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Enumeration of *Clostridium perfringens*— Microbiological Performance Testing of Membrane Filters in Accordance with EN ISO 7704:2023

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Abstract

The revised EN ISO 7704:2023 standard specifies the performance testing requirements of membrane filters used in water quality analysis. It mandates that manufacturers or microbiological laboratories using membrane filters demonstrate the suitability of each batch of membrane filters in conjunction with each lot of the respective solid culture medium. This study demonstrates the microbiological performance testing of membrane filters in accordance with EN ISO 7704:2023, using the EN ISO 14189 standard as a specific example. Additionally, the study investigates the impact of non-selective media from different manufacturers on the productivity ratio. The Sartorius high flow membrane filters were found to comply with the new EN ISO 7704:2023 standard.

Introduction

Assessing the number of microorganisms in water samples is a pivotal aspect of microbiological quality control. This process is essential for various types of water, from those expected to have few microorganisms, such as drinking and bottled water, to those likely to contain higher microbial counts, such as surface or industrial water. Membrane filtration stands out as the regulatory preferred method for the enumeration of these microorganisms. The revised EN ISO 7704:2023¹ provides guidelines for the performance testing of membrane filters, which are utilized to capture and enumerate microorganisms directly. As depicted in Figure 1, EN ISO 7704:2023 includes mandatory batch testing procedures (modules 1 and 2) and supplementary quantitative testing procedures (modules 3 and 4). By evaluating the results from modules 1 and 2, one can determine the productivity ratio, which reflects the effectiveness of the membrane filter in conjunction with the selective culture medium.

Various specific standards, including ISO 14189², ISO 7899-2, ISO 6461-2, ISO 9308-1, ISO 11731, and ISO 16266, refer to the revised EN ISO 7704:2023. EN ISO 14189 specifies the method for enumerating vegetative cells and spores of *Clostridium perfringens* in water samples by membrane filtration (Table 1). *C. perfringens* is a Gram-positive, sulfite-reducing bacterium and widely recognized as a key indicator organism for fecal pollution of water. Due to the spores' ability to survive in water for extended periods and their high resistance to chlorination and heat treatment, the detection and enumeration of *C. perfringens* is pivotal in microbiological quality control of water samples. In accordance with EN ISO 7704:2023, the release of cellulose nitrate membrane filters is demonstrated in this study using the specific standard EN ISO 14189 as an example.

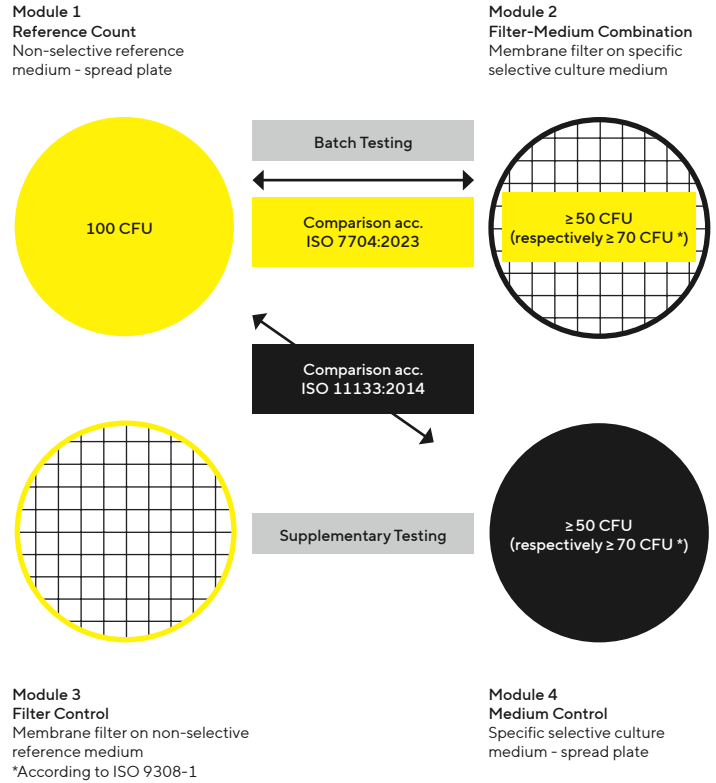


Figure 1: Schematic mandatory batch testing procedures (modules 1 and 2) and supplementary quantitative testing procedures (modules 3 and 4) according to the revised EN ISO 7704:2023 standard. For further information refer to our Best Practice Guide³.

Function	Incubation	Control strains	Method of control	Criteria	Characteristic reaction
Productivity	21 ± 3 h 44 ± 1 °C anaerobic	<i>C. perfringens</i> WDCM 00007*; <i>C. perfringens</i> WDCM 00080*; <i>C. perfringens</i> WDCM 00174*;	Quantitative	$P_R \geq 0.5$	Black colonies

Mandatory* and optional* strains according to EN ISO 11133

Table 1: Test microorganisms and performance criteria for culture media according to ISO 14189.

Materials and Methods

For the preparation and storage of culture media and diluents, the procedures have been adapted to the corresponding specific standards EN ISO 11133⁴ or EN ISO 8199.⁵ Each experiment was performed with three different lots of membrane filters in six replicates.

Product	Order No.
3 branch Microsart® Manifold	168M3-SS100
Microsart® e. jet pump	166MP-4
Cellulose Nitrate High Flow membrane filter (single-packed) - 0.45 µm	114H6--47----ACN
Tryptic Soy Agar (TSA)	Manufacturer A-C
Tryptose Sulfite Cycloserine (TSC)	Manufacturer A

Table 2: Consumables, media, and equipment used in this study.

Tryptic soy agar (TSA) sourced from three different well-known manufactures (A-C) was employed as a non-selective reference agar and one lot of tryptose sulfite cycloserine (TSC) agar was used as a specific selective agar for the recovery of *C. perfringens* (Table 2). All test microorganisms (Table 3) were diluted in sterile phosphate buffer (PBS) and incubated in an anaerobic atmosphere at 44 °C ± 1 °C for 21 h ± 3 h according to EN ISO 14189. The anaerobic conditions were monitored using indicator strips. .

Test microorganism	Function
<i>Clostridium perfringens</i> WDCM 00007	Productivity
<i>Bacillus subtilis</i> WDCM 00003	Selectivity

Table 3: Test microorganisms used in this study.

Filtration

To determine the reference count, an inoculum containing 50 – 150 CFU of *C. perfringens* WDCM 00007 was inoculated onto TSA and TSC agar using the spread plate technique. For assessing the productivity ratio, a membrane filter was placed on the manifold, a sterile funnel was mounted, and 10 mL of sterile PBS was poured into the funnel. The same inoculum volume, containing 50 – 150 CFU of *C. perfringens* WDCM 00007, was then added, followed by an additional 10 mL of sterile PBS to ensure even distribution of the inoculum prior the filtration. After filtration, the membrane filters were placed onto specific selective TSC agar and non-selective TSA. For membrane filters transferred to TSC, 10 mL of overlay agar were added directly on top of the membrane filters in accordance with EN ISO 14189. For selectivity testing, at least 10⁴ CFU of *Bacillus subtilis* WDCM 00003 inoculated onto TSC agar. All plates were placed into an anaerobic atmosphere.

The productivity ratio (P_r) is determined from the results of the quantitative tests from modules 1 and 2 (see Table 4).

$$P_r = \frac{N_s}{N_o}$$

N_s is defined by the total count of colonies obtained from membrane filters in intended use with the specific culture medium (TSC) according to module 2.

N_o is defined by the total count of colonies obtained from plates of non-selective reference culture medium (TSA) by direct inoculation without a membrane filter according to module 1.

Table 4: Calculation of the productivity ratio.

Results

Colonies of *C. perfringens* WDCM 00007 grown on TSC agar exhibit a black coloration on the membrane filter, while remaining pale on non-selective TSA (see Figure 2).

C. perfringens WDCM 00007

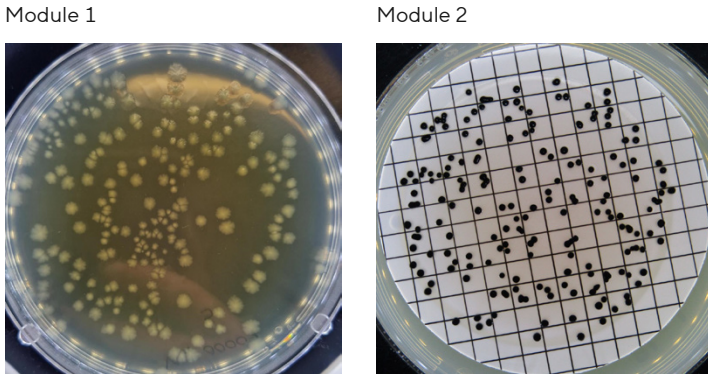


Figure 2: Exemplary results of *C. perfringens* WDCM 00007 grown on non-selective TSA (module 1) and on a membrane filter placed on specific selective TSC agar (module 2). The black colonies of *C. perfringens* are the result of the reduction of sulfite to sulfide, which subsequently reacts with ferric salts in the medium.

The productivity ratio of *C. perfringens* WDCM 00007 was found to be well above the minimum required P_r of ≥ 0.5 , when recovered on membrane filters in conjunction with TSC agar (Figure 3). All tested batches of membrane filters and the specific selective agar were in specification according to EN ISO 7704:2023.

C. perfringens WDCM 00007

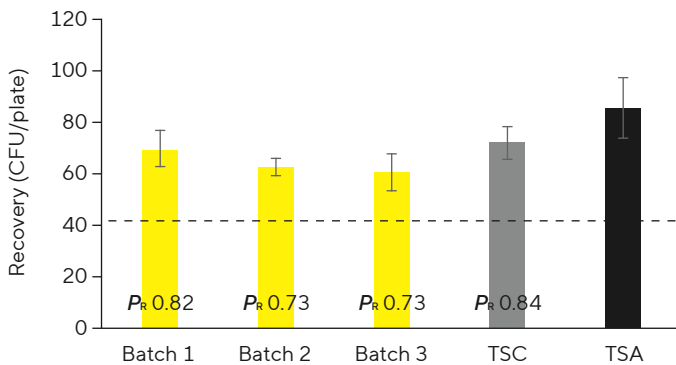
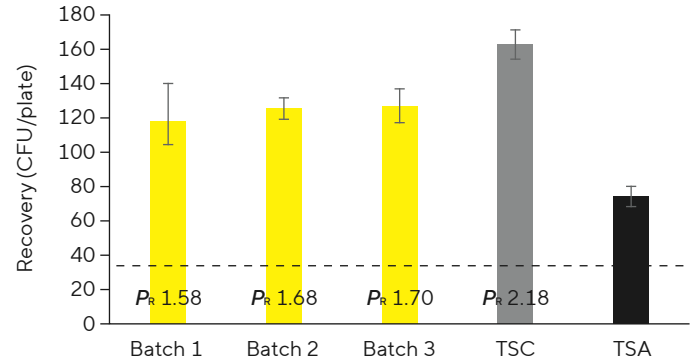


Figure 3: Reference count determination on non-selective culture medium (TSA from manufacturer A, as recommended by module 1) and productivity determination by using three different batches of 114H6 high flow membrane filters (ACN) with the specific selective culture medium (TSC, as required by module 2). The results for the productivity ratio (P_r) are depicted in the graph, with the dotted line indicating the minimum required P_r of ≥ 0.5 .

Figure 4 demonstrates the influence of the non-selective TSA on the performance testing results. Although the membrane filters and the specific selective agar in modules 2 and 4 achieve good recovery rates, the determination of the reference count on TSA of different manufacturers can lead to productivity ratios exceeding $P_r > 1.4$, necessitating a root cause analysis.

TSA Supplier B



TSA Supplier C

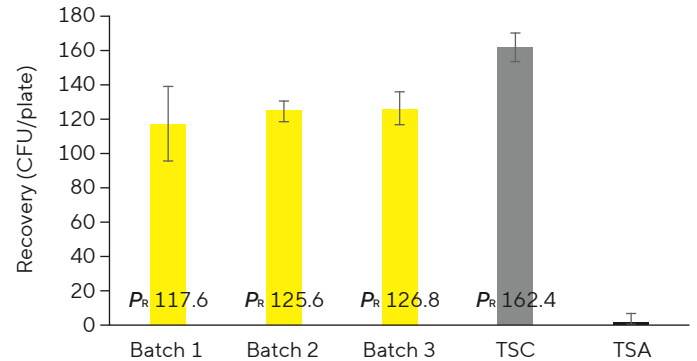


Figure 4: Impact of non-selective TSA from different manufacturers on reference count determination and subsequently on productivity ratios (P_r). The results for the productivity ratio are depicted in the graph, with the dotted line indicating the minimum required P_r of ≥ 0.5 .

Besides the determination of the productivity ratio, the performance testing according to module 2 also includes the examination of selectivity and specificity, if applicable. Qualitative selectivity testing was conducted by inoculation of *B. subtilis* WDCM 00003 on specific selective TSC agar (Table 5). After the indicated period, no growth of the test strain was observed on TSC agar.

Function	Incubation	Control strains	Method of control	Criteria	Results
Selectivity	21±3 h 44±1 °C	<i>B. subtilis</i> WDCM 00003	Quantitative	Total inhibition	Conform

Table 5: Performance testing of TSC agar for qualitative selectivity according to module 2.

Conclusion

The results of this study feature productivity ratios well above the minimum required P_R of 0.5 for each tested batch of membrane filters in conjunction with specific selective TSC media for *C. perfringens* WDCM 00007. Furthermore, the use of TSA from different manufacturers substantiates the impact of the non-selective media on the productivity ratio. The qualitative selectivity testing for TSC agar according to module 2 was successfully passed by using *B. subtilis* WDCM 00003. These results demonstrate EN ISO 7704:2023-compliant release of Sartorius cellulose nitrate high flow membrane filters, as exemplified by the specific standard EN ISO 14189.

For further information on EN ISO 7704:2023, please refer to our Best Practice Guide³ or to our App Note on performance testing using the standard EN ISO 7899-2⁶ as an example.

Literature

1. EN ISO 7704:2023 - Water quality – Requirements for the performance testing of membrane filters used for direct enumeration of microorganisms by culture methods
2. EN ISO 14189 - Water quality – Enumeration of *Clostridium perfringens* – Method using membrane filtration
3. Best Practice Guide - New DIN EN ISO 7704:2023 at a Glance
4. EN ISO 11133:2014 - Microbiology of food and animal feeding stuffs – Guidelines on preparation and production of culture media
5. EN ISO 8199:2018 - Water quality – General guidance on the enumeration of microorganisms by culture
6. Microbiological Performance Testing of Membrane Filters in Accordance with EN ISO 7704:2023

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