Abstract: The most probable number (MPN) method is a culture-based method commonly used in the field of environmental microbiology to examine microbial populations in liquid substrates. The MPN tests require a wide range of special laboratory equipment, a lot of laboratory space, and skilled staff, which together limit their applicability. This paper presents a modified MPN method, which reduces the experimental requirements by applying tissue plate as a carrier. The modified MPN method introduces a fast-filled tissue plate with $5 \times 5$ squares as agar carrier, instead of the commonly used set of glass tubes. Further, self-refilling automatic syringe Socorex® was implemented to apply samples to the plate. The response of the modified MPN method was tested on eight selected bacterial strains as well as on soil and water samples. Simultaneously, all the strains and samples were tested by standard spread plate method. High linear correlation between the two methods was found, which makes a new modified MPN method a useful alternative within the field of environmental microbiology.

Keywords: culture-based methods; soil; tissue plate; water

1. Introduction

Microbial characteristics are generally considered complex indicators of soil and water health because of clear relationships between microbiome, soil/water characteristics, plant quality, and environment sustainability [1]. A thorough understanding and the effective measurability of microbial characteristics such as biomass, activity, communication, and diversity are significant to support the beneficial effects of microorganisms to plants, animals, and humans, and to prevent their adverse effects on society. Soil and water microorganisms play a key role in the decomposition of organic matter residue, determining the release of mineral nutrients and nutrient cycling [2]. Moreover, microorganisms contribute to the transformation and degradation of waste materials and synthetic organic compounds. They also influence the physical properties of soil. Therefore, microorganisms may function as an excellent indicator of change in soil health, thereby providing an early sign of soil quality improvement or an early warning of soil degradation [3,4]. Most of the research effort applied now in the field of environmental microbiology is directed at culture-independent principles, such as molecular methods, dehydrogenase activity, and respiration activity [5]. However, these culture-independent techniques often do not allow detailed investigations that are possible with cultures. Without successful cultivation, it is difficult to detect and identify novel organisms, obtain phenotypic and functional information, and determine the functions of unknown genes [6].

Thus, the culture-based methods remain fundamental tools in environmental microbiology and help us to understand the microbial world and its contribution to sustainable practices in soil and water management.
Environmental samples generally contain an enormous number of microbial cells. This is especially true for soils with a typical range of $10^7 – 10^{12}$ cells per one gram of soil dry matter. Even if less than 1% of the microorganisms observed under the microscope may typically be cultivated [7], and irrespective of rapid development of molecular or genetic examination techniques, the culture-based methods remain a fundamental tool in environmental microbiology. Cultivability is a particularly important parameter when exploring contaminated matrices and preparing bioremediation systems.

Traditional culture-based methods include the pour plate method, spread plate method, and most probable number (MPN) method. All of these methods are widely applied and supported by a broad spectrum of guidelines. Still, the technical aspects exist, in which the culture-based methods may be improved.

When spread plate method is used with more than 10 s application of hand spreader, lower counts of gram-negative bacteria are mostly determined, which is related to mechanical forces sufficient to damage the cells [8]. Another limitation is a loss of viable bacteria as a result of bacteria adhesion to the spreader (irrespective of glass or plastic). The amount of bacteria adhered to the spreader varied according to volume of liquid being spread and the size of the spreader [9]. The spreader adhesion of bacterial cells also varied according to the organism species [10]. It was estimated that the loss would be approximately 10% [9].

Pour plate method may not be suitable to cultivate microorganisms, which are sensitive to the high temperatures at which mixing of sample and medium is typically carried out.

The MPN method is generally a means to estimate microbial population sizes in liquid substrate [11]. It relies on the detection of specific qualitative attributes of the microorganism of interest (bubbles, color, precipitates). Population estimates are derived from the pattern of attribute occurrence across a serial dilution from MPN tables. This method does not rely upon the population size within any single dilution or aliquot, but rather on the pattern of positive and negative test results following inoculation of a suitable test medium. It is considered suitable to estimate microbial population sizes when quantitative assessment of individual cells is not possible [12]. The MPN method is particularly useful when an estimate of numbers present in very low concentrations is required and the large volume of samples to be examined makes the plate count impracticable [13].

The most common form of the MPN method utilizes a system of multiple tubes containing the liquid medium. However, an alternative MPN method was developed that used an agar plate inoculated with drops, and the presence or absence of growth was recorded here [11].

Obviously, the MPN method is well suited for monitoring activities that produce a high number of samples, where a broad range of population sizes is expected and a cultivation examination strategy is preferred over molecular techniques. Processing a large number of samples with a broad concentration range automatically increased requirements on laboratory space, equipment, time, and staff experience.

Thus, the aim of this study was to develop a simplified modification of the MPN method for enumeration of indigenous microorganisms in environmental samples, represented here by the samples of soil and water. The modified MPN method was, in particular, intended for routine analysis of a heterotrophic population in environmental samples, and the connection of the MPN principle with the agar medium was identified as a promising strategy. The microbial recovery of the modified MPN method developed was compared with a spread plate method.

2. Materials and Methods

The following bacterial strains were used for testing: *Gordonia terrae* CCM 2633 (A), *Enterococcus faecalis* CCM 4224 (B), *Pseudomonas fluorescens* CCM 4796 (C), *Escherichia coli* CCM 3954 (D), *Pseudomonas aeruginosa* CCM 3963 (E), *Pseudomonas aeruginosa* CCM 1961 (F), *Pseudomonas aeruginosa* CCM 7930 (G), and *Pseudomonas fluorescens* CCM 2115 (H). Capital letters in parentheses indicate the particular points presented in Figure 1. All the strains originated from Czech Collection of Microorganisms, Brno. The received strains were subcultured at least three times in nutrient broth (Oxoid, Basingstoke, UK) to gain fresh culture before the target cultivation experiments.
was homogenized by shaking on a shaker for 30 min at 100 rpm at laboratory temperature. After
places (Prague city area), samples from contaminated site with chemical industry (Neratovice, Czech
Republic), and sediment samples. Sampling procedures followed the ISO 19458:2006 standard for the
soil and sediment samples.

A total of 10 aqueous and 13 soil samples were collected (Table 1). The first group included water
from public swimming pool, samples from drinking water wells and polluted pump-and-treat wells,
household wastewater, and mine water. The second group included soil samples from uncontaminated
places (Prague city area), samples from contaminated site with chemical industry (Neratovice, Czech
Republic), and sediment samples. Sampling procedures followed the ISO 19458:2006 standard for the
aqueous samples and ISO 18400-102:2017, together with ISO 18400-206:2018 standards for the soil and
sediment samples.

Table 1. The list of analyzed samples.

| Sample | Matrix | Description; GPS Position (Czech Republic Territory) |
|--------|--------|-----------------------------------------------------|
| 1      | Surface water | Public swimming pool; N 49°56.38670′, E 14°44.05240′ |
| 2      | Surface water | Creek bellow output of biological wastewater treatment unit; N 50°12.96942′, E 14°8.43233′ |
| 3      | Groundwater | Drinking water well; N 49°56.28245′, E 14°41.84592′ |
| 4      | Groundwater | Drinking water well; N 49°28.76600′, E 15°44.22397′ |
| 5      | Mine water | Kutná Hora; N 49°38.46962′, E 15°19.96935′ |
| 6      | Groundwater (contaminated) | Pump-and-Treat system, Crystal Bohemia factory, Poděbrady; N 50°8.71315′, E 15°7.65625′ |
| 7      | Groundwater (contaminated) | Pump-and-Treat system, Crystal Bohemia factory, Poděbrady; N 50°8.71542′, E 15°7.62857′ |
| 8      | Groundwater (contaminated) | Pump-and-Treat system, Crystal Bohemia factory, Poděbrady; N 50°8.72520′, E 15°7.61672′ |
| 9      | Groundwater (contaminated) | Pump-and-Treat system, Spolana, Neratovice; N 50°16.23263′, E 14°30.93505′ |
| 10     | Groundwater (contaminated) | Pump-and-Treat system, Karlovy Vary; N 50°13.73613′, E 12°50.73683′ |
| 11     | Soil (2 m depth) | Sampling borehole, Praha; N 50°2.92988′, E 14°19.43730′ |
| 12     | Soil (4 m depth) | Sampling borehole, Praha; N 50°2.92988′, E 14°19.43730′ |
| 13     | Soil (6 m depth) | Sampling borehole, Praha; N 50°2.92988′, E 14°19.43730′ |
| 14     | Soil (2 m depth) | Sampling borehole, Praha; N 50°2.91913′, E 14°19.34815′ |
| 15     | Soil (4 m depth) | Sampling borehole, Praha; N 50°2.91913′, E 14°19.34815′ |
| 16     | Soil (2 m depth) | Sampling borehole, Neratovice; N 50°16.23263′, E 14°30.93505′ |
| 17     | Soil (6 m depth) | Sampling borehole, Neratovice; N 50°16.23263′, E 14°30.93505′ |
| 18     | Soil (4 m depth) | Sampling borehole, Neratovice; N 50°16.23963′, E 14°30.93377′ |
| 19     | Soil (3 m depth) | Sampling borehole, Neratovice; N 50°16.23963′, E 14°30.93377′ |
| 20     | Soil (2 m depth) | Sampling borehole, Neratovice; N 50°16.23963′, E 14°30.93377′ |
| 21     | Sediment (0.15 m depth) | Lagoon Dobroutov; N 49°28.43400′, E 15°45.15958′ |
| 22     | Sediment (surface) | Lagoon Dobroutov; N 49°28.43400′, E 15°45.15958′ |
| 23     | Soil (5 m depth) | Sampling borehole, Neratovice; N 50°16.23963′, E 14°30.93377′ |

Figure 1. Regression line of most probable number (MPN) drop agar method counts vs. spread plate
method counts for eight selected strains.

Strains were cultivated at 22 °C for 2–3 days in nutrient broth before use. All strains were stored
at 4 °C and maintained at −20 °C in 30% v/v of glycerol.

All serial dilutions were performed using sterile saline (0.85% NaCl, ISO 8199:2007). The samples
of bacterial strains in nutrient broth and the collected aqueous samples were vortexed for 10 s. Soil
and sediment samples were processed as follows: 1 g of sample was introduced into glass reagent
bottle with glass stopper containing 9 mL of sterile saline with 15 glass balls of diameter 5 mm. Sample
was homogenized by shaking on a shaker for 30 min at 100 rpm at laboratory temperature. After
homogenization of samples, the serials of decimal dilutions were prepared.
Microbial counts were determined by cultivation on nutrient agar (Oxoid, Basingstoke, UK) using two methods: (1) spread plate method and (2) MPN drop agar method. Appropriate dilutions were regularly spread on pre-dried nutrient agar surface of a 9 cm plate by hand plating with a sterile bent glass rod until dry. Each dilution was plated in duplicate. The inverted plates were cultured at 22 °C. Time of incubation varied with the organism and type of sample tested. At the end of the incubation period, the grown colonies were enumerated and total count was calculated according to ISO 7218:2007.

Instead of classic tubes with liquid medium [13], a modified MPN method was applied with 5 × 5 polystyrene tissue plate as a carrier of media. Melted nutrient agar was poured in every square section of 5 × 5 polystyrene tissue plates using sterile self-refilling automatic syringe Socorex®. The volume of 1.5 mL was loaded in every square section (1.8 cm²). Pre-dried and pre-warmed nutrient agar was inoculated with 30 µL drops in the manner that five squares in line were used for each dilution level. The plate was tilted at an angle of about 30° to regularly distribute the poured dilution over the whole surface agar in the squares. The plates were inverted after sample drops had been absorbed into the agar and then incubated at 22 °C. Time of incubation varied with the bacterial strain and type of sample tested. The number of positive squares sections (where colony growth was observed) was recorded after incubation, and MPN index was calculated according to ISO 7218:2007.

Colony counts for both MPN drop agar method and spread method were transformed into Log10 values and interpreted, for each type of experiment, through linear regression model. The agreement between the experimental data provided by the two methods was verified here using the Student t test for paired samples at the 5% level of significance.

3. Results and Discussion

The first part of the results measured in this work is presented in Figure 1, where correlation between the counts of microbial strains obtained by MPN drop agar method and spread plate method for eight selected strains is shown. The results presented here cover a relatively broad range of concentrations of CFU, as evidenced by more than three orders of magnitude through which the experimental data were distributed. All the strains presented in Figure 1 are the typical subsurface or water inhabitants with high importance to the field of environmental science and technology [14].

Genus Pseudomonas was selected because it is known for swarming growth, especially at Pseudomonas aeruginosa [15]. This genus belongs to the group of soil generalists, and it is found, often in high numbers, in virtually every soil on the planet [16]. Pseudomonas is among the dominant culturable bacteria in soil [17].

Gordonia strain was included because it belongs to Actinomycetes. Actinomycetes is a nontaxonomic term for a group of the Gram-positive bacteria that exhibit filamentous growth and spore formation. Gordonia has been isolated not only from soil [17,18], where it is very common, but also from water. They have also been isolated from wastewater treatment bioreactors or biofilters. Gordonia degrade natural organic compounds that are not readily biodegradable, and are candidates for bioremediation processes (hydrocarbon degradation). Several species of Gordonia are known to cause infections, especially in humans [18]. Gordonia and Pseudomonas are known as biosurfactant producers, which is very important if hydrophobic organic pollutants are present.

Finally, Enterococcus and Escherichia strains are indicators for feces derived from human and animals and Enterococcus is an indicator for a “not so fresh” or recent contamination. Escherichia coli can also exhibit swarming growth [19].

Selection of the bacterial strains presented in Figure 1 followed, however, not only from their environmental importance but also from good cultivability and identifiability.

The value of correlation coefficient calculated for Figure 1 clearly indicates strong linear relationship between the two detection methods. Thus, for the eight bacterial strains, the results provided by modified MPN method were interchangeable for the results of a comparative spread plate method.
Figures 2 and 3 show correlations between the counts from environmental samples (listed in Table 1) obtained by a new MPN drop agar method and spread plate method. For all samples, there were no significant differences between these two methods. The correlation coefficients evidence again very high linear relationship between the two methods.

![Figure 2. Regression line of MPN drop agar method counts vs. spread plate method counts for 10 water samples.](image)

For all three types of experiments shown in Figures 1–3, the Student t-test for paired samples showed no significant difference between the colony counts at the 5% level of significance.

The applicability of the modified MPN drop agar method, as presented in this paper, might be limited if longer cultivation time is required, for example, in order of weeks. In such a case, we must often face a medium dehydration similar to traditional spread plate method.

The modified MPN drop agar method developed is fast to perform because of easy filling of squares with the autoclavable Socorex® self-refilling syringe with spring-loaded plunger and three-way valve system designed for serial precision injections. Melted agar can be poured into one plate divided into 25 square sections within 50 s. Moreover, the MPN drop agar method estimates results fast because only square sections giving visible growth are noted, rather than colony counting. However, the most important benefits are obtained in time required for sample handling, cost of material, and occupation of space in laboratory. This method is applicable for organisms with a swarming growth and a mycelial growth.
growth. The swarming growth was reported in some rhizobial strains [20], or in some strains with biosurfactant production used in bioremediation [21].

In this way, the modified MPN drop agar method provides significant benefit compared to current state of art [11,22–25], compared especially with drop agar methods cited in the literature. There, 96-well tissue plate was obviously used for dilution preparation and drops with dilutions were inoculated to one plate [26–28].

Material and cost assessments of both the modified MPN drop agar method and comparative spread plate method are shown in Tables 2 and 3. Table 2 summarizes consumable material required to examine one sample, where the MPN method is carried out in one tissue plate (5×5), while ten Petri dishes are considered for the comparative spread plate method. Table 2 further shows space and time requirements but does not include stable laboratory instrumentation (such as incubator, autoclave, and Vortex mixer) whose utilization is the same for the both techniques (the only difference being space volume occupied). The actual costs for consumable material collected for one Czech and one global supplier are summarized in Table 3.

**Table 2.** Calculation of material and labor requirements for the determination of heterotrophic microorganisms used for one sample by both methods.

| Method | MPN Drop Agar Method | Spread Plate Method |
|--------|----------------------|--------------------|
| **Nutrient agar (mL)** | 37.5 \(^a\) | 150 \(^b\) |
| **Quantity of carriers** | 1 tissue plate (5×5) | 10 Petri dishes |
| **Additional material** | 1 tip for pouring medium | 5 spreaders |
| **Dimensions of the plate with lid (mm)** | 20×101×101 height×width×depth | 90×160 diameter×height (10 plates) |
| **Space occupied (cm\(^3\))** | 202 | 1017 |
| **Weight of plates (g)** | 46.6 | 126.8 |
| **Mean weight of additional material (g)** | 5 | 9 |
| **Mean handling time \(^c\) (min)** | 5 | 20 |

\(^a\) for a set of 5 replicates 5 dilutions, 1.5 mL per square. \(^b\) for a set of 2 replicates 5 dilutions, 15 mL per plate. \(^c\) labeling of plates, absorption of water of the inoculum or spreading, and counting of plates.

**Table 3.** The actual costs for consumable material obtained from Thermo Fisher Scientific Inc. company and P-LAB company.

| Method | Carrier | Medium/per Sample/Unit | Additional Material | Sum |
|--------|---------|------------------------|---------------------|-----|
| MPN drop agar method | 2.3 EUR/1 tissue plate | 0.2 EUR/37.5 mL \(^a\) | 0.3 EUR/1 tip | 2.8 EUR |
| Spread plate method | 2.1 EUR/10 Petri plates | 0.8 EUR/150 mL \(^b\) | 1 EUR/5 spreaders | 3.9 EUR |
| MPN drop agar method | 1.5 EUR/1 tissue plate | 0.2 EUR/37.5 mL \(^a\) | 0.4 EUR/1 tip | 2.1 EUR |
| Spread plate method | 1.0 EUR/10 Petri plates | 0.8 EUR/150 mL \(^b\) | 1 EUR/5 spreaders | 2.8 EUR |

\(^a\) for a set of 5 replicates 5 dilutions, 1.5 mL per square. \(^b\) for a set of 2 replicates 5 dilutions, 15 mL per plate.

Comparison of the costs showed in Table 3 clearly evidenced direct economic benefit related to the modified MPN method, irrespective of national or global supplier of laboratory consumables. Significant time saving is another important advantage of the modified MPN method. Reduction in time demand is in particular connected with labeling of plates, absorption of water of the inoculum or spreading, and the counting of plates.

The economy of microbiological examination is also greatly influenced by indirect costs, which include the waste disposal expenses. All the waste materials from microbiological examination are, according to the international standards (ISO 7218:2007), autoclaved first, then stored in single-use plastic containers, and finally directed to waste incinerator. The weight reduction indicated in Table 2 for consumable material in the MPN method is of high logistic, environmental, and economic value.

The outputs presented in this paper contribute to the goals of the sustainable development strategy by:
- Providing a modified method that may be applied to microbiological examination of water and soil samples in simplified arrangement and at lower cost, thus preventing soil degradation and encouraging sustainable agricultural development;
- Reducing the amount of single-use plastic consumable material used within a laboratory microbiological examination;
- Reducing the amount of biohazardous waste, whose handling is typically related to complicated and expensive disposal procedures.

4. Conclusions

New modification of the MPN method was applied to enumeration of indigenous microorganisms in soil and water samples. The results measured by modified MPN drop agar method were highly correlated with the results from common spread plate method, which was used here as a standard cultivation technique. Besides providing comparable results with a common technique, the modified MPN drop agar method is advantageous due to lower costs, decreased requirements for laboratory space and equipment, and less staff time. All these aspects make a modified MPN drop agar method well-suited for extensive monitoring activities, where high numbers of samples are to be examined by means of the microorganism cultivation method. In a broader sense, better microbial control and reduced risks from contaminated soil and water can be reached this way.

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