# Optimization of the detection of the spores of aerobic spore-forming bacteria (ASFB) in environmental conditions

Clément Cartier, Benoit Barbeau, Marie Claude Besner, Pierre Payment and Michèle Prévost

## ABSTRACT

The measurement of spores of aerobic spore-forming bacteria (ASFB) is becoming a widely accepted method for validating the effectiveness of treatments applied in drinking water treatment plants. These bacteria have been proposed as indicators of soil intrusion in distribution systems. There are, nevertheless, some limitations to the measurement of ASFB spores by membrane filtration with respect to the rate of recovery of spores present in distributed water. This is mainly due to the low concentrations of these spores. Two factors may decrease spore recovery: the presence of a cake on the filter and the aggregation of spores. For distributed water, our data suggest limiting the volume of water filtered (V in ml) as a function of its turbidity, according to the relation: V = 500/turbidity. The addition of a surfactant, Tween 80®, can increase the recovery of spores very significantly (1000 X). The experimental results show that a triple manual inversion will ensure a significantly higher rate of recovery.

**Key words** | aerobic spores, ASFB, distribution system, drinking water, indicator, intrusion, spores

**Clément Cartier**
City of Montréal, Infrastructure, Transport and Environmental services,
Water Systems Strategic Planning, Montréal (Québec),
Canada H3A 3P1

**Benoit Barbeau**
NSERC Industrial Chair on Drinking Water,
École Polytechnique de Montreal,
Department of Civil, Geologic and Mining Engineering,
P.O. Box 6079, Succ. Centre Ville H3C 3A7,
Montreal (Quebec),
Canada

**Marie Claude Besner**
École Polytechnique de Montreal,
Department of Civil, Geologic and Mining Engineering,
P.O. Box 6079, Succ. Centre Ville
Montreal (Quebec),
Canada H3C 3A7

**Pierre Payment**
INRS – Institut Armand-Frappier,
531 boul. des Prairies, Laval (Québec),
Canada H7V 1B7

**Michèle Prévost** (corresponding author)
NSERC Industrial Chair on Drinking Water,
École Polytechnique de Montreal,
Department of Civil, Geologic and Mining Engineering,
P.O. Box 6079, Succ. Centre Ville
Montreal (Quebec),
Canada H3C 3A7
Tel.: 1(514) 340 4711 (5924)
Fax: 1 (514) 340 5918
E-mail: *michele.prevost@polymtl.ca*

## INTRODUCTION

The direct use of pathogenic microorganisms to measure microbial removal or inactivation during treatment and during drinking water distribution is a highly complex and costly endeavor. In addition, the low concentrations of pathogens found and their sporadic presence in the environment often make it necessary to work near, or even under, the detection limits of the available methods. Moreover, the detection of multiple pathogens necessitates the use of many specific measurement methods. Bacterial indicators, such as total and fecal coliforms and *Escherichia coli*, are widely used, but their

doi: 10.2166/aqua.2007.103

usefulness is limited as indicators of larger organisms and of organisms that are more resistant to chlorination (Rice *et al.* 1993). As an alternative solution, organisms such as the spores of aerobic spore-forming bacteria (ASFB) have been proposed as indicators of the effectiveness of treatment processes (Rice *et al.* 1996; Barbeau *et al.* 1997; Nieminski *et al.* 2000; Huertas *et al.* 2003; Galofré *et al.* 2004; Brown & Cornwell 2005; Mazoua & Chauveheid 2005). The main advantages of ASFB spores are their size, as it is similar to that of known pathogenic protozoa, their abundance in raw water and their high resistance to water disinfection treatments (Rice *et al.* 1996). Aerobic spores have also been accepted, in conjunction with other hydrogeologic data, as a surrogate for the site-specific removal of *Cryptosporidium* by bank filtration (USEPA 2005). More recently, these spores have been proposed as indicators of contamination in distribution systems, primarily to detect intrusion events (Karim *et al.* 2003; Besner *et al.* 2005). Interest in their use for this application is based on the hypothesis that they are abundant in soils, but not in treated drinking water in distribution pipes. Their detection in water taken from a distribution system could therefore indicate the intrusion of soils and/or water into the distribution system and the potential for fecal contamination (Besner *et al.* 2005; Cartier *et al.* submitted).

Two main methods have been proposed for the recovery of ASFB spores in water by membrane filtration on a 0.45 μm filter (Rice *et al.* 1996; Barbeau *et al.* 1997). The principal difference between the two methods is that in one of them a pasteurization step is performed prior to filtration in a liquid medium (Rice *et al.* 1996) and in the other it is applied directly on Petri plates (Barbeau *et al.* 1997). Other methods for measuring ASFB spores (Nieminski *et al.* 2000; Francis *et al.* 2001) are derived from these two methods. Conventional treatment processes, such as chemically assisted filtration and disinfection, remove and inactivate ASFB spores found in source water (Rice *et al.* 1996; Galofré *et al.* 2004; Brown & Cornwell 2005), resulting in low concentrations in treated and distributed water. To enable the precise enumeration of these lower spore concentrations, larger volumes of water must be filtered.

Three factors that can influence the enumeration of bacteria (ASFB and others) by membrane filtration in water samples can be considered. These factors are especially relevant to waters requiring higher filtered volumes and which are loaded with particles.

### Interference caused by competing microorganisms

When the membrane filtration method is used in the presence of competing microorganisms evaluated by the heterotrophic plate count (HPC) method, a reduction in the number of coliforms can be observed (Geldreich *et al.* 1978; Herson & Victoreen 1980). The pasteurization step required for the detection of ASFB spores leaves the environment free of competing bacteria, which could reduce the growth of ASFB spores. This factor is therefore not considered significant for the enumeration of these spores in samples with a high concentration of non-spore-forming bacteria.

### The impact of the accumulation of particles on the filter

The filtration of water containing particles can lead to the formation of a thin layer of particles on the filter, referred to hereafter as a "cake". This layer may constitute a diffusion barrier ($O_2$, nutrients, etc.) or interfere with growth in some other way (metals, for example). As well, the accumulation of turbidity can lead to an underestimation of the coliform concentration in water samples when membrane filtration is used, because of the formation of a cake on the membrane (Geldreich *et al.* 1978; Herson & Victoreen 1980; LeChevallier *et al.* 1981). The least severe impact was observed by LeChevallier *et al.* (1981), who maintains that a turbidity of 5 NTU is acceptable for the measurement of total coliforms in 100-ml samples. The impact of the cake on the detection of ASFB spores is not well established, however. Some testing has been done on relatively small volumes of raw water (500 ml) inoculated with spores of *Bacillus subtilis* (Nieminski & Bellamy 2000) or with sulfate-reducing *Clostridia* in large volumes (>50 L) of high-quality treated water (Hijnen *et al.* 2000). These studies suggest that the impact of particle accumulation on recovery is not significant (either not significant or more than 75% recovery). Nevertheless, no direct verification of the interference of turbidity in the enumeration of environmental ASFB spores in distributed water is available.

In the case of distributed water, the presence of metals such as copper and lead, in both soluble and particulate forms, is common and could reduce the recovery rate of ASFB spores because of their inhibiting effect on bacterial growth. Specifically, when copper is added to the culture medium, the growth of sulfate-reducing bacteria (Utgikar *et al.* 2003), *Bacillus thuringiensis* (Hassen *et al.* 1998) and *Aeromonas spp.*, total coliforms and HPC (Versteegh *et al.* 1989) is limited. Moreover, copper can limit the development of biofilm at the surface of pipes for microorganisms such as non tuberculous mycobacteria (Williams & Donlan 2005), *Flavobacteria sp.*, *Pseudomonas sp.*, *Xanthomonas maltophilia* (Critchley *et al.* 2002) and *Legionella pneumophila* (Rogers *et al.* 1994). However, the impact of the presence of copper on the growth of the ASFB spores present in distribution systems, such as *Bacillus subtilis*, is not known.

### Aggregation in response to adverse environmental conditions

Drinking water treatment and distribution systems are likely to generate various stresses on the microorganisms present in the liquid phase of the water and in the biofilm. Microorganisms offering the best resistance to major stress, like ASFB spores, use particular methods of adaptation, such as aggregation, colonization of particles by bacteria or biofilm formation (Morin *et al.* 1997).

There is some clear indication that the aggregation of spores, or their attachment to particles in distribution systems, may be common. It has been shown, for example, that spore aggregation occurs during treatment (Gale *et al.* 1997; Barbeau *et al.* 2005), and the colonization of sediments present in distribution systems by bacteria has been established for coliform bacteria (Herson *et al.* 1991; Oliver & Harbour 1995), for fungi, for actinomycetes and for total bacteria (Ridgway & Olson 1982; Zacheus *et al.* 2001). Gibbs *et al.* (2003, 2004) report significant colonization of the biofilm by endospores ($2.5 \times 10^4$ CFU/cm$^2$) following the injection of wastewater into a pilot distribution system. Moreover, the presence of *Bacillus sp.* in the biofilm of full-scale distribution systems has been documented by Rompré *et al.* (1998). While soil constitutes the principal habitat of ASFB spores (Frobisher 1968; Demain & Solomon 1985), the extent of aggregation of these spores in a natural environment is not well documented.

Culture-based enumeration techniques cannot assess the presence of aggregation, since the presence of a colony does not indicate whether it is the result of the growth of an aggregate of bacteria or of a single organism. Not taking into account aggregation could lead to a significant underestimation. A dispersing agent (such as a surfactant) can be added or a homogenization treatment performed, or both, to disaggregate the spores, or to liberate the spores associated with particles. However, the addition of a surfactant and the application of homogenization treatments may inhibit bacterial growth and spore recovery. When isolating organisms from soil samples, the samples are often homogenized in order to destabilize the aggregates (Germida 1993). Barbeau *et al.* (1997) have shown that homogenization has no influence on spore counts in raw water samples. In contrast, other studies show the usefulness of mechanical methods of bacterial dispersion (Camper *et al.* 1985; Madge 2002; Borst & Selvakumar 2003; Ormeci & Linden 2005). However mixing at high sheer stress may result in the destruction of a portion of the microorganisms in the sample (Ormeci & Linden 2005). The use of a chemical dispersant, such as Zwittergent 3–12 (Sigma-Aldrich, MO), shows an increase in the recovery rate of coliforms in some conditions (Camper *et al.* 1985; Ormeci & Linden 2005), and a reduction in recovery rate in others (Borst & Selvakumar 2003), which makes the use of this substance debatable. Another surfactant, Tween 80®, has been used to study the extent of the distribution of ASFB spores in coagulated waters (Gale *et al.* 1997). Their results offer somewhat muted conclusions, which suggest that verification for each type of water and each strain of microorganism will be required.

## OBJECTIVES

The objectives of this study are:

1. To verify the impact of the concentration of large volumes of distributed water on the recovery of ASFB spores by membrane filtration. Two aspects capable of

modifying the recovery of ASFB spores are studied: the presence of cakes in terms of their constituent particles, and the nature of the cake particles.

2. To confirm the presence of aggregates and to estimate the impact of their dispersion on the recovery of spores in soil samples and in samples of water flushed from distribution systems.

## MATERIAL AND METHODS

### Influence of suspended solids and metals on the recovery of *Bacillus subtilis*

The culture of *Bacillus subtilis* spores was performed with the method described by Barbeau *et al.* (1999). A suspension of this bacterium (ATCC, no. 6633, USA) was prepared from a culture on R2A media (Difco, 1826-17-1) at 35°C, then left for 10 days in the incubator to sporulate. Spores were collected by rinsing the agar with sterile phosphate buffer. The final spore suspension was heated at 75°C for 15 min and subsequently maintained at 4°C. *B. subtilis* suspensions were diluted in a physiologically sterile water (phosphate buffer, pH 6.5). The method described by Barbeau *et al.* (1997) was used to measure the *B. subtilis* spores. Trypticase soy broth (pH $7.3 \pm 0.2$; Becton Dickinson, MD, USA) was used as the culture medium. Enumeration in test water was performed after pasteurization at 75°C for 15 mins. Cellulose acetate filters (0.45 μm, Millipore, Billerica, MA- HAWG047S3) were used. The concentration of injected spores ($N_0$) was validated by the use of control filters on which only the spore inoculum was filtered.

Tests on the influence of particles on the recovery of ASFB spores were carried out on tap water with a typical load in terms of particles and metals. Two water samples were collected from a tap located on a main pipe. Water characteristics include turbidity of $<0.5$, pH of 7.5, TOC of 2.13 and alkalinity of 83 mg/L $CaCO_3$. Samples were sterilized at 121°C for 15 mins, cooled and seeded with spore suspensions of *Bacillus subtilis*. Three types of seeding were carried out:

- Case A, for which *B. subtilis* spores were homogeneously distributed in the cake, with the spore inoculum being added to the sterile tap water, homogenized and then filtered.
- Case B, to examine the growth of spores on the cake surface by adding the *B. subtilis* spore inoculum following filtration of the sterile tap water.
- Case C, to show the growth of spores under the cake. In this case, the spore inoculum was filtered before the sterile tap water.

A schematic representation of the three types of seeding is presented in Figure 1. Every measurement was taken in triplicate. After incubation, the number of colonies per Petri plate was counted, up to a maximum of 400 colonies, a higher number being considered "too numerous to count" (TNTC).

Suspended solids (SS) and volatile suspended solids (VSS) measurements were taken, according to Method 2540 (Standard Methods for the Examination of Water & Wastewater 1998), using 0.7-μm fiberglass filters (Whatman 47 mm, No. 1825047, Maidstone, England).

The metal content of the cake (Cu, Pb, Mn, Fe, Zn) was measured on the filters used for spore enumeration. Metal analyses were performed following mineralization by digestion of the filter in a Teflon vial containing 1 ml

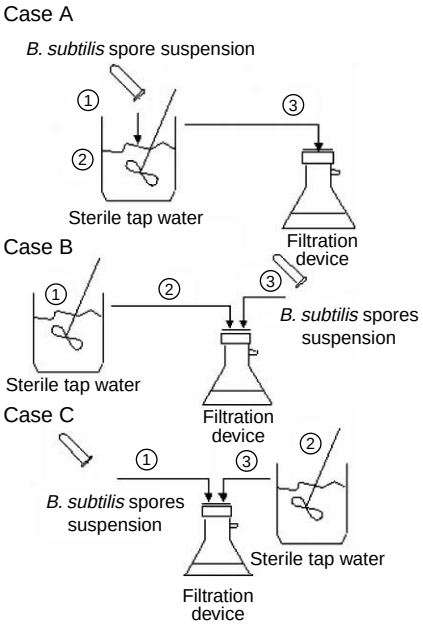


**Figure 1** | Schematic representation of the three types of *B. subtilis* seeding.

of HCl, 0.5 ml of $HNO_3$ and 0.5 ml of $H_2O_2$. The concentrate was then diluted and analyzed for these metals using atomic absorption flame spectrometry (A-Analyst 200, PerkinElmer, MA, USA). In order to determine the impact of copper on the growth of *B. subtilis* spores, copper sulfate (C493-500, Fisher Scientific, NJ, USA) was added to the culture medium (trypticase soy broth) prior to sterilization. The concentrations of copper added were: 0.9; 1.6; 4.1 and 7.7 mM. Actual concentrations were verified by analyzing the copper content in the culture medium by atomic absorption flame spectrometry. Four replicates of plate counts were performed.

## Influence of aggregation on the recovery of ASFB spores

Tests were carried out to evaluate the impact of the aggregation of a natural strain of ASFB spores by applying various dispersion techniques. ASFB spores were dispersed by means of a chemical surfactant, Tween 80® (Fisher Scientific, NJ, USA) and an anti-foaming agent, Polypropylene glycol (PPG, Sigma Aldrich, Belgium). Tween 80® was chosen as the surfactant because it is not harmful to the growth of spores of the *Bacillus* type (Brar *et al.* 2005). Slow homogenization (< 1,000 rpm) was selected in order to minimize the potential reduction in the number of viable spores. Dispersion tests were carried out on the samples containing a soil suspension, as well as on the samples of flushing water taken from the hydrants of a distribution system. The water was sampled during the first two minutes of flushing at a hydrant attached to a cast-iron pipe. The soil was sampled on university grounds. The soil suspensions were prepared by adding 10 mg of soil to 995 ml of sterile saline water (0.85%). The suspension was then agitated gently and continuously with a magnetic stirrer in a 2-L beaker. For the dispersion tests, two 50-ml samples were removed from this suspension using 60-ml syringes (Becton Dickinson, NJ, USA). In the same way, 100-ml samples of the hydrant flushing water were taken from a 4-L beaker subjected to gentle agitation with a magnetic stirrer.

The reagents (Tween 80®, PPG) were first filtered (0.22 μm, Millex GS, Millipore, Ireland) to prevent microbiological contamination. The following combinations of reagents and their respective concentrations (v/v) were used: i) Tween 80® 0.0% and PPG 0.0%; ii) Tween 80® 0.1% and PPG 0.1%; and iii) Tween 80® 1% and PPG 1%. Finally, three modes of homogenization were tested for the water samples (soil suspension and flushing water) to which the two reagents had been added:

1. A manual inversion (3 ×) (MI) of a 100-ml sample placed in a 125-ml polypropylene bottle (Nalgene, NY, USA), followed by the removal with a pipette (Nichipet EX, Nichiryo, Japan) of a 10-ml sample to be filtered for spore counting.
2. A vortex homogenization (V) for 10 s of a 10-ml sample of water to be analyzed in a 15-ml tube (05-539-5, Fisherbrand, PA, USA), followed by filtration of the contents of the tube for spore counting.
3. Mixing of a 100-ml sample in a 125-ml polypropylene bottle (Nalgene, NY, USA) for a period of 15 minutes by mechanical agitation at 300 rpm (Innova 2300, New Brunswick Scientific, NJ, USA) with 25 glass beads (Fisher Scientific, Czech Republic- 11-312A), as prescribed for soil analysis (MB) (Germida 1993). Then, 10 ml was sampled using a pipette. This volume of water was used directly for spore counting by filtration.

For soil suspensions, three replicates were used for each dispersion method and 15 replicates served to establish the concentration of ASFB spores without dispersion. For the hydrant water, each pretreatment (MI, V, MB with T0%, T0.1% and T1%) is the mean concentration calculated for triplicate samples (bottles A, B and C), for each of which 9 (bottles A and B) and 7 (bottle C) measurements were repeated for spore counting, which corresponds to a total goal of 25 spore measurements per pretreatment category. The tests were performed on the same day, in order to avoid changing the nature of the spores in the soil suspension or the flushing water.

Results were analyzed by non-parametric tests using the software Statistica V7 (version 7.0.61.0, StatSoft, USA). For the Mann-Whitney U-Test, the p-values considered were those obtained for an adjusted value of Z. P-values of $< 0.05$ were considered significant unless otherwise noted.

## RESULTS

### Influence of water quality on *B. subtilis* spore recovery

The effects of water quality on the recovery of spores in tap waters are summarized in Table 1. These results are expressed as a function of the direct count of the *B. subtilis* inoculum without the addition of sterile tap water ($N_0$). Mann-Whitney tests were carried out based on the replicates used for enumerating the *B. subtilis* spores. The two tap water samples (Samples 1 & 2) were collected from the same tap on different days.

Significantly different results from those for the controls were observed for all the tests for which volumes of 5 L were filtered. For volumes of less than 1 L, five tests out of six were also significantly different from those for the controls ($p < 0.05$). Independent of the type of treatment (A, B or C) and the volume filtered (1 L or 5 L), the extent of spore recovery is less in distribution system water than it is in the sterile physiological solution used for the recovery of the controls.

Spore recovery was significantly less for 5 L than for 1 L for the first of the duplicate samples taken from the distribution system water. For the second sample, spore recovery was not influenced by the increase in the filtered volume from 1 L to 5 L. For the first sample, spore recovery on the cake and under the cake with a volume of 5 L was significantly less than when the spores were inside the cake, which was not the case for the second sample. The impact of the volume of water filtered identified for Sample 1 was greater when the spores were located either under the cake or on the surface of the cake.

The SS concentrations (13.8 and 5.0 $\mu g/cm^2$ for 1 L) differ significantly between the two samples. The effect of the filtered volume on the recovery of ASFB spores is dependent on the quantity of solids present in suspension. This effect appears above a certain threshold of particles having accumulated on the filter. Table 1 shows the composition of the filter cakes. Note that the concentrations of soluble metals in the distribution system water studied met the standards of quality for drinking water at all

**Table 1** | Percentage recovery of *B. subtilis* spores; particle composition and accumulation

| | | | Mean | Mann-Whitney U-test | Solids accumulated on the filter | Metal accumulation in the filter cake ($\times 10^{-3}\ \mu g/cm^2$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample | Case | Volume (L) | $N/N_0$ | p value <0,05 | SS ($\mu g/cm^2$) | Cu | Zn | Fe | Mn | Pb |
| 1 | A | 1 | 78% | S | 13.8 | 11 | 41 | 69 | 9 | <6 |
| | A | 5 | 40% | S | 69.2 | 233 | 79 | 817 | 24 | <6 |
| | B | 1 | 63% | S | – | 16 | 43 | 79 | 8 | <6 |
| | B | 5 | 11% | S | – | 349 | 52 | 817 | 18 | <6 |
| | C | 1 | 55% | S | – | NM | NM | NM | NM | NM |
| | C | 5 | 18% | S | – | NM | NM | NM | NM | NM |
| 2 | A | 1 | 61% | S | 5.0 | 35 | 38 | 128 | 8 | <6 |
| | A | 5 | 70% | S | 25.2 | 192 | 81 | 692 | 19 | <6 |
| | B | 1 | 66% | S | – | 38 | 46 | 242 | 12 | <6 |
| | B | 5 | 67% | S | – | 399 | 53 | 629 | 14 | <6 |
| | C | 1 | 71% | NS | – | ND | ND | ND | ND | ND |
| | C | 5 | 73% | S | – | ND | ND | ND | ND | ND |

Mann-Whitney U-Test, null hypothesis: [spores] Petri dish = [spores] injected. S: significant; NS: Non significant;-Not required; NM not measured * Turbidity of sample is 0.36 NTU.

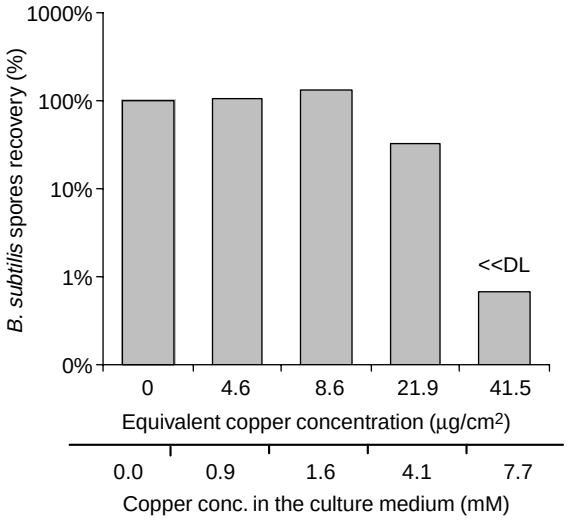


**Figure 2** | Recovery of *B. subtilis* spores as a function of the concentration of copper in the nutrient medium. n = 4 for each concentration of copper DL = under the detection limit.

times. In contrast, the accumulation of metals in particulate form by volume of filtered water volume is quite variable.

Preliminary tests had suggested that the concentration of copper on the filter (indicated by a green color) could cause significant reductions in ASFB spore recovery. In order to investigate the impact of copper on the growth of *B. subtilis* spores, increasing concentrations of copper sulfate were added to the culture medium. The results in Figure 2 show, that, from a copper concentration of 4.1 mM, the recovery of *B. subtilis* spores decreases significantly. For purposes of future comparison, the concentration of copper in the culture medium is also expressed in µg Cu/cm$^2$.

## Influence of the mode of homogenization and the surfactant concentration on the recovery of ASFB spores

As illustrated in Figure 3, the rate of recovery is reduced following the addition of Tween 80®, when mixing with beads. The results for the mixing-with-beads category without surfactant are significantly higher (Mann-Whitney U-Test $P < 0.05$) than those with Tween 80® (0.1% and 1%). There is no other significant difference between the other categories. The variability of the results can be seen in Figure 3, suggesting that the heterogeneous distribution of spores in the samples is revealed by dispersion. This is supported by the homogeneous results from the 15 replicates not subjected to dispersion or homogenization.

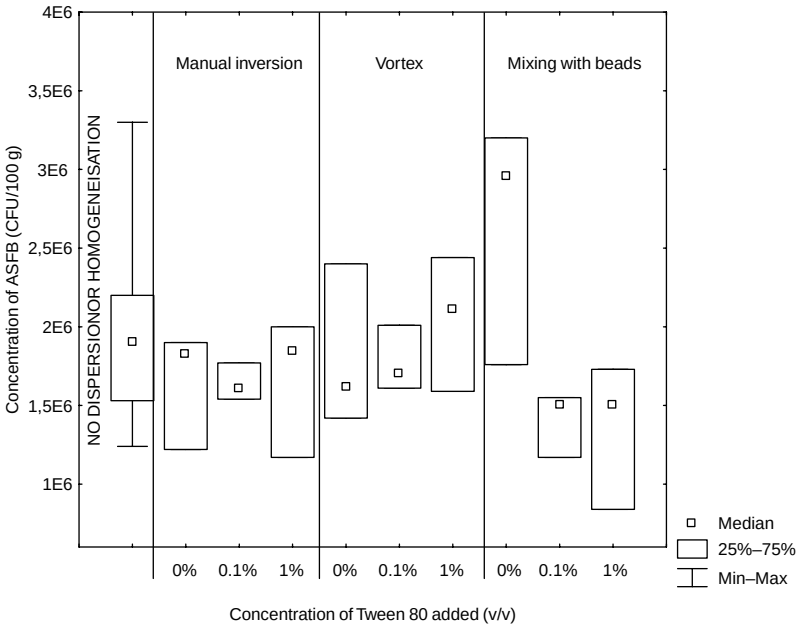


**Figure 3** | Number of ASFB spores in a soil suspension according to the type of homogenization and the concentration of Tween 80® added. Homogenization: MI = manual inversion, V = vortex, MB = mixing with beads Tween 80® concentration (v/v): 0 (0%); 0.1% (T 0.1%); 1% (T 1%) (n = 3 for each category, except n = 15 for the category without surfactant and without mode of homogenization).

Figure 4 illustrates the results obtained for the same type of test, carried out on a sample of hydrant flushing (turbidity of 46 NTU, SS of 37 mg/L and VSS of 13 mg/L).

With the exception of the vortex mixing, the results obtained for the three concentrations of surfactant (Tween 80®: 0%; 0.1%; 1%) are significantly different (Kruskall-Wallis; $p < 0.001$). The addition of surfactant increases the rate of recovery of ASFB spores in this type of water for homogenization by manual inversion and mixing with beads. The results obtained for all three methods of homogenization are also significantly different for concentrations of surfactant of 0.1% and 1% (Kruskall-Wallis $p < 0.0001$). These results show that the homogenization methodology applied significantly influences the results. The mean concentration of ASFB spores recovered was maximized for the MI-T1% category (Mann-Whitney U-Test). For the flushing water, the addition of Tween 80® at the time of application of the mixing by manual inversion method has a significant impact, even at a low concentration. When using a combination of mixing with beads and adding a surfactant, a concentration of Tween 80® of 1% is needed to obtain a significantly higher count. Finally, the addition of Tween 80® at the time of application of the vortex method has no impact.

The results presented in Figure 4 are the mean concentration of aggregated results. Figure 5 shows detailed

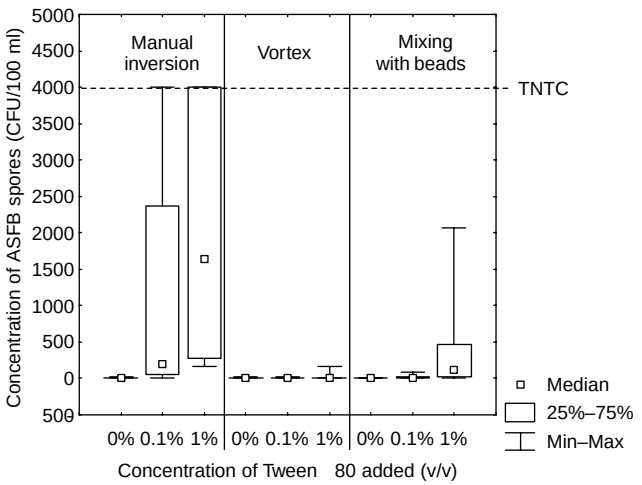


**Figure 4** | Number of ASFB spores in the flushing water according to the type of homogenization and the concentration of Tween® 80 added. Homogenization: MI = manual inversion, V = vortex, MB = mixing with beads Tween® 80 concentration (v/v): 0% (T 0%); 0.1% (T 0.1%); 1% (T 1%) (n = 25 for each category) – TNTC: too numerous to count.

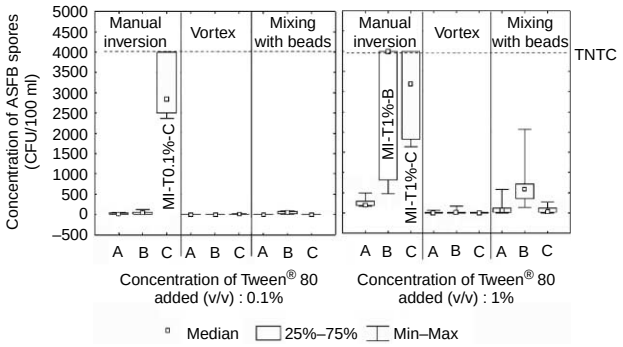


**Figure 5** | Number of ASFB spores in the flushing water according to the type of mixture and a concentration of Tween® 80 (v/v) of 0.1% and 1%. Homogenization: MI = manual inversion, V = vortex, MB = mixing with beads Tween® 80 concentration (v/v): 0.1% (T 0.1%); 1% (T 0.1%) (n = 9 for bottles A and B and n = 7 for bottles C).

results for each bottle with concentrations of Tween 80® of 0.1% and 1%. Results show that, at the time the surfactant was added, the distribution of the spores in the three bottles undergoing the same pretreatment was not uniform.

A single sample (MI-T0.1%-C) stands out as significantly different from the other 0.1% concentrations samples (Mann-Whitney U-Test). This sample is responsible for the higher concentration of ASFB spores observed when the manual inversion mixing method is used with the addition of Tween 80® (0.1%). For the 1% concentration of Tween 80®, two samples (MI-T1%-B and MI-T1%-C) are noticeably different from the others (Mann-Whitney U-Test). This suggests a higher concentration of ASFB spores for these three samples (MI-T0.1%-C, MI-T1%-B and MI-T1%-C) than for the other six samples treated by manual inversion with surfactant. In fact, there is no significant difference between the three samples (Kruskall-Wallis $p > 0.3$). These results are a good illustration of the wide variability in the concentration of ASFB spores in the samples.

## DISCUSSION

### Presence of a diffusion barrier and interference caused by metals

When copper is incorporated into the media, the growth of *Bacillus subtilis* spores is inhibited (70% of the reduction with a concentration of copper sulfate is 4.1 mM or 21.9 $\mu g/cm^2$). Similar inhibitions were observed for *Bacillus thuringiensis* by Hassen *et al.* (1998) with a

concentration of 200 mM and for sulfate-reducing bacteria with a concentration of 0.11 mM (Utgikar *et al.* 2003).

The concentration of copper that had accumulated on filters from tap water (0.35 $\mu g/cm^2$) is significantly lower than that reported. It is also highly probable that particulate copper is less bioavailable than the copper sulfate incorporated into the media. This suggests that the presence of copper on the filters is not the cause of the observed reduction in the recovery of *B. subtilis* spores. Therefore, it appears to be more important to take into account the amount of accumulated solids, rather than the nature of the suspended solids, in order to limit the influence of the cake on recovery.

The impact of the cake on recovery has been shown. When using a 5-L volume of filtered water (Sample 1), the recoveries of *B. subtilis* spores are significantly lower when these spores are located under the filter cake (18%) or on the surface of the cake (11%) than when they are distributed within the cake (40%). The negative impact of the cake may be attributed to two phenomena: (i) greater difficulty in reaching the nutrient medium (Geldreich *et al.* 1978; LeChevallier *et al.* 1981); or (ii) reduced oxygen content under the cake, or a combination of both. When spores are distributed within the cake, those phenomena are still present, but they are less pronounced.

The experimental results suggest limiting the volume of water filtered as a function of the quality of the water, as is the case for coliform detection (Geldreich *et al.* 1978; Herson & Victoreen 1980; LeChevallier *et al.* 1981). The extent of the impact of the cake can be approximately estimated by a factor relating the filtered volume to the turbidity. Our results suggest that the recovery of *B subtilis* spores by membrane filtration is less affected by the influence of the cake than the recovery of coliforms (Geldreich *et al.* 1978; Herson & Victoreen 1980; LeChevallier *et al.* 1981). Taking into account the low concentrations of ASFB spores typically found in treated or distributed water ($<10$ spores/L), the use of larger volumes than those suggested in the literature is desirable. Increasing the volume of filtered water may cause a reduction in the recovery of ASFB spores if the turbidity of the water is too high. In the case of coliform measurement by membrane filtration, it is suggested that the sample be split when turbidity is high; but specific values are not proposed (Standard Methods for the Examination of Water & Wastewater 1998). It is also desirable to avoid increasing the number of filtrations and unduly burdening the analyst. Using the acceptable value of recovery of 75% proposed by Hijnen *et al.* (2000), it is proposed, based on our results, that the volume filtered (V) be limited as a function of turbidity (NTU), according to the following relation: Volume (ml) X Turbidity (NTU) = 500. This limit corresponds to 5 NTU for a 100-ml sample, a value which has already been validated for coliforms (LeChevallier *et al.* 1981). It should be noted that the above criterion constitutes a limit that is specific to the waters studied.

## Aggregation or colonization phenomenon requiring dispersion

Results in soil suspensions show a great deal of heterogeneity in the distribution of ASFB spores (Figure 3). For this reason, a large number of replicates ($>3$) is desirable in order to determine the concentration of ASFB spores in a soil. The use of Tween 80® does not improve the recovery of ASFB, which is in agreement with the results of Germida (1993), which suggest that ASFB spores were not significantly aggregated in the soil suspension studied.

The impacts of homogenization and surfactant on the recovery of ASFB spores in the hydrant flushing water samples differ from those observed in soil suspensions. The addition of surfactant reveals wide variability in spore distribution in the water from hydrants, greater than that observed by Gale *et al.* (1997) in coagulated waters. The results observed are in agreement with those of Morin *et al.* (1997), who suggest that aggregation phenomena increase in the distribution system. The experimental results show that a triple manual inversion will ensure a significantly higher recovery. For the hydrant flushing water used, the addition of a surfactant maximizes recovery. But the absence of a significant difference between samples MI-T0.1%-C, MI-T1%-B and MI-T1%-C (Mann-Whitney U-Test $p > 0.5$) suggests that a Tween 80® concentration of 0.1%, as used by Gale *et al.* (1997), is sufficient. Increasing the concentration of surfactant beyond 0.1% does not increase the recovery of *Aspergillus spp.* either (Gomez-Lopez *et al.* 2005). Moreover, a concentration of Tween 80® of more than 0.1% is more likely to cause foaming. Finally, the high variability in the dispersed samples, especially the distribution of ASFB spores in triplicate bottles, strongly suggests the presence of one or more aggregates or particles

colonized by ASFB spores. This substantial heterogeneity is similar to that observed for *Cryptosporidium* (LeChevallier *et al.* 2003).

## CONCLUSION

A number of parameters influence the recovery of ASFB spores in soil suspensions and water from flushed hydrants. Particle load has a significant effect, and one which can result in a major reduction in spore recovery. Copper can also inhibit the growth of bacteria, if it is available. The impact of the presence of a cake is difficult to predict based on its composition, and so, for tap water, it is suggested that the volume filtered be limited as a function of the turbidity of the water, according to the relation: Volume (ml) X Turbidity (NTU) = 500.

The presence of aggregates or colonized particles can also lead to an underestimation of the number of ASFB spores in a sample of flushing water. The beneficial effects of a dispersion method which combines homogenization and the addition of a surfactant are apparently specific to the type of water involved. The influential factors studied are dependent on the use of a culture method for counting ASFB spores. In spite of these limitations, it should be possible to compare counts from different samples when pretreatment procedures are kept constant. Improvements to the detection method could, however, be considered. Lower temperatures, as are used for environmental HPCs, could also be used. Finally, the development of a counting method using solid phase cytometry (Chemscan®) or microflow imaging (MFI) particle analysis (Brightwell®) could be of interest, and permit the direct observation of aggregates.

## ACKNOWLEDGEMENTS

This research was supported by the Canadian Water Network. The author would like to thank the staff of the NSERC Industrial Chair on Drinking Water, especially Mélanie Rivard and Yves Fontaine, for their valuable assistance. The authors would like to acknowledge the assistance of Manon Leduc and Christian Dallaire of École Polytechnique for the metal analysis and Jean-François Therrien of the City of Laval for the field work. Finally, assistance from Dr. Bernard Clement for statistical analyses was greatly appreciated.

## REFERENCES

Barbeau, B., Boulos, L., Desjardins, R., Coallier, J., Prévost, M. & Duchesne, D. 1997 A modified method for the enumeration of aerobic spore-forming bacteria. *Canadian Journal of Microbiology* **43**, 976–980.

Barbeau, B., Boulos, L., Desjardins, R., Coallier, C. & Prévost, M. 1999 Examining the use of aerobic spore-forming bacteria to assess the efficiency of chlorination. *Wat. Res.* **33**, 2941–2948.

Barbeau, B., Desjardins, R., Mysore, C. & Prévost, M. 2005 Impacts of water quality on chlorine and chlorine dioxide efficacy in natural waters. *Wat. Res.* **39**, 2024–2033.

Besner, M.-C., Cartier, C., Morissette, C., Aubin, A., Payment, P. & Prévost, M. 2005 Water quality variations during repairs of pipe leaks. In: *Proceedings of the AWWA Water Quality Technology Conference*, Quebec City, Quebec, Canada, November 2005. AWWA, Denver, Co.

Borst, M. & Selvakumar, A. 2003 Particle-associated microorganisms in stormwater runoff. *Wat. Res.* **37**, 215–223.

Brar, S. K., Verma, M., Barnabe, S., Tyagi, R. D., Valero, J. R. & Surampalli, R. 2005 Impact of Tween 80 during Bacillus thuringiensis fermentation of wastewater sludges. *Process Biochemistry* **40**, 2695–2705.

Brown, R. A. & Cornwell, D. A. 2005 Utilizing spore removal to monitor plant performance for Cryptosporidium removal. In: *Proceedings of the AWWA Annual Conference*, San Francisco, California, June 2005. AWWA, Denver, Co.

Camper, A. K., LeChevallier, M. W., BroadAway, S. C. & McFeters, G. A. 1985 Evaluation of procedures to desorb bacteria from granular activated carbon. *J. Microbiol. Met.* **3**, 187–198.

Cartier, C., Prévost, M., Barbeau, B., Besner, M. C. & Morissette, C. (Submitted) Evaluation of ASFB spores as indicators of intrusion in full-scale distribution systems. *J. Am. Wat. Wks. Assoc*.

Critchley, M. M., Cromar, N. J., McClure, N. & Fallowfield, H. J. 2002 Biofilms in copper plumbing systems: sensitivity to copper and chlorine and implications for corrosion. *Wat. Sci. Technol.: Water Supply* **2**, 81–87.

Demain, A. L. & Solomon, N. A. 1985 *Biology of Industrial Microorganisms*. Benjamin-Cummings Publishing Company, Stoneham, MA, USA.

Francis, C. A., Lockley, A. C., Sartory, D. P. & Watkins, J. 2001 A simple modified membrane filtration medium for the enumeration of aerobic spore-bearing bacilli in water. *Wat. Res.* **35**, 3758–3761.

Frobisher, M. 1968 *Fundamentals of Microbiology. An introduction to the Microorganisms with Special Reference to the*

*Procaryons*, 8th edition. W.B. Saunders Company, Philadelphia, USA.

Gale, P., van Dijk, P. A. H. & Stanfield, G. 1997 Drinking water treatment increases micro-organism clustering; the implications for microbiological risk assessment. *Journal of Water Supply: Research and Technology-AQUA* **46**, 117–126.

Galofré, B., Israel, S., Dellunde, J. & Ribas, F. 2004 Aerobic bacterial spores as process indicators for protozoa cysts in water treatment plants. *Water Science and Technology* **50**, 165–172.

Geldreich, E. E., Allen, M. J. & Taylor, R. H. 1978 Interferences to coliform detection in potable water supplies. In: in Hendricks, C. W. (ed.) *Evaluation of the Microbiology Standards for Drinking Water*. United States Environmental Protection Agency, Washington, DC, USA, pp. 13–20.

Germida, J. J. 1993 Cultural methods for soil microorganisms. In: in Carter, M. R. (ed.) *Soil Sampling and Methods of Analysis*, pp. 263–272, Canadian Society of Soil Science, Ann Arbor Tokyo Lewis Publishers, Boca Raton.

Gibbs, S. G., Meckes, M. C., Green, C. F. & Scarpino, P. V. 2004 Evaluation of the ability of chlorine to inactivate selected organisms from the biofilm of a drinking water distribution system simulator following a long-term wastewater cross-connection. *Journal of Environmental Engineering and Science* **3**, 97–105.

Gibbs, S. G., Meckes, M. C. & Scarpino, P. V. 2003 The effect of long-term wastewater cross-connection on the biofilm of a simulated water distribution system. *Journal of Environmental Engineering and Science* **2**, 85–98.

Gomez-Lopez, A., Aberkane, A., Petrikkou, E., Mellado, E., Rodriguez-Tudela, J. L. & Cuenca-Estrella, M. 2005 Analysis of the influence of Tween concentration, inoculum size, assay medium, and reading time on susceptibility testing of Aspergillus spp. *Journal of Clinical Microbiology* **43**, 1251–1255.

Hassen, A., Saidi, N., Cherif, M. & Boudabous, A. 1998 Resistance of environmental bacteria to heavy metals. *Bioresource Technology* **64**, 7–15.

Herson, D. S., Marshall, D. R., Baker, K. H. & Victoreen, H. T. 1991 Association of microorganisms with surfaces in distribution systems. *J. Am. Wat. Wks. Assoc.* **83**, 103–106.

Herson, D. S. & Victoreen, H. T. 1980 United States Environmental Protection Agency. Municipal Environmental Research Laboratory. Office of Research and Development, Cincinatti, OH.

Hijnen, W. A. M., Van Veenendaal, D. A., Van Der Speld, W. M. H., Visser, A., Hoogenboezem, W. & Van Der Kooij, D. 2000 Enumeration of faecal indicator bacteria in large water volumes using on site membrane filtration to assess water treatment efficiency. *Wat. Res.* **34**, 1659–1665.

Huertas, A., Barbeau, B., Desjardins, C., Galarza, A., Figueroa, M. A. & Toranzos, G. A. 2003 Evaluation of Bacillus subtilis and coliphage MS2 as indicators of advanced water treatment efficiency. *Wat. Sci. Technol.* **47**, 55–259.

Karim, M. R., Abbaszadegan, M. & LeChevallier, M. 2003 Potential for pathogen intrusion during pressure transients. *J. Am. Wat. Wks. Assoc.* **95**, 134–146.

LeChevallier, M. W., Di Giovanni, G. D., Clancy, J. L., Bukhari, Z., Bukhari, S., Rosen, J. S., Sobrinho, J. & Frey, M. M. 2003 Comparison of method 1623 and cell culture-PCR for detection of Cryptosporidium spp. in source waters. *Appl. Environ. Microbiol.* **69**, 971–979.

LeChevallier, M. W., Evans, T. M. & Seidler, R. J. 1981 Effect of turbidity on chlorination efficiency and bacterial persistence in drinking water. *Appl. Environ. Microbiol.* **42**, 159–167.

Madge, B. A. 2002 *Evaluation of Wastewater Solids and their Relevance in Ultraviolet Disinfection*. Thesis, University of New York at Buffalo.

Mazoua, S. & Chauveheid, E. 2005 Aerobic spore-forming bacteria for assessing quality of drinking water produced from surface water. *Wat. Res.* **39**, 5186–5198.

Morin, P., Gauthier, V., Saby, S. & Block, J.-C. 1997 Bacterial resistance to chlorine through attachment to particles and pipes surfaces in drinking water distribution systems. *Proceedings of the Royal Society of Chemistry*, Warwick, United Kingdom, April 1997.

Nieminski, E. C. & Bellamy, W. D. 2000 *Application of Surrogate Measures to Improve Treatment Plant Performance*, *American Water Works Association Research Foundation and American Water Works Association No. 90811*. AWWA, Denver CO.

Nieminski, E. C., Bellamy, W. D. & Moss, L. R. 2000 Using surrogates to improve plant performance. *J. Am. Wat. Wks. Assoc.* **92**, 67–78.

Oliver, E. D. & Harbour, J. D. 1995 Speciation of coliforms isolated from sediments in brown water released during a flushing program undertaken by the Seattle water department. *Proceedings of the AWWA Water Quality Technology Conference*. AWWA, Denver, CO, New Orleans, Louisiana, November 1995.

Ormeci, B. & Linden, K. G. 2005 Comparison of physical and chemical methods for extraction of coliform from wastewater particles and flocs. *Environmental Engineering and Science* **22**, 459–471.

Rice, E. W., Covert, T. C., Wild, D. K., Berman, D., Johnson, S. A. & Johnson, C. H. 1993 Comparative resistance of escherichia coli and enterococci to chlorination. *Journal of Environmental Science and Health, Part A* **28**, 89–97.

Rice, E. W., Fox, K. R., Miltner, R. J., Lytle, D. A. & Johnson, C. H. 1996 Evaluating plant performance with endospores. *J. Am. Wat. Wks. Assoc.* **88**, 122–130.

Ridgway, H. F. & Olson, B. H. 1982 Chlorine resistance patterns of bacteria from two drinking water distribution systems. *Appl. Environ. Microbiol.* **44**, 972–987.

Rogers, J., Dowsett, A. B., Dennis, P. J., Lee, J. V. & Keevil, C. W. 1994 Influence of temperature and plumbing materials selection on biofilm formation and growth of Legionella pneumophila in model potable water systems contaminating complex microbial flora. *Appl. Environ. Microbiol.* **60**, 1585–1592.

Rompré, A., Prévost, M., Coallier, J. & Servais, P. 1998 Impact of reducing biodegradable organic matter on fixed and suspended biomass in distribution systems (Chapter 4). *Microbial Impact of Biological Filtration*. American Water Works Association

Research Foundation and American Water Works Association, Denver, Colorado, USA, pp. 57–120.

Standard Methods for the Examination of Water and Wastewater 1998 20th edition. American Public Health Association/ American Water Works Association/Water Environment Federation, Washington, DC.

United States Environmental Protection Agency (USEPA) 2005 *National Primary Drinking Water Regulation: Long Term 2 Enhanced Surface Water Treatment Rule (Final Rule)*, Washington, DC, USA; 40 CFR Parts 9, 141, and 142 (EPA-HQ-OW-2002-0039; FRL-RIN 2040-AD37).

Utgikar, V. P., Tabak, H. H., Haines, J. R. & Govind, R. 2003 Quantification of toxic and inhibitory impact of copper and zinc on mixed cultures of sulfate-reducing bacteria. *Biotechnology and Bioengineering* **82**, 306–312.

Versteegh, J. F., Havelaar, A. H., Hoekstra, A. C. & Visser, A. 1989 Complexing of copper in drinking water samples to enhance recovery of Aeromonas and other bacteria. *Journal of Applied Bacteriology* **67**, 561–566.

Williams, M. M. & Donlan, R. M. 2005 Mycobacterium fortuitum biofilm formation on water distribution pipe materials. *Proceedings of the AWWA Water Quality Technology Conference*. AWWA, Denver, CO, Quebec City, Quebec, Canada, November 2005.

Zacheus, O. M., Lehtola, M. J., Korhonen, L. K. & Martikainen, P. J. 2001 Soft deposits, the key site for microbial growth in drinking water distribution networks. *Wat. Res.* **35**, 1757–1765.

First received 5 May 2006; accepted in revised form 21 December 2006