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Performance characteristics of the temperature-modified ISO 9308-1 method for the enumeration of *Escherichia coli* in marine and inland bathing waters

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ABSTRACT

This study defines performance characteristics of the temperature-modified ISO 9308-1 method for *E. coli* enumeration in bathing water. After a 4-hour resuscitation period at 36 ± 2 °C, the incubation temperature was changed to 44 ± 0.5 °C. Elevated incubation temperature significantly suppressed the growth of thermo-intolerant bacteria, and enhanced the selectivity of Chromogenic Coliform Agar (CCA) up to 49.5% for inland and up to 66.0% for coastal water. Consequently, most of the selectivity-related performance characteristics are improved.

Relative recovery was determined by comparing an alternative method against the reference, ISO 9308-1:2014 method, following the criteria set out in ISO 17994:2014. Temperature modification did not significantly alter the results and the methods were evaluated as "not different" for both, coastal and inland waters.

Chromogenic Coliform Agar was assessed as a suitable medium for reliable *E. coli* enumeration in bathing water when incubated for 17–19 h at 44 ± 0.5 °C after the 4–5 h resuscitation period at 36 ± 2 °C.

1. Introduction

Croatia was one of the first Mediterranean countries to begin monitoring the microbiological quality of bathing water, back in 1989. Because of many advantages of the membrane technique over the Most Probable Number (MPN) methods, such as fast and simple procedure, less preparation and laboratory space occupied, and the most important ones, the concentration of large sample volumes and significantly better relative precision, the membrane filtration methods were applied for the enumeration of all required indicator microorganisms.

The revised European Union Bathing Water Directive (BWD) (2006/7/EC) references new parameters and methods to use in bathing water microbiological quality monitoring. Annex I of the Directive defines two analytical methods for *E. coli* enumeration in bathing water samples, ISO 9308-3 (Miniaturized Most Probable Number (MMPN) method, liquid medium) and ISO 9308-1 (membrane filtration (MF) method, solid medium). The former version of ISO 9308-1 (ISO 9308-1, 2000) included two tests: Standard test using TTC Tergitol® 7 agar and

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Rapid test using TSA/TBA agar. Prior to selective incubation at 44 ± 0.5 °C, the Rapid test included 4–5 hour resuscitation period at 36 ± 2 °C, to enhance the recovery of viable bacteria. Both tests were suitable for waters with low bacterial numbers but could be applied to the other types of water provided that suspended matter or background bacterial flora did not interfere with filtration, culture and counting. Since the Rapid test was more selective and faster than Standard test (obtaining results within 24 h), some Member States, including Croatia, successfully used it for *E. coli* enumeration under the scope of the BWD.

As directives are revised, so are the standard methods, in order to follow progress in science. This means that the reference methods referenced in a directive may change also. A revised edition of the ISO 9308-1 method, which came into force in 2014, is drastically different from the previous standard version of year 2000. Among other changes, the recently revised reference method does not include other kinds of water except drinking water in its scope, thus questioning the use of this method for monitoring of bathing water quality. The main technical difference of the new method is that it is based on the Chromogenic Coliform Agar (CCA), meeting a new *E. coli* definition based on β-D-glucuronidase activity, and that the rapid test (using TSA/TBA) is no longer included in the standard. In ISO formulated CCA, the growth of Gram-positive bacteria, as well as certain Gram-negative bacteria, is inhibited by the presence of Tergitol®7, which has no negative effect on the growth of coliform bacteria. In a routine analysis, Gram-negative bacteria other than coliform bacteria are not inhibited sufficiently, resulting in low selectivity of the CCA media. Consequently, ISO recommended this method primarily for waters where low bacteria numbers are expected (< 100 total colonies per plate), such as drinking water. Therefore, there is an urgent need to amend Annex I of the BWD, since the reference method (ISO 9308-1) is no longer valid for bathing water monitoring, or to modify the current reference method so as to be suitable for *E. coli* enumeration in bathing water. Microbiological EN ISO standard methods for water quality monitoring purposes are being prepared by the ISO Technical Committee 147 - Subcommittee 4 (TC147/SC4). According to its recommendations, TC147/SC4 welcomes proposals from national member bodies as “preliminary work item proposals” or “new work item proposals”. The need for new method proposals has been noted especially for the ISO 9308 series (Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria), which is currently lacking a membrane filtration method suitable for bathing water *E. coli* monitoring (EMEG, 2016). With the exception of low selectivity, the use of CCA offers many advantages: rapid colony growth (even for sub-lethally injured coliform bacteria), high recovery rate, good color contrast that facilitates interpretation (especially for *E. coli*), no need for confirmation of *E. coli*, low cost, simple procedure, etc. Because of all the above-mentioned advantages, in our previous study (Vukić Lušić et al., 2016) we modified the reference method by changing the incubation temperature to 44 ± 0.5 °C, after a 4-h resuscitation period at 36 ± 2 °C. An equivalence trial was carried out following the criteria set out in ISO 17994 (2014). The modified method was compared against the reference ISO 9308-1 (2014) method. Based on the results of equivalence testing, the methods were evaluated as “not different” (equivalent). Because of the positive effect of the resuscitation period on the recovery, temperature modification did not significantly alter the results, but it greatly inhibited background bacterial flora growth and simplified the reading of the plates. Besides thermo-intolerant non-coliform bacteria, the elevated incubation temperature also inhibited thermo-intolerant coliform bacteria, without a negative effect on *E. coli* recovery. Since *E.coli* is the only coliform bacteria required for bathing water quality monitoring, temperature modification could be considered as successful. Prior to being officially proposed as an alternative method or an amendment to the ISO 9308-1 standard, performance characteristics of the temperature-modified method have to be established in accordance with the current ISO 13843 standard.

The aim of this study was to perform full validation of a temperature-modified ISO 9308-1 (2014) method and to evaluate its suitability for the enumeration of *E. coli* in coastal (marine) and inland bathing water, thus offering a choice to countries where the membrane filtration method has been traditionally used for monitoring the microbiological quality of bathing water.

2. Materials and methods

2.1. Sampling and spiking

2.1.1. Determination of relative recovery

The study was carried out in two phases. The first phase was performed in order to determine relative recovery of the temperature-modified method in coastal (marine) water samples (Vukić Lušić et al., 2016). In brief, data were obtained during the 2015 bathing season. Seven laboratories participated in the comparison trial by parallel analysis of samples using the reference and modified method. The samples were collected at > 100 official and additional sampling sites (additional monitoring), covering the entire Croatian Adriatic coastline. To obtain higher count data, one laboratory that covers the area with traditionally very clean bathing water prepared additional samples by spiking clean seawater with wastewater from different sources. To simulate environmental conditions as much as possible, glass bottles with spiked initial samples were placed in the sea (at 30 cm depth) near the official sampling sites and kept there for 3 h (9:00–12:00 AM – the period of very intensive bathing activity) in order to expose the content to natural sunlight.

The second phase was performed in order to determine relative recovery in inland water samples. Four geographically distant laboratories participated, analyzing naturally contaminated river water samples collected at 22 different sampling sites.

All samples were routinely collected in glass bottles and delivered to the laboratories in accordance with a routine procedure.

2.1.2. Establishment of the performance characteristics of the modified method

The study was carried out in the last quarter of the bathing season 2017. Because of very extensive work and relatively short period till the end of the bathing season, the study was carried out by two laboratories: the Institute of Oceanography and Fisheries (IOF) performed the study on coastal (marine) water, while the Teaching Institute of Public Health of Primorje-Gorski Kotar County (IPHR) performed the study on inland water.

All test samples, except the samples used for determining categorical performance characteristics of the modified method, were prepared in the same way. Naturally contaminated initial bathing water samples were collected in glass bottles, targeting the areas where moderate contamination was expected. In the laboratory, two decimal dilutions were prepared and analyzed (100 mL) in duplicate, using the modified method. The rest of the initial samples were stored at 4 °C overnight. After reading the plates, the concentrations of *E. coli* in the initial samples were determined by multiplying the number of *E. coli* colonies (average of two replicates of the same dilution that gave 20–80 targeted colonies per membrane) with the reciprocal value of sample dilution. To achieve targeted *E. coli* concentrations, a test sample was prepared by diluting the initial sample with clean water (seawater). Clean spring water and seawater were collected earlier, tested and kept at 4 °C. When determining the working range and recovery rate (with pure cultures) of the method, clean water was additionally sterilized by autoclaving.

2.2. Sample analysis

All samples were filtered through a 0.45 μm pore size, 47 mm in diameter membrane filters (Sartorius, Whatman) using 6-place manifold filtration apparatus. After filtration, the funnels were rinsed twice