in Table 2. These results show that, in spite of a relatively poor quality of the raw river water (DOC = 3.6 mg l^{-1}; total coliform counts = 3000 to 6000 per 100 ml) the finished and distributed water always complied with EEC regulations for drinking water.

Efficiency of the treatment plant for removing and inactivating culturable or VBNC bacteria

A typical example of the removal and inactivation of bacteria cultured on PCA (Afnor 1990) and R_2A media (Reasoner & Geldreich 1985), and CTC-stained bacteria, is given in Figure 3.

Several conclusions can be drawn from these results.

Cultures on PCA medium (3-day incubation period at 20°C, pour-plate technique) largely underestimated the numbers of viable bacteria in water (1 to 2 $\log_{10}$ orders of magnitude per ml) for all types of water. During the experimental period this technique failed to detect the presence of bacteria after all oxidative treatments in ozonated, post-chlorinated and distributed water at the Méry sur Oise treatment plant.

In comparison, cultures on R_2A medium (11-day incubation period at 20°C, pour-plate technique) detected small numbers of bacteria (per ml) after ozonation and post-chlorination, but detected more than 100 culturable bacteria per ml in GAC-filtered water samples.

For river water samples, the numbers of respiring bacteria (detected after CTC staining) were very close to the colony count obtained after cultivation on R_2A medium. However, this observation was not true for the partially or fully treated water samples, for which the CTC-stained bacteria counts were higher by 1 or 2 $\log_{10}$ orders of magnitude: CTC counts yielded 10 bacteria ml^{-1} in the ozonated water and more than 100 bacteria ml^{-1} in the finished water, whereas the heterotrophic plate counts for R_2A medium remained lower than 5 bacteria ml^{-1} for both types of water.

Neither ozonation (0.4 mg l^{-1} residual ozone after 10 min contact time) nor post-chlorination (2 mg l^{-1} applied dose; 1.2 mg l^{-1} residual free-chlorine after about 120 min contact time) were able to inactivate all bacteria which had passed through or were released from sand or GAC filters as seen by microscopic counts of respiring bacteria. The reduction efficiencies were 2 $\log_{10}$ ml^{-1} for ozone and 1 $\log_{10}$ ml^{-1} for chlorine, respectively.

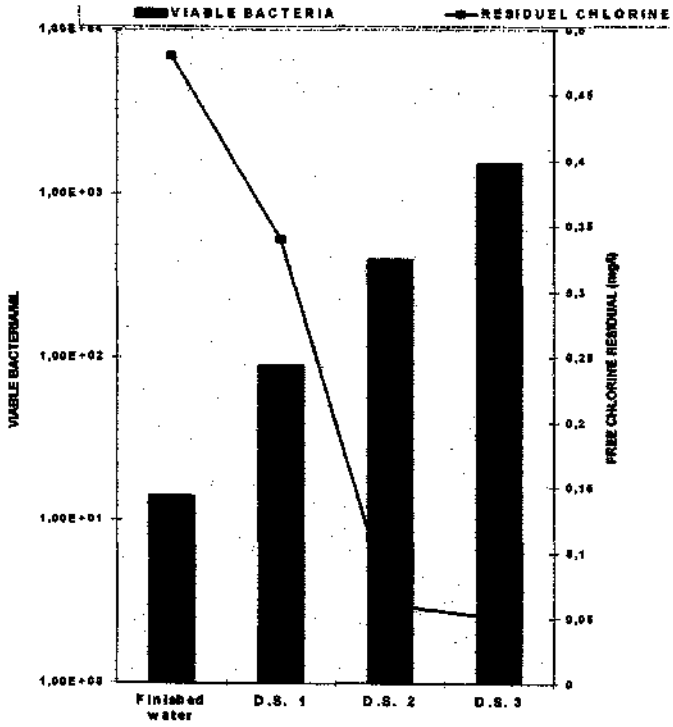

Figure 4. Regrowth of bacteria along the water distribution system at Méry sur Oise, observed by counting viable CTC-staining bacteria (histograms showing numbers l^{-1} from 0 to 10^4) in finished water and at sampling points DS1, DS2 and DS3. Points joined by the solid line indicate the concentrations of residual free-chlorine (mg l^{-1}).

Whilst inactivating large numbers of bacteria, ozone leads to the formation of large quantities of bacterial growth-promoting components (biodegradable organic matter) as previously described (Joret *et al.* 1988; Van der Kooij *et al.* 1989; Volk *et al.* 1993). As a consequence, for this treatment plant, large numbers of bacteria were released from the biologically active GAC filters. This phenomenon has to be balanced against the beneficial effects of ozone: i.e. the inactivation of viruses (Joret *et al.* 1986), *Giardia* cysts (US EPA 1989) and *Cryptosporidium* oocysts (Peeters *et al.* 1989; Joret *et al.* 1992; Finch *et al.* 1994), the destruction of pesticides, and the reduction of the formation potential of disinfection by-products in biologically-treated water (after GAC filtration).

Regrowth of VBNC bacteria along the distribution system

Neither total coliforms nor classical faecal indicators (thermotolerant coliforms; enterococci) were detected in 100 ml samples from the different sampling points of the distribution system that we studied (sampled weekly from March to September 1995). Heterotrophic plate-count determinations using PCA (3 days incubation at 20°C) were always 10 times lower than the EEC guidelines (100 bacteria ml^{-1}).

However, a bacterial regrowth phenomenon along this distribution system was noted by direct measurements of respiring bacteria (typical results are given in Fig. 4 for week 22) or by the culture technique using R_2A medium. CTC counts increased from 10 bacteria ml^{-1} (finished water) to 10^3 bacteria ml^{-1} (DS3) while residual free-chlorine decreased from 0.48 to 0.05 mg l^{-1}. For the same sampling dates, counts of bacteria (ml^{-1}) on R_2A medium after 11 days at 20°C were 0 (finished water), 25 (DS1), 570 (DS2) and 66 (DS3). Clearly, the importance of the CTC technique is that it rapidly detects (in less than 1 day), with a high degree of sensitivity, degradation of the microbiological quality of water along the network. A similar bacterial regrowth phenomenon (with isolation of coliforms) was previously reported for the same distribution system (Volk & Joret 1994).

Efficiency of chlorine for inactivating indigenous bacteria in water at 20°C

Most of the results reported in the literature describing the inactivation of bacteria with chlorine have been obtained by using non-injured laboratory strains of bacteria, and the level of inactivation is recorded by classical cultivation techniques. As an example, for a 2 $\log_{10}$ reduction in numbers of *E. coli*, contact-time values as low as 0.006 to 0.02 mg min^{-1} l^{-1} have been reported (Katzenelson 1974). More recent studies have shown that pure strains of bacteria (*Klebsiella pneumoniae*) under a nutritional stress or growing in biofilms on GAC can be 100 to 10,000 times more resistant (Lechevallier *et al.* 1988a,b).

Based on these data and taking into account the results obtained during this study at the industrial scale (Fig. 3), an extensive bench-scale study was designed to estimate the contact-time values necessary for inactivating 90, 99 and 99.9% of indigenous bacteria from GAC-filtered water samples with chlorine at pH 7.5 to 7.7, and at different temperatures, using CTC techniques. Typical results of inactivation obtained at 20°C for different chlorine doses are shown in Figure 5.

The characteristics of the GAC-filtered waters used in this study are given in Table 3; dissolved organic carbon (DOC) levels ranged from 1.6 to 2.3 mg l^{-1}, and free-chlorine consumption in 15 min ranged from 0.17 to 0.6 mg l^{-1}. For these samples, the level of disinfection was mainly determined by the initial applied dose of chlorine.

The decrease in numbers of CTC-reducing bacteria obtained after 5 min contact time was *ca.* 1.2 $\log_{10}$ (relative numbers) for the lower applied dose (C_1 = 0.25 mg l^{-1}), 1.9 $\log_{10}$ for the intermediate dose (C_2 = 1.44 mg l^{-1}) and 2.5 $\log_{10}$ for the higher dose (C_3 = 6.6 mg l^{-1}). Increasing the contact time to 15 min had only a slight effect on the level of inactivation of

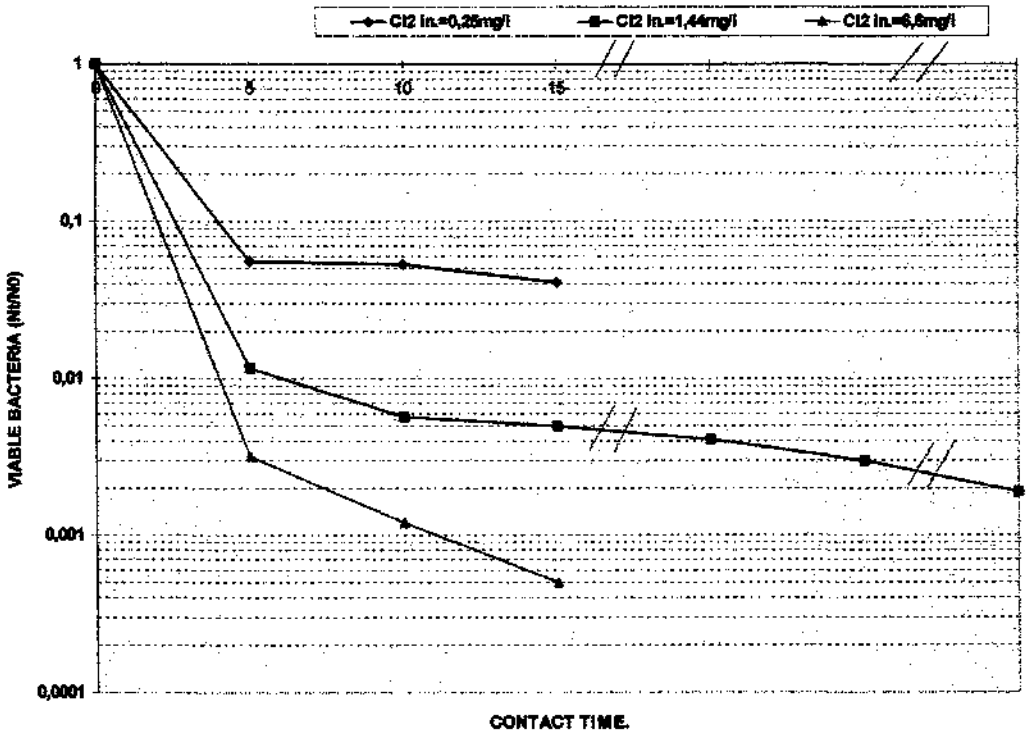

Figure 5. Relative numbers of CTC-staining bacteria as a function of contact time (minutes) and three different initial doses of residual free-chlorine (Cl_1 = 0.25 mg l^{-1}, top line; Cl_2 = 1.44 mg l^{-1}, centre line; Cl_3 = 6.6 mg l^{-1}, bottom line) in bench-scale experiments at 20°C.

Table 3. *Effects of chlorine on the inactivation of indigenous viable but non-culturable bacteria (VBNC) from GAC-filtered water samples at 20°C.*

Experiment number	DOC (mg l^{-1})	Initial dose of residual chlorine	Chlorine demand (mg l^{-1} in 15 min)	Log_{10} reduction bacteria ml^{-1}) (15 min)	Estimated contact-time value (mg min^{-1} l^{-1})
1	2.3	0.25	0.17	1.3	2
2	2.3	1.44	0.26	2.3	19
3	2.1	1.73	0.20	1.9	23
4	2.1	1.71	0.18	1.6	23
5	2.1	1.76	0.22	1.8	23
6	1.6	6.60	0.60	3.3	98

bacteria for the three doses: an additional 0.1 log_{10} reduction in numbers for C_1, 0.4 log_{10} for C_2 and 0.8 log_{10} for C_3.

Compared with the 1.9 log_{10} reduction of bacteria obtained after 15 min for an initial free-chlorine dose of 1.44 mg l^{-1} (mean 15 min contact-time value = 19 mg min^{-1} l^{-1}), the numbers of indigenous bacteria were reduced by only 2.7 log_{10} after a contact time of 2 hours (mean 15 min contact-time value = 126 mg min^{-1} l^{-1}). In comparison, a very similar level of inactivation (2.5 log_{10} reduction) was obtained after 10 min for an initial free-chlorine dose of 6.6 mg l^{-1} (mean 15 min contact-time value = 34 mg min^{-1} l^{-1}).

These results demonstrate the limits of the contact-time concept, the initial applied dose of free-chlorine being the determining factor for the level of inactivation recorded.

Table 3 summarizes the results of six disinfection experiments with chlorine at 20°C. The results of experiments 2 to 5 (initial applied doses of free-chlorine at 1.44 to 1.76 mg l^{-1}), done on GAC-filtered water samples with relatively similar chemical characteristics (DOC = 2.1 to 2.3mg l^{-1}; chlorine demand in 15 min = 0.18 to 0.26 mg l^{-1}), show levels of bacterial inactivation varying from 1.6 to 2.3 $\log_{10}$ numbers ml^{-1} after 15 min contact. These differences in bacterial reduction are not very large and could be due to uncontrolled physicochemical variables (e.g. particles) or microbiological variables (e.g. nature of the bacterial population) in the water samples.

References

AFNOR NF 90-402 (1990). Normes Francaises d'analyse microbiologique des eaux: Essais des eaux-dénombrement des microorganismes revivifiables à 20°C (Méthode d'incorporation en gélose). *Recueil des Normes Francaises*, 4th edition, 466-471.

Brock, T. D. (1987). The study of microorganims *in situ:* progress and problem. *Symposium Society General Microbiology*, **41**, 1-17.

Finch, G. R., Black, K. & Gyurek, L. L. (1994). Ozone and chlorine inactivation of Cryptosporidium. *Proceedings of the American Water Works Association & Water Quality Treatment Council*, San Francisco, California.

Joret, J. C., Levi, Y., Dupin, T. & Gibert, M. (1988). Rapid method for estimating bioeliminable organic carbon in water. In *Proceedings of the American Water Works Association Annual Conference*, pp. 19-23, Orlando.

Joret, J. C., Perrine, D. & Langlais, B. (1992). Valeurs minimales et maximales de C.t d'inactivation de *Cryptosporidium* (oocystes) par l'ozone, à diverses témperatures. In *Microbiologie des Eaux d'Alimentation* (eds C. Haslay & H. L. Leclerc), Chapter 3.2, p. 274. Tech. & Doc-Lavoisier, 1993.

Joret, J. C., Hassen, A., Bourbigot, B. B., Agbalika, F. *et al.* (1986). Inactivation des virus dans l'eau sur une filière de production à ozonation étagée. *Water Research*, **20**, 871.

Katzenelson, E. (1974). Inactivation of viruses and bacteria in water by use of ozone. *Journal of the American Water Works Association*, **66**, 725-729.

Lechevallier, M., Cawthon, C. D. & Lee, R. G. (1988a). Factors promoting survival of bacteria in chlorinated water supplies. *Applied Environmental Microbiology*, **54**, 649-654.

Lechevallier, M., Cawthon, C. D. & Lee, R. G. (1988b). Inactivation of biofilm bacteria. *Applied Environmental Microbiology*, **54**, 2492-2499.

Morita, R. Y. (1985). Starvation and miniaturisation of heterotrophs, with special emphasis on maintenance starved viable state. In *Bacteria in their Natural Environments* (eds M. Fletcher & G. D. Floodgate), pp. 11-130. Academic Press Inc., London.

Peeters, J. E., Ares Mazas, E., Masschelein, W. J., Villacorta M. de Maturana & Debacker, E. (1989). Effects of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. *Applied Environmental Microbiology*, **55**, 1519-1522.

Reasoner, D. J. & Geldreich, E. E. (1985). A new medium for enumeration and subculture of bacteria from potable water. *Applied Environmental Microbiology*, **49**, 1-8.

Rodriguez, G., Phipps, D., Ishiguro, K. & Ridgway, F. (1992). Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Applied Environmental Microbiology*, **58**, 1801-1808.

Schaule, G., Flemming, H. C. & Ridgway, F. (1993). Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. *Applied Environmental Microbiology*, **59**, 3850-3857.

US EPA (1989). *Guidance Manual for the Compliance with the Filtration and Disinfection Requirements for Public Water Systems Using Surface Water Supplies.* Washington DC, USA.

Van der Kooij, D., Hijnen, A. M. & Kruithof, J. C. (1989). The effects of ozonation, biological filtration and distribution on the concentration of easily assimilable organic carbon (AOC) in drinking water. *Ozone Scientific Engineering*, **11**, 297-311.

Volk, C., Roche, P., Renner, C., Paillar, H. & Joret, J. C. (1993). Effect of ozone on the production of Biodegradable Dissolved Organic Carbon (BDOC) during water treatment. *Ozone Scientific Engineering*, **15**, 405-417.

Volk, C. & Joret, J. C. (1994). Paramètres prédictifs de l'apparition des coliformes dans les réseaux de distribution d'eau d'alimentation. *Revue des Sciences de l'Eau*, **7**, 131-152.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. *et al.* (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology*, **37**, 463-364.

Yu, F. P. & McFeters, G. A. (1994). Rapid *in situ* assessment of physiological activities in bacterial biofilms using fluorescent probes. *Journal of Microbiological Methods*, **20**, 1-10.

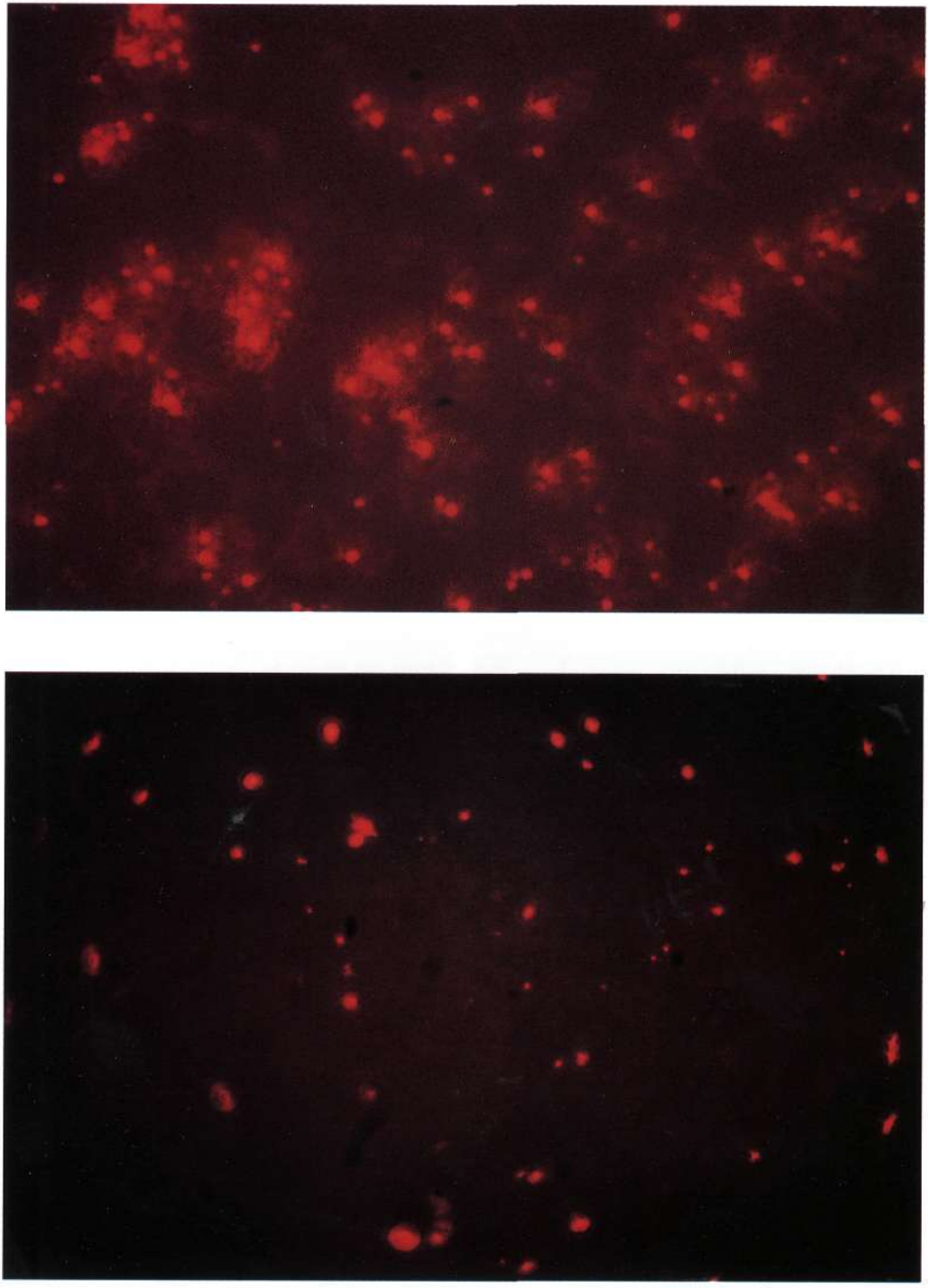

Plate 1. Epifluorescence photomicrographs of CTC-reducing bacteria. *Above:* Culture of *Escherichia coli. Below:* Indigenous bacteria from GAC-filtered water. (See Cervantes *et al.*, pp. 54-62).