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Use of bacterial spores in monitoring water quality and treatment

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Abstract

Because *Clostridium perfringens* spores are both specific to sewage contamination and environmentally stable, they are considered as possible conservative indicators of human fecal contamination and possible surrogates for environmentally stable pathogens. This review discusses the reasons and summarizes methods for monitoring spores in water. Cultural methods are still preferred over qPCR for routine water quality monitoring because of their low costs. Membrane filter (MF) methods are preferred over the more laborious and less accurate most probable number methods. The most commonly used MF media are TSC medium and mCP medium. TSC normally allows higher recoveries than mCP. TSC produces fewer false-positive results than mCP; however, it does produce more false-negatives. Two newer methods have substantial potential, CP Chromo Select agar, which allows better recoveries and greater specificity than mCP, and the Fung double tube method, which creates anaerobic conditions and allows enumeration of colonies in tubes in 5–6 hours. Aerobic spores are not associated with fecal contamination but they can be surrogates for environmentally stable pathogens in monitoring water for treatment efficacy; *Bacillus cereus* spores are normally measured on nutrient agar by the MF method.

Keywords

Bacillus; *C. perfringens*; indicators; monitoring; spores; water quality

INTRODUCTION

Since pathogens of fecal origin are numerous and the occurrence of any particular pathogen is random over time and space, water quality is normally assessed by way of indicators of fecal contamination. Basic historical characteristics of the ideal water quality indicator described by Griffin *et al.* (2001) and Yates (2007) are: (i) occurs where pathogens do, (ii) cannot grow in the environment, (iii) more resistant to disinfectants than are pathogens, (iv) easy to isolate and count, (v) only found in sewage, (vi) found in higher numbers than pathogens, (vii) correlated with health risks and (viii) densities of indicators should relate to degree of contamination. *Clostridium perfringens* spores have been proposed as indicators of fecal pollution because they are entirely of fecal origin, widely distributed in sewage and consistently associated with human feces (Bonde 1963; Bisson & Cabelli 1980). Although rapid molecular methods are now available, cultural methods for measuring indicator organisms are still preferred in routine monitoring because of their relatively low costs. This

review focuses primarily on comparing the various methods used to detect and enumerate bacterial spores to help laboratory personnel choose which method is most appropriate for their studies.

C. PERFRINGENS SPORES AS CONSERVATIVE INDICATORS OF FECAL CONTAMINATION

C. perfringens has rarely been used as a water quality indicator in the USA. Cabelli (1977, 1982) believed that the lack of interest in *C. perfringens* in the USA was mainly due to its perceived ubiquity and extraordinary persistence in nature. However, he also believed that the refractory nature of the spores and the lack of evidence that *C. perfringens* can multiply in water may provide some specific applications for use of the *C. perfringens* indicator system.

The exceptional longevity of spores implies that *C. perfringens* in water environments in the absence of other indicators, such as *Escherichia coli*, is a useful indication of remote fecal contamination, a potential conservative tracer in field studies and a conservative tracer in pathogen die-off studies (Cabelli 1977). Lisle *et al.* (2004) also observed that *C. perfringens* spores survived longer than other fecal indicator bacteria (FIB) in marine systems and were higher in number than other FIB in sediments and at greater distances from the discharge sites. Ferguson *et al.* (1996), in their study of relationships between indicators and pathogens in an estuarine system, reported that *C. perfringens* spores were the best indicators of fecal pollution and were the only indicators significantly correlated to any of the pathogen groups in the water column. Payment & Franco (1993) suggested using *C. perfringens* as an alternate indicator for the evaluation of wastewater, drinking water and environmental waters. The absence of *C. perfringens* in some reasonable quantity of drinking water is considered to indicate the absence of recent or remote fecal contamination (Cabelli 1977) and the absence of enteric viruses and bacterial pathogens in drinking water (Bisson & Cabelli 1980).

The persistence of *C. perfringens* spores in the environment and their absence in streams with no human sewage led Fujioka & Shizumura (1985) to suggest that *C. perfringens* is a better indicator to use in tropical waters. They reported that the concentrations of *C. perfringens* were consistently low in Hawaiian streams not receiving wastewater, whereas the concentrations of fecal coliforms and fecal streptococci were consistently high, which they attributed to non-point pollution due to animals and growth of these indicators in Hawaiian soils. Conversely, stream samples below effluent discharge sites consistently contained 56–2,100 *C. perfringens* per 100 mL. Sorensen *et al.* (1989) also reported higher concentrations of *C. perfringens* spores in streams which had input of sewage effluents upstream. Fujioka (2001) also suggested *C. perfringens* for use as an indicator for use in recreational waters in tropical environments; although *C. perfringens* has not been associated with illness in epidemiological studies (Wade *et al.* 2003, 2010).

Sorensen *et al.* (1989) reported that concentrations of *C. perfringens* were relatively high through the wastewater treatment unit processes at the San Jose/Santa Clara plant but were reduced after filtration. Fujioka & Shizumura (1985) observed that chlorination reduced the

concentrations of fecal coliforms (FC) and fecal streptococci (FS) by 1–4 logs. Reductions were from 1.1–1.5 × 10^7 CFU/ 100 mL in raw sewage to 1.8–2.8 × 10^3 CFU/100 mL in receiving waters for FC and from 1.6–2.4 × 10^6 CFU/ 100 mL in raw sewage to 1.9–5.3 × 10^3 CFU/100 mL in receiving waters for FS. However, *C. perfringens* CFU were only reduced by 0.2 log, from 2.8–5.3 × 10^4 CFU/100 mL to 3.4–9.4 × 10^4 mL. Added support for *C. perfringens* as a more stable indicator was provided by Harwood *et al.* (2005), who observed that 100% of influent samples contained FC and FS while only 93% of these samples were positive for *C. perfringens.* In contrast, only 27% of disinfected effluent contained FC and FS while 61% contained *C. perfringens*.

In spite of the evidence that *C. perfringens* is a conservative indicator, it has not always been found in waters in which other indicators are present. Till *et al.* (2008) performed a freshwater microbiology survey in which *E. coli* were detected in 99% of all samples and coliphage were detected in 89% of the samples but *C. perfringens* spores and F-RNA phages were present in only 58 and 52% of the samples respectively. In addition, there was a lack of correlation between human viruses and *C. perfringens* spores.

C. perfringens spores have been proposed as indicators of *Cryptosporidium parvum* oocyst presence in river water due to their slower die-off rates versus those of *E. coli* and enterococci (Medema *et al.* 1997) and as surrogates for *Cryptosporidium* oocysts in water treatment studies (Venczel *et al.* 1997). Within the limits of precision and accuracy of the infectivity methods for *Cryptosporidium* oocysts, a mixed oxidant solution generated by electrolysis of a solution of sodium chloride produced similar inactivation of *C. parvum* oocysts and *Clostridium* endospores. However, *C. perfringens* spores were somewhat more susceptible than *Cryptosporidium* oocysts to inactivation by free chlorine (Venczel *et al.* 1997). Hijnen and colleagues also reported that *C. perfringens* spores were a potential surrogate for efficiency of removal of protozoan (oo)cyst in treatment processes. They noted that it was necessary to enlarge the sampled water volumes to determine the concentration of spores after every treatment stage, which they could do by using more than one membrane for volumes larger than 100 mL (Hijnen *et al.* 2000). They also observed that inactivation kinetics of *C. perfringens* and *C. parvum* were in the same order of magnitude (Hijnen *et al.* 2002).

METHODS USED FOR DETECTING *C. PERFRINGENS*

Although the most probable number (MPN) procedure has been used for many years in liquid media, such as iron-milk medium, the MPN method is laborious and less accurate than are methods utilizing nitrocellulose membrane filter (MF) and solid media. Hence, solid media have usually been preferred. In general solid media represent progressive attempts to increase both selectivity and productivity (Mead 1985). The sulfite-polymyxin-sulfadiazine (SPS) medium of Angelotti *et al.* (1962) was said to provide quantitative recovery of *C. perfringens* from foods and to provide reduced interference from facultative anaerobes. As with most *C. perfringens* media, the differentiation of *C. perfringens* on SPS is based on the well-known blackening reaction which arises from the ability of these organisms to reduce sulfite to sulfide in the presence of an appropriate iron salt, causing the precipitation of black ferrous sulfide around individual colonies (Mead 1985). Unfortunately, SPS also permitted

growth and black colony formation by a variety of other *Clostridium* species and provided poor growth by some strains of *C. perfringens* (Mead 1985).

Trypticase-sulfite-neomycin (TSN) agar was developed as a modification of SPS agar. Neomycin sulfate was substituted for sulfadiazine and the concentration of polymyxin was increased to enhance selectivity at a growth temperature of 46 °C without impairing recovery of *C. perfringens* (Marshall *et al.* 1965). Although TSN agar has not been widely used, there is evidence that it is more selective than SPS. In spite of this improved selectivity, TSN still allows the growth of many facultative anaerobes (Harmon *et al.* 1971). Egg yolk (EY) was included in TSN to differentiate lecithin-positive *C. perfringens* strains from other sulfite reducers; yet this could be seen as a disadvantage because not all *C. perfringens* strains produce the characteristic halos around the black colonies on this medium (Hauschild & Hilsheimer 1974a).

Bisson & Cabelli (1979) developed a method specifically for water. Recognizing the disadvantages of the imprecise MPN method, they developed a two-step MF medium (mCP) to allow for the specific enumeration of *C. perfringens* colonies, rather than all spore forming, sulfite-reducing anaerobes. This method could be performed without the need for sub-culturing. They reported that recoveries of *C. perfringens* by mCP were comparable to those by the Bonde pour tube method (BPT) (Bonde 1963). They also reported that the recovery frequency by mCP was 93% while that by BPT was only 79%. Fujioka & Shizumura (1985) reported that mCP was reliable in recovering *C. perfringens*. Eighty nine of the 98 colonies (91%) they recovered by mCP were positive by all eight confirmatory tests used and they were subsequently determined to be true *C. perfringens.* Only one of the 29 colonies (3%) presumptively negative for *C. perfringens* was subsequently identified as *C. perfringens*.

The introduction of Shahidi, Ferguson perfringens medium (SFP) (Shahidi & Ferguson 1971) overcame the problem of *C. perfringens* inhibition partly via substituting metabisulfite for sulfite in SPS agar that was supplemented with kanamycin, polymyxin and EY. Nevertheless, SFP failed to provide adequate suppression of facultative anaerobes, some of which produced EY reactions. Tryptose-Sulfite-Cycloserine (TSC) agar was then developed as a modification of SFP agar by incorporating 400 μg of D-cycloserine as the selective agent (Harmon *et al.* 1971). TSC agar and the EY-free modification introduced by Hauschild & Hilsheimer (1974a, 1974b) are highly selective and permit quantitative recovery of *C. perfringens* while inhibiting most of the facultative anaerobes that cause interference in other media. Both TSC agars (with and without EY) produced the non-specific counts (total counts minus confirmed *C. perfringens* counts) of the same order of magnitude. Sartory *et al.* (1998) compared recoveries of *C. perfringens* on egg-yolk-free TSC containing 400 mg/L cycloserine with those on mCP and reported that cycloserine is extremely selective against facultative anaerobes. TSC was as selective as mCP, produced higher *C. perfringens* counts and was a cheaper alternative. Confirmation of colonies as *C. perfringens* was still necessary because TSC detects all sulphite reducing clostridia (Sartory 1986). Modification of TSC medium by addition of neomycin sulfate resulted in significant reduction in recovery of *C. perfringens.* Another advantage to using TSC over mCP for detecting and isolating *C. perfringens* from water samples was that the colonies on TSC were unambiguously black,

making them easier to count and subculture for confirmation. Color differentiation of presumptive colonies on mCP after incubation and exposure to ammonia fumes was very subjective. Burger *et al.* (1984) reported that TSC and TSC minus egg yolk (TSC-EY) both recovered more *C. perfringens* than mCP; however, they also reported high false-positives on both TSC and TSC-EY agars. This was possibly due to the fact that they did not heat the samples to kill the vegetative cells.

Wohlsen *et al.* (2006) observed that recoveries of *C. perfringens* on oleandomycin polymixin sulphadiazine perfringens (OPSP) agar (Oxoid) were not statistically different from recoveries on TSC and that OPSP agar was more selective than TSC. They eliminated mCP from consideration due to the difficulty in marking which yellow colonies turned pink/ magenta after exposure to ammonium hydroxide because the color change lasted only for a short time. In addition, *C. perfringens* colonies were more discrete on OPSP than on TSC. They also had safety concerns regarding the usage and disposal of the ammonium hydroxide reagent needed for mCP. Manafi *et al.* (2013) had the same concern about the difficulty in differentiating the presumptive yellow colonies on mCP and those which had turned pink following exposure to ammonia fumes. They also noted that the presence of the ammonia prevented sub-culturing of the *C. perfringens* colonies. They found that a new medium, CP Chromo Select Agar, allowed better recoveries and had greater specificity than mCP. A summary of studies comparing recoveries of *C. perfringens* by TSC and mCP, along with other MF media comparisons, is presented in Table 1.

Emerson & Cabelli (1982) developed extraction and separation procedures for use in conjunction with the mCP membrane filter method for the enumeration of spores in estuarine sediment. This method was developed because Labelle *et al.* (1980) had observed that measurements of indicators in sediments may be a better indication of water quality over a long period of time than the number of bacteria and viruses in the overlying water.

The MF methods required several confirmation tests: Gram staining, inoculation into buffered motility nitrate medium (Harmon & Kautter 1978) and lactose gelatin medium (Hauschild & Hilsheimer 1974a, 1974b). All Gram-positive spore forming bacilli that were non-motile, reduced nitrate to nitrite, fermented lactose and liquified gelatin within 48 hours were recorded as *C. perfringens*. A rapid confirmation test for *C. perfringens* colonies using chromogenic and fluorogenic substrates was developed by Adcock & Saint (2001). This test was based on the previous observation that detection of acid phosphatase was reported to be a useful diagnostic tool for identifying *C. perfringens* (Bisson & Cabelli 1979). *C. perfringens* can metabolize 4-methylumbelliferyl phosphate (MUP) using acid phosphatase to produce 4-methylumbelliferone, which fluoresces when placed under long-wavelength (365 nm) ultraviolet light (Schallehn & Brandis 1973). Addition of MUP to TSC agar was used successfully by Araujo *et al.* (2001, 2004). *C. perfringens* also utilizes β-galactosidase to ferment lactose to lactic acid and gas; this reaction has also been successfully used for the confirmation of *C. perfringens* by detecting the hydrolysis of *ortho*-nitrophenyl-β-galactopyranoside (ONPG), which yields the yellow chromogenic product ortho-nitrophenol. Combining the detection of acid phosphatase and β-galactosidase using a MUP-ONPG assay improved confirmation to a level superior to the methods used by Harmon & Kautter (1978) or Hauschild & Hilsheimer (1974a, 1974b) and yielded results within 4

hours. MUP and ONPG substrates were incorporated in HEPES buffer in the presence or absence of 0.2% (wt/vol) sodium lauryl sulfate and 1% (wt/vol) sodium chloride. Presumptive *C. perfringens* isolates were emulsified in the MUP-ONPG mixtures and overlaid with sterile mineral oil to eliminate evaporation. The reaction tubes were placed in a heating block at 35 °C and checked hourly over a 4 hour period. A species-specific polymerase chain reaction (PCR) method based on the 16S rRNA gene identity (Mueller-Spitz *et al.* 2009) and a real-time PCR (rtPCR) method based on the *C. perfringens* alpha toxin gene (*cpa*) are also available to confirm colonies isolated from mCP. In addition, the rtPCR assay detects as few as one *C. perfringens* per 100 mL of drinking water (Maheux *et al.* 2013).

Fung *et al.* (2007) developed a rapid test for *C. perfringens* spores that does not require the use of an anaerobic chamber. This method, called the Fung double tube (FDT) method, creates anaerobic conditions and allows the enumeration of *C. perfringens* colonies in tubes within 5–6 hours. The FDT and an experimental medium called CP AnaSelect Oxyplate, developed by Oxyrase Inc. (Mansfield, OH), are the only media that create anaerobic conditions without reliance on an anaerobic chamber; all of the other methods on solid media require incubation in an anaerobic chamber (Vijayavel *et al.* 2009). Vijayavel *et al.* (2009) made three modifications to the FDT method to increase the sensitivity and selectivity of the method. The modifications were: (i) pretreatment of the water samples using a microwave to attain high temperatures (70 °C) for a short time (2.5 min.); (ii) the addition of phosphatase reaction to allow confirmation of the enumerated colonies as *C. perfringens*; and (iii) an increase in the sample volume from 5 to 10 mL. The second modification was accomplished by adding MUP to SFP medium, as Sartory *et al.* (2006) had done with TSC medium. Black colonies that fluoresced in blue were enumerated as confirmed *C. perfringens* colonies. It should be stated, however, that Sartory *et al.* (2006) reported that testing for acid phosphatase is as reliable as the conventional (ISO 2002) MNLG procedure (testing for motility, nitrate reduction, lactose fermentation and gelatin liquefaction) and that incorporation of MUP, which allows for detection of both acid and alkaline phosphatases in media with a neutral pH, into TSC does not reliably improve the identification of *C. perfringens.* Further evidence that testing for acid phosphatase is a reliable alternate method for confirming presumptive *C. perfringens* was provided by Ryzinska-Paier *et al.* (2011) who found that the percentage of correct identification for 127 environmental strains was 92% for the acid phosphatase test but only 83% for the ISO_LGMN procedure (ISO 2002), which is comparable to the confirmation results of 94% found by Eisgruber *et al.* (2000). A multilaboratory study by Watkins & Sartory (2014) showed that their new tryptose cycloserine agar (TCA) medium, which lacks sodium metabisulfate but contains sodium pyruvate to improve recovery, was equivalent to the UK reference and ISO 14189 TSCA medium. This method also incorporates a membrane filter transfer procedure onto reagent-soaked filters for the immediate testing for acid phosphatase production.

AEROBIC SPORES AS SURROGATES FOR ENVIRONMENTALLY STABLE PATHOGENS

Unlike *C. perfringens*, aerobic spores are not associated with fecal contamination. Their value has been to assess drinking water treatment plant performance and to serve as surrogates for the more environmentally stable pathogens such as viruses and protozoa. Particle counting and turbidity measurements are two of the most valuable water quality parameters used in assessing plant performance. Monitoring for spore removal coupled with monitoring for turbidity and particle counts was shown by Rice *et al.* (1996) to allow utilities to optimize unit processes and thus provide more efficient treatment. Spore removals closely paralleled particle and turbidity removal in response to coagulant dosage under all water quality conditions examined. Spores are smaller in size than cysts or oocysts and thus are a conservative indicator of removal efficiency. Aerobic spores appear to occur in most surface waters at concentrations that would permit their use as a microbial surrogate for evaluating treatment plant efficiency; their presence throughout the treatment train allows actual removal rates to be calculated (Rice *et al.* 1996).

The results of the chlorine inactivation of indigenous spores demonstrated that the indigenous aerobic spores exhibited a chlorine resistance greater than that reported for *Giardia duodenalis* (formerly *G. lamblia*) cysts but considerably less than that of *Cryptosporidium parvum* oocysts (Rice *et al.* 1996). A comparative inactivation of several microorganisms in natural water by ozone was evaluated in a pilot study performed by Owens *et al.* (2000). The results demonstrated that endospores were the only organisms more resistant to ozone than *Cryptosporidium* oocysts. Relatively low ozone CTs reduced the viable populations of indigenous endospores by approximately 0.5 logs, while similar ozone CTs had little effect on *Bacillus subtilis* endospores, suggesting that some species among the indigenous endospores were more susceptible to ozone than *B. subtilis* endospores. Once the susceptible endospores were inactivated, ozone appeared to have had similar effect on both spiked *B. subtilis* and indigenous endospores at higher CTs.

Inactivation of *B. subtilis* spores by application of UV light was studied by Chang *et al.* (1985), who found that *B. subtilis* spores were 400 times more UV resistant than enteric bacteria but less resistant than *Acanthamoeba castellanii* cysts.

METHODS USED FOR MEASUREMENT OF AEROBIC SPORES

The protocol for enumeration of aerobic spores used by Rice *et al.* (1996) was a modification of the procedure recommended for the detection of bacterial spores in milk. Samples were collected and aseptically placed in closed heat-resistant glass bottles or flasks. Each individual bottle or flask was labeled in such a manner that the identification markings were not removed during the heat treatment. The flasks were placed in a thermostatically controlled water bath equipped with a shaker and equilibrated at 82 °C. The temperature of the water bath was lowered to 80 °C when the temperature in the flasks reached 79 °C. The timing sequence was then begun and samples were kept in the bath for an additional 12 minutes. After cooling, appropriate dilutions were filtered onto 0.45-um porosity MFs. The MFs were placed on the surface of nutrient agar containing trypan blue dye. The plates were

inverted and incubated at 35 °C for 20–22 hours. All colonies were considered to be derived from bacterial spores. A quality control check was provided by examining at least two replicates from each dilution and testing them for heat-resistance and to determine if they were Gram-positive rods. These same procedures were used by Owens *et al.* (2000). This method was modified by Francis *et al.* (2001), who substituted bromothymol blue for trypan blue because trypan blue colored the membrane filters making colony delineation difficult. The incorporation of bromothymol blue at a concentration of 0.005% (w/v) into nutrient agar facilitated colony counting without inhibiting *B. cereus* colony formation. The procedure used by Facile *et al.* (2000) was based on membrane filtration of diluted samples. The 0.45 μm nominal pore size filters were placed on Petrie dish pads saturated with trypticase soy broth (TSB), which were placed in a water bath at 75 °C for 15 minutes. The pads were then incubated at 35 °C for 24 hours. Chang *et al.* (1985), in their study of UV light inactivation, enumerated *B. subtilis* spores by the pour plate technique with nutrient agar. These methods are summarized in Table 2.

SUMMARY

The association of *C. perfringens* with human feces, its wide distribution in sewage and the stability of *C. perfringens* spores in the environment, have led to the consideration of *C. perfringens* spores as conservative indicators of human fecal contamination in the environment. *C. perfringens* spores have also been considered as possible surrogates for environmentally stable pathogens such as viruses and protozoan cysts or oocysts. Cultural methods are still widely used because of their low costs relative to molecular methods. MF methods are preferred over the more laborious and less accurate MPN methods, although neither of these methods produce rapid results. The two most commonly used MF media are TSC medium and mCP medium. When these two media have been compared, TSC has normally produced higher recoveries than mCP. TSC produced fewer false-positive results than mCP did but it produced more false-negatives. CP Chromo Select Agar also allowed better recoveries and had greater specificity than mCP (Manafi *et al.* 2013). Although it has not been tested extensively, the FDT method, as modified by Vijayavel *et al.* (2009), has potential as a replacement for MF methods. The FDT creates anaerobic conditions and it allows enumeration of *C. perfringens* colonies within 5–6 hours rather than 18 hours.

Aerobic spores are not associated with feces and, therefore, are not considered as fecal indicators. Nevertheless, they are still useful as surrogates for environmentally stable pathogens. Indigenous aerobic spores exhibited a chlorine resistance greater than that reported for *Giardia lamblia* cysts but considerably less than that of *C. parvum* oocysts. However, they have been used successfully in monitoring removal of protozoan pathogens by filtration during drinking treatment (Rice *et al.* 1996). The only organisms more resistant to ozonation than *C. parvum* oocysts were *B. subtilis* endospores. Some species among the indigenous endospores were more susceptible to ozone than *B. subtilis* endospores. Once the susceptible endospores were inactivated, ozone appeared to have had a similar effect on both spiked *B. subtilis* and indigenous endospores at higher CTs. Aerobic spores are easily measured by treating the samples at 79 °C for 12 minutes, filtering the appropriate dilutions, placing the MFs on nutrient agar containing trypan blue dye, and counting the colonies after incubating the plates overnight (Rice *et al.* 1996).

CONCLUSIONS

C. perfringens spores are useful as conservative indicators of sewage contamination and as surrogates for environmentally stable fecal pathogens. TSC medium provides better recovery of *C. perfringens* spores than mCP and produces fewer false-positive results. The newer CP Chromo Select Agar also allows better recovery and has greater specificity than mCP. The FDT method is very rapid and specific and should be tested more extensively. It creates anaerobic conditions and allows enumeration of *C. perfringens* colonies within 5–6 hours. Aerobic spores are not specific to fecal contamination but they are good surrogates to use in monitoring the efficacy of various drinking water treatments. Aerobic spores are easily monitored by inactivating vegetative cells by heat treatment and growing them on nutrient agar with added trypan blue.

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Table 1

Comparison of MF methods used to detect *C. perfringens* spores in water

Comparison	Accuracy	Specificity	Disadvantages	Heat treatment	Reference
Egg yolk-free TSC vs mCP	TSC >mCP	mCP >TSC; both > 90%	Overlaying the MFs with TSC did not improve recovery and sometimes caused blackening in the overlays	75 °C, 20 min	Sartory (1986)
mCP vs MmCP[a]	mCP: 95% ± 4 MmCP: 94% ± 4	No difference	None reported	Not done	Armon & Payment (1988)
TSC vs mCP	TSC: 82.5%, mCP: 60%	mCP more selective than TSC	Poor color differentiation on mCP following exposure to ammonia fumes	75 °C, 20 min	Sartory *et al.* (1998)
TSCF[b] vs mCP	TSCF: 80.8%, mCP: 53.5%	TSC:14.8% false +, 17.2% false −; mCP: 41.6% false +, 1.9% false −	mCP preparation was complex and mCP was cumbersome to use	Not done	Araujo *et al.* (2001)
TSC vs TSCF vs mCP	TSC: 58%; TSCF: 88.5%; mCP: 58.2%	TSCF is more specific for spore recovery	mCP performed poorly if water samples contained predominantly spores	80 °C, 5 min	Araujo *et al.* (2004)
TSC vs TSC-EY[c] vs mCP	TSC >TSC-EY >mCP	false +: mCP 6%; TSC: 74% TSC-YE: 76%	mCP was labor intensive and time consuming	Not done	Burger *et al.* (1984)
FDT[d] vs CP AnaSelect[e] vs mCP vs SFP	All were equivalent	CP AnaSelect more selective than mCP	CP AnaSelect slower (12–15 hr) than FDT (5–6 hr)	70 °C, 2.5 min	Vijayavel *et al.* (2009)
CCP[f] vs mCP	CCP >mCP	CCP more specific than mCP	Poor color differentiation on mCP following exposure to ammonia fumes	Not done	Manafi *et al.* (2013)

[a]Modified mCP (MmCP) with indoxyl-β-D-glucoside reduced from 600 to 60 mg/L.

[b]Fluorogenic TSC supplemented with 4-methylumbelliferylphosphate (MUP) disodium salt.

[c]TSC agar without egg yolk.

[d]Fung double tube method.

[e]CP AnaSelect Oxyplate (Oxyrase Inc., Mansfield, OH).

[f]CP Chromo Select Agar (CCP Sigma-Aldrich).

Table 2

Methods used to detect and quantify aerobic spores in water

Indicator/Surrogate	Medium	Heat treatment	Performance	Reference
Aerobic spores	Nutrient agar + trypan blue	80 °C, 12 min	Spore removal closely parallels particle removal	Rice et al. (1996)
Indigenous aerobic endospores and B. subtilis endospores	Nutrient agar + trypan blue	80 °C, 12 min	Indigenous aerobic endospores were a good indicator of ozone treatment effectiveness	Owens et al. (2000)
Indigenous aerobic endospores	Pads saturated in TSB	75 °C, 15 min	Indigenous aerobic endospores were a good indicator of ozone treatment effectiveness	Facile et al. (2000)
B. subtilis aerobic endospores	Nutrient agar pour plates	80 °C, 10 min	Spores were 400 times more resistant to UV than enteric bacteria but less resistant than A. castellanii cysts	Chang et al. (1985)