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Comparison of mCP and TSC Media to Enumerate *Clostridium perfringens* in Surface Water Samples

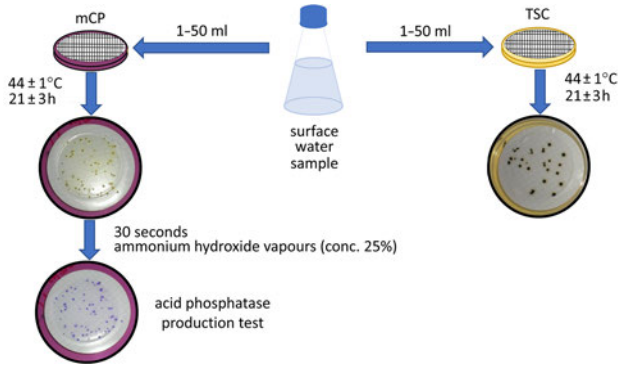
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Abstract

The *Clostridium perfringens* bacteria are used to assess water quality as an indicator parameter. If detected, it can confirm the occurrence of past fecal contamination. Tests determining *C. perfringens* in water samples are usually performed by membrane filtration where filters are incubated on selective media under anaerobic conditions. Available media include mCP and TSC. The aim of this study was to compare the relative recovery of *C. perfringens* (including spores) from surface water samples and to determine the performance characteristics of the membrane filtration method using both media. The results showed that, although the procedure using the mCP medium was more sensitive and specific, higher recoveries were obtained in the tests based on the TSC medium.



K e y w o r d s: *Clostridium perfringens*, mCP medium, membrane filtration, surface water, TSC medium

Introduction

The *Clostridium perfringens* bacteria, besides *Escherichia coli* and intestinal enterococci, are indicators of fecal water contamination (Ashbolt et al. 2001; Harwood et al. 2005; WHO 2022). *C. perfringens* is a Gram-positive anaerobic spore-forming rod-shaped bacterium, which in the context of this method, reduces sulphite to sulphide at 44°C within 24 hours (Schlegel 2000; WHO 2022). *C. perfringens* reduces nitrate, is non-motile, ferments lactose, and liquefies gelatin. *C. perfringens* also produces the enzyme acid phosphatase, a diagnostic characteristic for this species amongst the clostridia. These bacilli are a constant component of the human intestinal microflora (13–35%) and warm-blooded animals, but compared with other bacteria acting as fecal contamination indicators, the number of their cells excreted in feces is significantly lower than that of *E. coli* or intestinal enterococci (Payment and Franco 1993; Schlegel 2000; Ashbolt et al. 2001; WHO 2022). The presence of *C. perfringens* (both spores and vegetative forms) in water, with the absence of *E. coli* and intestinal enterococci, may indicate remote or intermittent fecal pollution. The spores of these bacteria can dwell for a long time in extremely adverse conditions without losing their ability for subsequent growth. The exceptional resistance of endospores to unfavorable environmental conditions in a water environment, including temperature and pH extremes, disinfection processes, UV radiation, and various chemical agents, has led to their use, among other things, to validate the effectiveness of treatment processes for water intended for human consumption and as an indicator of the presence of enteric viruses or protozoa (e.g., *Cryptosporidium*) in treated water (Payment and Franco 1993; Ashbolt et al. 2001; Harwood et al. 2005; Matuszewska et al. 2013; WHO 2022).

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The detection and enumeration of *C. perfringens* (spores and vegetative forms) in routine tests is usually performed through membrane filtration, where filters are incubated on a selective medium under strictly anaerobic conditions (Sartory 1986; Sartory et al. 2006; Stelma 2018). For the quantitative determination of *C. perfringens* in water samples, suggested methods included the use of the TSC medium and the procedure described in the draft standard ISO/CD 6461-2:2002 Water Quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration (2002). Later, a test procedure dedicated to testing drinking water samples and described in Council Directive 98/83/EC (1998) was introduced, which was based on the mCP medium, also using the membrane filtration method.

Although the method included in the directive has been withdrawn, it is used by, among others, water suppliers for water control tests due to the short time it takes to obtain results. In this case, it is worth noting what differences in the quantification of *C. perfringens* may occur on the two media compared. The aim of the works carried out was to compare the relative recovery and performance characteristics of the membrane filtration method using the TSC and mCP media in the testing of surface water samples for the enumeration of *C. perfringens* (including spores).

Experimental

Materials and Methods

Nineteen surface water samples were collected to detect and enumerate *C. perfringens* (including spores). Surface water samples were collected from three different standing water reservoirs in Warsaw. The water samples were not heated. The tests were performed by membrane filtration (MF-Millipore™ Membrane Filter, 0.45 µm pore size, 47 mm diameter, Merck KGaA, Germany). Depending on the expected contamination, 1 ml to 50 ml water samples were cultured. Filters were placed on selective media and incubated under anaerobic conditions at 44 ± 1°C for 21 ± 3 hours. According to the methodology of ISO/CD 6461-2:2002 (2002), water samples were inoculated onto the TSC agar (Tryptose Sulphite Cycloserine agar without yolk; Oxoid, USA) consisting of basic medium (enzymatic digest casein 15.0 g; enzymatic digest soya 5.0 g; yeast extract 5.0 g; sodium disulphate (IV) anhydrous 1.0 g; iron (III) ammonium citrate 1.0 g; agar 9 g to 18.0 g; water 1,000 ml) and cycloserine supplement 10 ml (D-cycloserine 4.0 g; water 100 ml). After 24 h incubation on the TSC medium, all characteristic colonies were quantified as presumptive *C. perfringens*. From the colonies that grew on the TSC, black- and grey-colored colonies were selected and transferred onto nutrient agar and then confirmed based on biochemical test results, where tests were conducted to determine motility, reduction of nitrates to nitrites, ferments lactose with acid formation, and gelatine liquefaction. According to the procedure from Council Directive 98/83/EC (1998), water samples were inoculated onto mCP agar consisting of basic medium (tryptose peptone 30 g; yeast extract 20 g; sucrose 5 g; L-cysteine hydrochloride 1 g; magnesium sulphate 0.1 g; bromocresol purple 40 mg; agar 15 g; water 1,000 ml) and supplement (D-cycloserine 400 mg; polymyxin-β-sulphate 25 mg; indoxyl β-D-glucoside 60 mg; 0.5% phenolphthalein diphosphate solution 20 ml; 4.5% solution $FeCl_3 \times 6H_2O$ 2 ml). After 24 h incubation on the mCP medium, all characteristic colonies were determined as suspected *C. perfringens*. Colonies grown on the medium were confirmed directly on it for their ability to produce the enzyme acid phosphatase. Colonies were treated with ammonium hydroxide vapors (at a concentration of 25%) for 30 seconds to confirm their ability to produce acid phosphatase. The test results were expressed as the number of *C. perfringens* CFU/100 ml.

Test methods. The performance characteristics of the method using the TSC and mCP media were determined according to PN-EN ISO 13843:2017 (2017). The characteristics of the methods included the following parameters: sensitivity – the fraction of the total positives correctly assigned in the presumptive counts; specificity – the fraction of the total negatives correctly assigned in the presumptive counts; selectivity – the ratio of the target colonies number to the total number of colonies in the sample volume; false-positive rate – the fraction of positive results (e.g., typical colonies) that are subsequently shown to be due to non-target organisms; false-negative rate – the fraction of negative results (e.g., atypical colonies) shown to be target organisms; efficiency – the fraction of total colonies correctly assigned in the presumptive counts. The tests included 847 isolated and confirmed bacterial strains, including 466 isolates from the TSC medium and 381 isolates from the mCP medium.

A comparison of relative bacterial recovery (including statistical analysis) was carried out according to PN-EN ISO 17994:2014 (2014) using parallel tests of water samples according to the procedure ISO/CD 6461-2:2002 (2002) and according to the procedure described in Council Directive 98/83/EC (1998).

Results and Discussion

Categorical method performance characteristics. Method performance characteristics on the substrates compared were determined using natural surface water

samples. The water samples and isolated bacterial strains were tested according to appropriate procedures, including confirmatory testing.

Categorical performance characteristics were determined based on the analysis of 466 suspected *C. perfringens* strains isolated on the TSC and 381 suspected *C. perfringens* strains isolated on the mCP. In order to confirm the results, confirmatory tests were performed using biochemical tests according to ISO/CD 6461-2:2002 (2002) or an acid phosphatase production capacity test according to the methodology set out in the directive (Council Directive 98/83/EC 1998), respectively. Characteristic black- or grey-colored colonies transferred from the TSC to nutrient agar and showing growth under only anaerobic conditions were confirmed for *C. perfringens*, whereby this species was differentiated from other sulphite-reducing clostridia based on no motility (Table I).

For colonies that grew on the mCP, those that caused its yellow discoloration (sucrose fermentation) were considered characteristic. The plate with the colonies grown was placed in ammonium hydroxide vapors to confirm the ability of the bacteria to produce acid phosphatase. Positively confirmed colonies changed color from yellow to pink (Table II). A summary of the results of confirmatory tests for bacterial strains determined towards *C. perfringens* is shown in Table III.

The following categorical performance characteristics were determined: sensitivity, specificity, selectivity, false-positive rate, false-negative rate, and efficiency. For the mCP and TSC media, the determined method sensitivity reached a high value of 90.7% and 82.0%, respectively. At the same time, it turned out that, for the surface water samples tested, the method according to Council Directive 98/83/EC (1998) with the mCP medium is more specific (70.0%) than the method according to ISO/CD 6461-2:2002 (2002) based on the TSC medium (30.7%). Method specificity is determined, among other things, through error introduced by false-positive colonies growing on the media used (mCP – 42.5%, TSC – 56.6%) and error resulting from non-detection of typical colonies (mCP – 0.09, TSC – 0.18). The results presented in Table IV show that the false-positive rate values, i.e., the fraction of observed positive (typical) colonies selected for confirmation that were not confirmed to be *C. perfringens*, were similar, with a higher value of this parameter recorded for the TSC-based method than for the mCP-based method.

According to literature data, the false-positive rate is usually lower for the TSC medium because it is

Table I
Confirmatory tests for *Clostridium perfringens* according to ISO/CD 6461-2:2002.

Feature	*Clostridium perfringens*
Reduction of sulphites to sulphides	black or grey colonies
Motility	none
Reduction of nitrate to nitrites	orange/red-colored medium after the addition of the Nessler reagent
Lactose fermentation	orange or yellow-colored medium with lactose (acidification of the medium and gas formation)
Protease formation	gelatine liquefaction

Table II
Confirmatory tests for *Clostridium perfringens* according to Directive 98/83/EC.

Feature	*Clostridium perfringens*
Sucrose fermentation	sucrose-positive – yellow and matte colonies
Possess acid phosphatase	change of color of the colony from yellow to pink in ammonium hydroxide vapors

Table III
Comparison of the results of confirmation tests for *Clostridium perfringens*.

Characteristics of *Clostridium perfringens* presumptive confirmed colonies	TSC ISO/CD 6461-2:2002	mCP Directive 98/83/EC
Number of all confirmed colonies	466	381
Typical colonies, confirmed positive (true positive)	150	107
Atypical colonies, confirmed positive (false negative)	33	11
Typical colonies, confirmed negative (false positive)	196	79
Atypical colonies, confirmed negative (true negative)	87	184
Typical colonies, non-detection error	0.18	0.09

Table IV
Comparison of performance parameters of procedures used for detection of *Clostridiun perfringens* in water samples.

Performance parameters	TSC ISO/CD 6461-2:2002	mCP Directive 98/83/EC
Sensitivity	82.0%	90.7%
Specificity	30.7%	70.0%
Selectivity	32.2%	28.1%
False negative rate	27.5%	5.6%
False positive rate	56.6%	42.5%
Efficiency	50.9%	76.4%

considered more selective than the mCP medium, leading to fewer false-positive results (Lamy et al. 2020). During the present study, non-typically-looking colonies, which, in part, were confirmed as *C. perfringens* colonies, were observed more frequently on the TSC than on the mCP. The present study reflects this in the false negative rate, which was higher on the TSC (27.5%) than on the mCP (5.6%). Other researchers also reached similar study conclusions when they observed that more false negative results were obtained on the TSC than the mCP. The higher value of false negative results for TSC may be since this medium selects for all sulphite-reducing clostridia (Sartory 1986; Sartory et al. 2006; Stelma 2018 Lamy et al. 2020). At the same time, the lower specificity of the mCP towards the *C. perfringens* species is, according to some researchers, caused by the very subjective analyst-performed differentiation of the color of presumptive colonies after incubation and after exposure to ammonia vapors (Sartory et al. 1998; Araujo et al. 2001; Araujo et al. 2004). Published data also indicated that significant numbers of non-typical (false negative) colonies, identified as *C. perfringens*, grew on this medium (Sartory et al. 1998). This could be due to the neutral pH of the medium, which resulted in the counting of other non-target colonies that could produce acid phosphatase (Eisgruber et al. 2003). It was also observed that yellow non-colored colonies could sometimes remain on the mCP after exposure to ammonia vapors, which was considered a negative result. However, this was not confirmed by the test results presented, while the error arising from non-detection of typical colonies was low and amounted to 0.09. It is worth noting that, according to some researchers, both media may have a low confirmation rate depending on the contamination level of the water samples tested (Sartory et al. 1998).

Regarding selectivity, for both media compared, a similar value was obtained for this parameter, which was 28.1% (Council Directive 98/83/EC 1998) and 32.2% (ISO/CD 6461-2:2002 2002), respectively. Available literature data indicate that, when testing real samples, the mCP usually performs worse than the TSC medium. However, the authors point out possible differences in results that are caused by different types of water samples tested (groundwater, surface water) and the testing direction, i.e. determination of the number of spores or vegetative forms (Bisson and Cabelli 1979; Sartory 1986; Sartory et al. 1998; Araujo et al. 2004; Sartory et al. 2006).

As part of the study, the efficiency of the methods was also determined, and was expressed as the number of colonies correctly selected for confirmatory testing. In this case, a higher efficiency was achieved by the method based on the mCP (76.4%) compared with the method based on TSC (50.9%). This parameter is strongly influenced by the experience of the analyst and the degree of contamination of the sample tested.

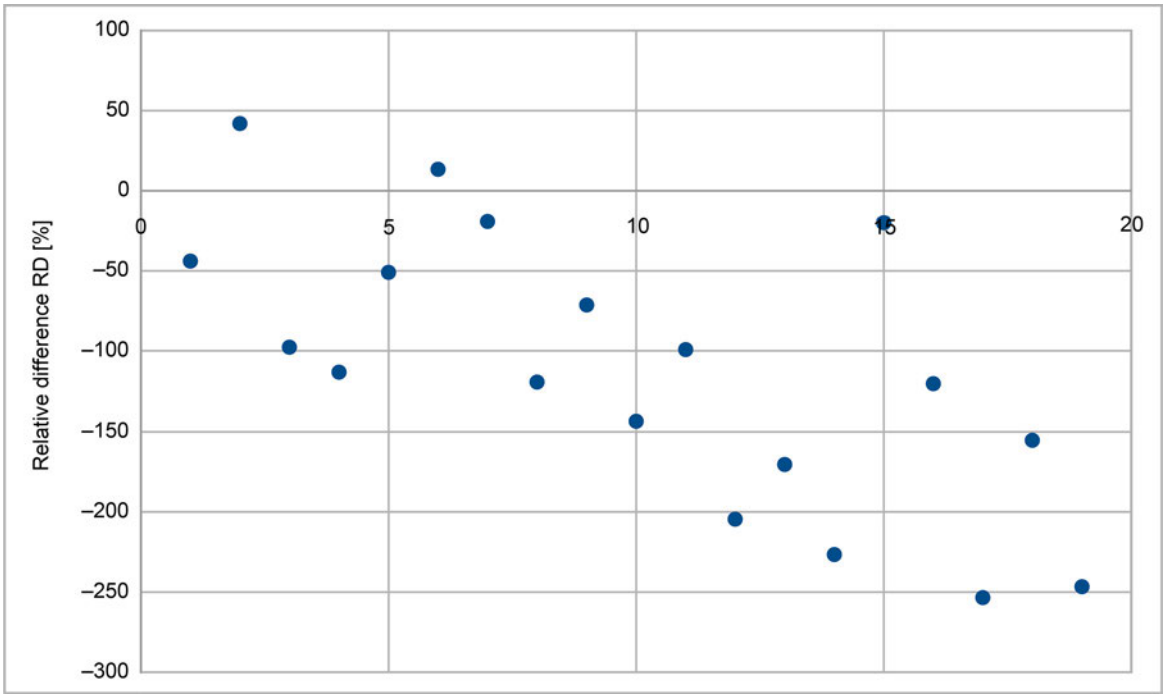
Assessment of relative bacterial recovery. Methods for detecting and enumerating of *C. perfringens* (including spores) in water samples using the TSC and mCP media rely on the ability of these bacteria to reduce sulphites to sulphides (Bisson and Cabelli 1979). In order to limit the growth of accompanying microflora, cycloserine is added to the TSC medium, while cycloserine and polymyxin B are added to the mCP medium. Both antibiotics inhibit the growth of various species of facultatively anaerobic bacteria, including *Bacillus* (Hatheway and Johnson 1998; ISO/CD 6461-2:2002 2002; PN-EN ISO 14189:2016 2016). In both cases, confirmatory tests were required to achieve final results. A procedure that provides higher recovery of confirmed target organisms is preferable when confirmation is required for routine use. Sometimes, however, a method that gives a slightly lower recovery but does not require confirmation may be preferred.

The results were analyzed using the approach in PN-EN ISO 17994:2014 (2014). A relative difference (RD) was determined for each pair of confirmed counts, i.e., the difference between the two results, a and b, measured on a relative (natural logarithmic) scale. The equation used was:

$$x = [\ln(\alpha) - \ln(b)] \times 100\%$$

where ln(a) is the natural logarithm of a count performed using the method tested on the mCP medium (according to Council Directive 98/83/EC 1998) and ln(b) is the natural logarithm of a count performed using the method on the TSC medium (according to ISO/CD 6461-2:2002). In order to establish the confidence interval, the limit of 2 l = 20% suggested for testing environmental water samples was used. A paired *t*-test for the result showed that the mean difference is significant, i.e. the methods are statistically different. The result of comparing relative recovery expressed as relative difference RD (%) is shown in Fig. 1.

The tests performed to detect *C. perfringens* (including spores) confirmed the presence of these bacteria in



Relative difference	Mean value	X_L (95%Cl)	X_U (95%Cl)	n (number of observations)
RD	−110.56	−148.96	−72.16	19

Fig. 1. Variability summary for RD [%].

all the surface water samples tested. In the majority of samples, 89.5% (17/19), the determined bacterial counts on the TSC were higher than on the mCP, both in samples with lower contamination (< 100 CFU/100 ml: from 21 CFU/100 ml to 93 CFU/100 ml) and in samples with high contamination (≥ 100 CFU/100 ml; from 101 CFU/100 ml to 1031 CFU/100 ml). Comparable or higher recovery on the TSC in tests of different types of water samples (groundwater, surface water) was also obtained by other researchers (Sartory et al. 1998; Araujo et al. 2001; Araujo et al. 2004; Stelma 2018; Lamy et al. 2020). In their studies, some of them suggest that mCP detects comparable levels of *C. perfringens* concerning other media when vegetative cells are predominantly present in samples tested. However, when spores are tested, the mCP may be unsuitable because underestimated results are obtained (Araujo et al. 2001; Araujo et al. 2004). In addition, they pay attention to the type of samples tested, and, in this case, the mCP is indicated for testing pure water samples with relatively low bacterial counts, such as e.g., groundwater partially purified water. The TSC medium, on the other hand, is more widely used for all types of water samples with different degrees of contamination (Schlegel 2000; Sartory et al. 2006; Lamy et al. 2020). The results of a comparative study of mCP and TSC conducted by Sartory et al. (1998) for the recovery of *C. perfringens* from surface water samples and partially treated drinking water indicate that the number of *C. perfringens* was higher on TSC than on mCP (Sartory et al. 1998). In contrast, in another study, involving highly contaminated water samples, Sartory et al (1998) reported comparable recovery values and confirmation rates for mCP and TSC (Sartory 1986). The above observations are important for testing drinking water because *C. perfringens* is mainly found as spores, and using this medium may underestimate the bacterial count determined, giving a false sense of safety (Araujo et al. 2004). The analysis of the above study results indicates that TSC may be a more suitable medium for routine water testing (including drinking water) towards *C. perfringens* emphasizing water intakes based on surface water. In this case, the confirmation step becomes crucial, as the TSC medium is not selective enough to enumerate *C. perfringens*, especially in contaminated waters (Sartory et al. 2006).

Furthermore, it is worth paying attention to the technical aspects and the speed of obtaining results in the procedures compared. A significant advantage of the method for detecting *C. perfringens* on the mCP medium is the possibility of obtaining a result within 18–24 hours from the time of water sample inoculation and the use of a rapid test for the enzyme-specific to the bacteria to be determined (Bisson and Cabelli 1979;

Ryzinska-Paier et al. 2011). Literature data indicate that the acid phosphatase test correctly identified environmental strains in 92% of cases, compared with 83% of cases identified using a short biochemical pathway according to ISO/CD 6461-2:2002 (2002) (Ryzinska-Paier et al. 2011). At the same time, the expression of acid phosphatase is very specific for the *C. perfringens* species compared to other species belonging to the sulphite-reducing clostridia (Eisgruber et al 2003; Sartory et al. 2006; Ryzinska-Paier et al. 2011). The procedure based on the mCP medium eliminates the need to transfer isolated colonies and laborious confirmatory tests using a biochemical pathway.

In contrast to the procedure from the Directive, it is not possible to obtain the final results of tests carried out on the TSC medium according to ISO/CD 6461-2:2002 (2002) until after 72 hours, and sometimes even after 96 hours. This is mainly due to the need to carry out confirmatory tests requiring two successive steps: transferring isolated colonies onto blood medium or nutrient agar and performing culture on the appropriate media that make up the biochemical pathway after another day of incubation. However, this procedure is supported by the higher recovery achieved, which is essential in health safety, especially for drinking water samples. In this case, testing as part of ongoing surveillance for *C. perfringens* should be carried out according to PN-EN ISO 14189:2016 (2016). The same procedure should be followed by water suppliers when the results are presented to the sanitary inspection unit.

Conclusions

The results showed that some performance characteristics for the procedure based on the mCP medium were significantly better than those using the TSC medium. At the same time, a statistical analysis of the results showed that the recovery of target bacteria from the samples tested using the TSC medium was significantly higher. Based on literature data and the presented results, it can be concluded that the procedure using the TSC medium may be more suitable for testing surface water samples for the enumeration of *C. perfringens* (vegetative forms including spores).

In summary, when it comes to legal regulations, at the level of the laws of Europe and Poland, the requirement for the enumeration of *C. perfringens* (including spores) has been defined for water intended for human consumption (Council Directive 98/83/EC 1998; Commission Directive (EU) 2015/1787 2015; Directive (EU) 2020/2184 2020; Regulation of the Minister of Health of 29 March 2007; Regulation of the Minister of Health of 20 April 2010; Regulation of the Minister of Health of 13 November 2015; Regulation of the Minister of Health of 7 December 2017). In 2013, the ISO committee approved the standard PN-EN ISO 14189:2016 "Water quality – Enumeration of *Clostridium perfringens* – Method using membrane filtration" and the presented procedure for testing water samples was based on the TSC medium and a confirmatory test for acid phosphatase (PN-EN ISO 14189:2016 2016). In the legally regulated area, the standardized methodology was introduced in 2015 by way of Commission Directive (EU) 2015/1787 of 6 October 2015 amendment of Annexes II and III to Council Directive 98/83/EC on the quality of water intended for human consumption (Commission Directive (EU) 2015/1787 2015, Directive (EU) 2020/2184 2020, Regulation of the Minister of Health of 13 November 2015, Regulation of the Minister of Health of 7 December 2017).

It can therefore be considered that the current standardized method for the determination of *C. perfringens* has drawn upon both procedures and used those solutions that allow the determination of the bacterial forms that are most frequently found in natural water samples, which guarantees adequate recovery as well as simplification and shortening of the microbiological analysis.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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