J Ind Microbiol Biotechnol (2011) 38:241–247
DOI 10.1007/s10295-010-0819-1

ORIGINAL PAPER

Automated measurement and quantification of heterotrophic bacteria in water samples based on the MPN method

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Received: 24 March 2010 / Accepted: 26 July 2010 / Published online: 12 September 2010

Abstract Quantification of heterotrophic bacteria is a widely used measure for water analysis. Especially in terms of drinking water analysis, testing for microorganisms is strictly regulated by the European Drinking Water Directive, including quality criteria and detection limits. The quantification procedure presented in this study is based on the most probable number (MPN) method, which was adapted to comply with the need for a quick and easy screening tool for different kinds of water samples as well as varying microbial loads. Replacing tubes with 24-well titer plates for cultivation of bacteria drastically reduces the amount of culture media and also simplifies incubation. Automated photometric measurement of turbidity instead of visual evaluation of bacterial growth avoids misinterpretation by operators. Definition of a threshold ensures definite and user-independent determination of microbial growth. Calculation of the MPN itself is done using a program provided by the US Food and Drug Administration (FDA). For evaluation of the method, real water samples of different origins as well as pure cultures of bacteria were analyzed in parallel with the conventional plating methods. Thus, the procedure described requires less preparation time, reduces costs and ensures both stable and reliable results for water samples.

This article is part of the BioMicroWorld 2009 Special Issue.

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Keywords Heterotrophic bacteria · Most probable number (MPN) · Automated measurement · Water analysis

Introduction

The heterotrophic plate count (HPC) is a relevant and widely used analytical tool in water quality assessment [1, 12] despite the advancing developments of molecular biological methods [2, 18, 21, 29]. For day-to-day drinking water analysis, specific tests for pathogenic microorganisms such as *E. coli, Legionella* spp. and *Mycobacterium* spp. tend to replace the HPC, but for industrial applications, particularly for validation and verification of water treatment procedures, determination of HPC bacteria is an important tool [27]. Furthermore, for establishing and testing of new methods, the HPC serves as a reference method [12].

Basically, the method for determination of HPC bacteria has not changed for more than 100 years. At that time, Robert Koch postulated how to cultivate bacteria on solid media and set the limit for tolerable contamination of drinking water to 100 CFU (colony-forming units)/ml. In addition, the pour plate method is still the official reference method for the enumeration of cultivable microorganisms for drinking water analysis [7, 12]. However, the pour plate method comes along with several difficulties such as the possibility of heat shock when psychrophilic bacteria are covered with molten agar [24, 25], uneven sample distribution from delayed mixing of sample and media, or uncertainties of inexperienced users when reading the plates. In general, the pour plate method is labor-intensive in preparing, and handling has to be carried out by well-trained personnel. This induces the need for alternatives such as the spread plate method, which also uses

agar-based media for cultivation, and the MPN (most probable number) method using liquid media.

The MPN is a cultivation technique to estimate the number of cultivable bacteria based on statistical analysis. According to the European standard EN ISO 8199 [9], it can be used to count microorganisms in water samples. The MPN method has been widely used and adapted for multiple applications since it was first described [20]. Recently, the use of micro-titer plates for incubation has become exceedingly popular. Rowe et al. [22] stated that the MPN in micro-titer plates showed a significant correlation to the tube technique. This leads to the conclusion that the micro-titer plate method can be used in place of the tube technique, which is also mentioned in the European standard EN ISO 8199 [9].

There are MPN methods using micro-titer plates for quantification of thermophilic bacteria [3], fecal coliforms [16, 19], hydrocarbon-degrading bacteria [5, 15, 28], denitrifying microorganisms [11], anaerobic denitrifiers [23], and many more. Walser [26] described an MPN method for aerobic mesophilic count, *E. coli* and Enterococci in 96-well plates using a chromogenic growth indicator as a basis for photometric detection and a pipetting robot for loading of the samples. A drawback of this method is the limitation of the bacterial load to a maximum of 2×10^4 cells per ml, which would prevent the analysis of surface waters and many groundwaters. Sartory et al. [24] compared the commercially available kit Quanti-Disc™ (IDEXX Laborartories, Westbrook, ME, USA) to the reference method for enumeration of cultivable microorganisms, EN ISO 6222 [7]. The Quanti Disc™ system is based on the MPN method and three fluorogenic enzymes are used for detection of positive signals for microbial growth [24]. As they mainly analyzed chlorinated drinking water samples, one can assume that this method is also not suitable for higher contaminated waters.

The objective of the present study was to find a method for measurement of cultivable bacteria of various sources of water, including bottled water, tap water, groundwater and different kinds of surface waters. Another important criterion was to ensure that this method complies with the standard method according to the European Drinking Water Directive, but overcomes complications that may occur during implementation of the pour plate method [12]. Furthermore, it was desired to enable high throughput of the method using limited laboratory space and facilities. Additionally, it shall be less user-dependent in terms of conducting the procedure as well as data analysis. Thus, potential sources of errors shall be minimized as well as preparation time, reagents and incubation space.

In this paper, a method for total cultivable bacteria count is described and tested, which uses 24-well micro-titer plates for testing of water samples. Assessment of microbial growth is not performed visually but photometrically by measurement of absorbance using a micro-titer plate reader. In compliance with a certain threshold level, data are transformed into positive or negative signals for microbial growth and can then be analyzed with a software tool for computation of the MPN [4], which also provides basic statistics and allows great flexibility concerning sample volume, number of dilutions, and replicates within certain dilutions [13].

Materials and methods

Cultivation techniques

Pour plates and spread plates

Preparation of pour plates as well as spread plates was conducted according to the current European standard for enumeration of microorganisms by culture, EN ISO 8199 [9]. The test volumes used for pour plates were 1-ml aliquots (or the same volume of certain dilutions), whereas for the spread plates 100-µl aliquots were used. After incubation, reading of the plates was in all cases independently done by two experienced laboratory staff members.

MPN

The MPN method was done in 24-well titer plates using 1-ml sample aliquots or corresponding dilutions and 1-ml aliquots of double concentrated cultivation media. After preparation of the dilution series (usually tenfold dilutions until 10^{-7}), media was provided in each well of the titer plates by using a dispenser for exact and swift distribution of the media. Then, the diluted samples were again vigorously mixed and pipetted into the corresponding wells. After incubation, visual evaluation of microbial growth was in all cases independently done by two laboratory staff members.

Sample material, dilution series, incubation conditions and reagents

Sampling of groundwater as well as surface water and drinking water was done according to the European standard [8]. For testing of pure cultures, overnight cultures of *Bacillus* ssp. and *Alcaligenes* ssp. were grown in single concentrated yeast extract media (6 g/l tryptone, 3 g/l yeast extract) at 36°C shaking. Blank samples consisted either of autoclaved double-distilled water or various diluents.

The composition of all reagents used were in accordance with the European standards EN ISO 6222 [7] and EN ISO 8199 [9].

For the establishment of dilution series, peptone water (1 g/l tryptone) as well as a salt solution (0.85% NaCl) were used as diluents. The diluent was provided in 50-ml centrifugation tubes by use of a dispenser to ensure an exact dilution ratio of the samples. Depending on the number of desired replicates for the MPN, the volume of the dilutions was adjusted correspondingly. For 3 or 6 replicates, 10 ml were prepared, whereas for 12 and 24 replicates the volumes were 20 and 40 ml, respectively. As peptone water is required as a diluent for the reference method [7], after preliminary tests with the salt solution, which showed no difference in results (data not shown), peptone water was solely used for further analyses.

Yeast extract agar (6 g/l tryptone, 3 g/l yeast extract, 15 g/l agar) [7] was used as a cultivation media for pour plates as well as spread plates; for the MPN, double concentrated liquid yeast extract media, composed of 12 g/l tryptone and 6 g/l yeast extract, was used, which is also in accordance with the current European standard EN ISO 8199 [9].

Incubation conditions were the same for all samples and all methods, as are according to the drinking water directive at $36 \pm 2°C$ for 44 ± 4 h and at $22 \pm 2°C$ for 68 ± 4 h.

In order to detect the possibility of cross contamination, blank samples and 1-ml aliquots from pure cultures were distributed randomly on 24-well titer plates which were incubated at $36 \pm 2°C$ for 5 days and never showed any contamination at all.

Measurement parameters

OD (optical density) measurement was done with a micro titer plate reader (Tecan Spectrafluor Plus) which was positioned in a laboratory air-conditioned to $22 \pm 1°C$. The measurement mode absorbance was selected, the measurement filter wavelength was 610 nm and no reference filter was used. The number of reads was set to 1 as well as the number of flashes per read. When the shaking option was used, shaking duration was set to 10 s in orbital mode at intermediate intensity with 2 s of settle time preceding the measurement.

Computation of MPN and statistics

The computation of the MPN was done according to the US Food and Drug Administration online manual Appendix 2: Most Probable Number from Serial Dilutions [4].

Statistics were compiled using SigmaPlot software (release 9.01; Systat Software).

Results

Threshold level

The first important step for the establishment of the new detection method was the definition of a threshold level to distinguish between sterile wells and wells with microbial growth. Incubation and measurement of more than 800 blank samples showed that their absorbance values never exceeded 0.045 OD. The mean of the collected blank measurements was 0.034 OD with a standard deviation of 0.002, regardless of whether peptone water, the salt solution, water or nothing at all had been used for inoculation of the media. The box plot in Fig. 1 shows the distribution of these blank values. Thus, the threshold level was set to 0.05 OD and the data were tested against 2,500 collected values designated as positive for microbial growth. A normality test confirmed the normal distribution with a p value of <0.0001 for both datasets, respectively. The results of an independent t test stated a p value of 0 and a Student's t statistic of -44.9273, which confirms the validity of the assumption of 0.05 OD as a threshold level. Experiments with different kinds of cultivation media showed that this threshold level is applicable for many commercially available media, provided they are clear. Varying the adjustable measurement parameters of the photometer did not show to have any effects on the outcome (data not shown), thus the quickest way of measuring was chosen. Furthermore, homogenization by shaking of the plates using the shaking function of the photometer immediately before measuring did not have any effects on the OD values (data not shown). The only thing really

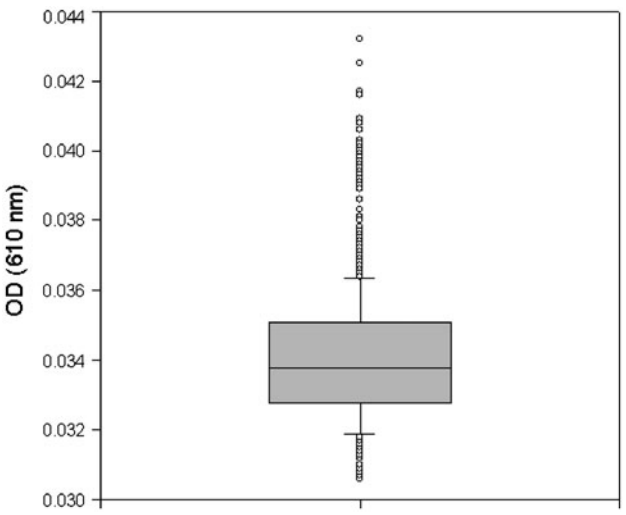

Fig. 1 Box plot of 864 blank samples measured at 610 nm showing the median, 25 and 75th percentiles (*boundaries* of the *box*) and *whiskers* indicating the 10 and 90th percentiles. Outlying points are shown as *circles*

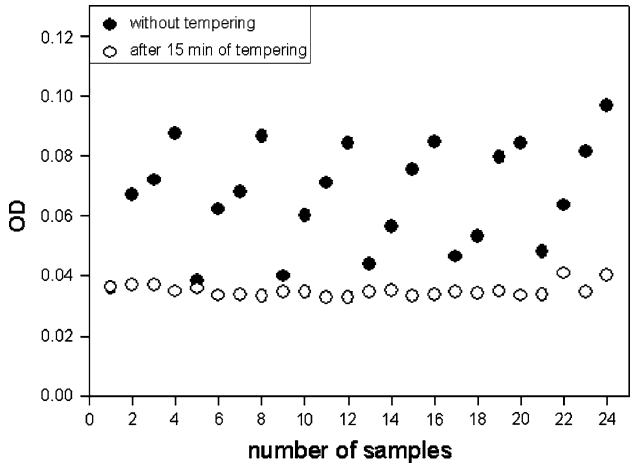

Fig. 2 Scatter plot of OD values at 610 nm of 24 blank samples measured immediately after incubation at 36°C (*filled circles*) and after 15 min of tempering at 22°C (*circles*)

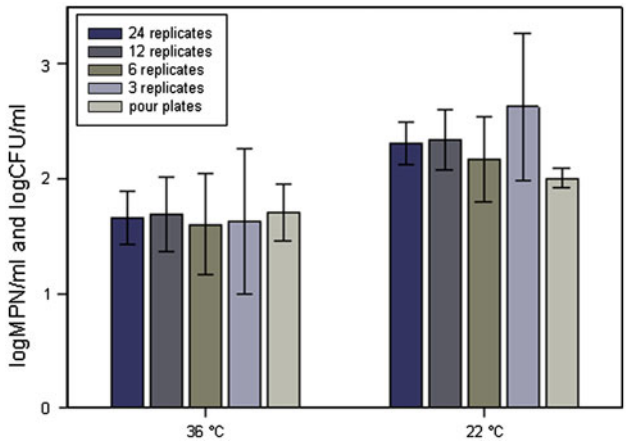

Fig. 3 LogMPN and logCFU values with 95% confidence intervals of a groundwater sample with differing numbers of replicates, after incubation at 36°C as well as 22°C

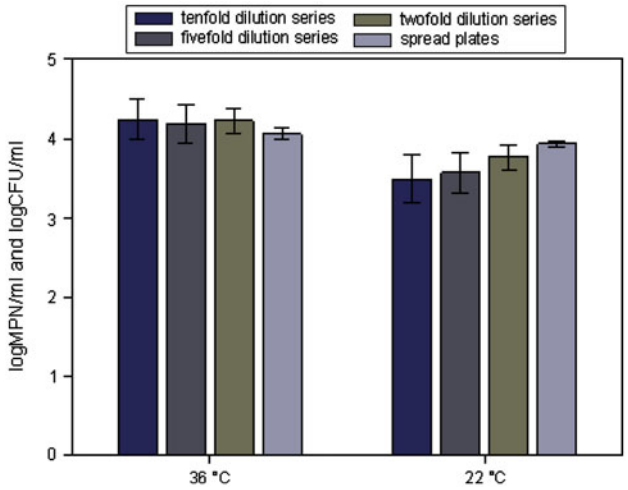

Fig. 4 LogMPN and logCFU values with 95% confidence limits of a surface water sample, diluted at different ratios, after incubation at 36 and 22°C

affecting the outcome was the temperature of the plates when measuring their ODs. Blank samples incubated at 36°C showed great scattering whereas those incubated at 22°C did not. It turned out that cooling the plates to the temperature of the photometer solved that problem, which is illustrated in Fig. 2.

Different numbers of replicates

Figure 3 shows the results of a typical groundwater sample experiment, which was done in parallel with the pour plate method as a reference. The MPN was done with 24, 12, 6 and 3 replicates per dilution in a tenfold dilution series starting from the original sample until 10^{-7}. The pour plates were done in 5 replicates using 1-ml aliquots of the original sample. All samples were incubated at 36°C as well as 22°C. Figure 3 demonstrates that the 95% confidence limits of the logMPN/ml values decreased with increasing numbers of replicates per dilution but were in all cases higher than the confidence limits of the pour plate method. In numerous experiments, the MPN yielded significantly higher bacteria counts at 22°C than the pour plate method. Due to that effect, and the high effort involved in the production of pour plates, for further experiments the spread plate method was used as a reference.

Dilution series with different ratios

To enhance the accuracy of the MPN method, it was tested using different dilution ratios in parallel with spread plates as a reference. The number of replicates for the experiment presented in Fig. 4 was 12, whereas the spread plates were done in triplicates. Figure 4 shows the effect of the different dilution ratios on the 95% confidence limits of the logMPN values. The 95% confidence limits for the incubation temperature 36°C did not differ significantly for tenfold (0.26) and fivefold (0.25) dilution series, whereas they were significantly lower for twofold dilutions (0.15). At 22°C there was an apparent difference in 95% confidence limits for all three dilution ratios, being again highest for the tenfold dilution series and smallest for the twofold dilution series. However, confidence limits of the plating technique were in both cases substantially lower.

Visual versus photometric detection

For several series of experiments, detection of microbial growth was done visually as well as by photometric absorbance measurement at 610 nm. In most cases, visual

Table 1 Visual assessment of microbial growth in 24-well titer plates compared to photometric detection at 610 nm as a reference in absolute numbers and percentages

Visual observation	OD measurement (600 nm)	
381 positive (100%)	375 positive (98.4%)	6 negative (1.6%)
276 negative (100%)	25 positive (9.1%)	251 negative (90.9%)

detection was in agreement with the OD values (Table 1). In some cases, samples actually positive for microbial growth would not have been perceived as turbid without comparison to the measured OD values. Less frequently, wells seemed to be slightly turbid at first sight, but could be identified as negative after the photometric evidence of the values ranging below the threshold level. Table 1 shows that careful visual detection without the aid of OD values would not lead to a large error, but can still be misleading. As execution of the visual detection was both user-dependent and tedious, further experiments were evaluated by photometric measurement at 610 nm only.

MPN of pure cultures

Pure cultures of *Alcaligenes* ssp. and *Bacillus* ssp. were grown overnight in liquid yeast extract media and then counted using a Thoma counting chamber of 0.01 mm depth. Due to the high cell numbers (Table 2), a tenfold dilution series was made until 10^{-11}. The MPN was done in 12 replicates per dilution. For the spread plate method, dilutions 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were plated in triplicates each. The results of this experiment are presented in Table 2.

Discussion

For the establishment of this MPN method with photometric detection of microbial growth in 24-well titer plates, experiments with blank samples, real water samples as well as pure cultures of waterborne bacteria were done. Experiments with blank samples showed that the corresponding OD values all ranged distinctly below the defined threshold level of 0.05 OD as long as the plates were allowed to temper (Fig. 1). The cooling of the plates to the temperature of the photometer turned out to be essential as the extent of scattering could not be predicted or compensated (Fig. 2). Adaptation of the threshold level to 0.10 OD would classify wells as negative, which could be clearly identified as positive for microbial growth by visual examination. The outliers, which can be seen in Fig. 1, could result from insufficient tempering of the plates preceding the measurement, or from finger prints on the bottom of the plates which could also lead to an increase in OD. However, these deviations are small enough to remain beneath the defined threshold level and have no impact on its validity (Fig. 1). Normal distribution of both the negative and the positive group of OD measures allowed the implementation of a *t* test which confirmed the hypothesis for both groups being significantly different and thus the threshold level to being valid.

The MPN method cannot quantify absolute numbers of bacteria present in water samples, as do cultivation-independent methods [2, 18, 21, 29], but it can estimate the density of microorganisms cultivable in the respective media without direct counting [4, 10, 13, 20, 30]. Although cultivation-independent methods are known to include a much greater diversity of microorganisms, official government guidelines [12, 27] require bacteria counts based on cultivation techniques such as the MPN or plating methods. Contrary to the MPN, plating techniques offer the advantage of easy access to the grown colonies for isolation of bacteria and establishment of pure cultures with subsequent identification of bacterial strains. In addition, the MPN is known to have a lower precision than direct counting methods [10, 25], which was reproduced in the present study (Figs. 3 and 4). Nevertheless, we think that the complex and tedious handling in preparation of the tests, especially for numerous samples as well as difficulties in reading of the plates, place the MPN method in favor of the plating techniques. The lower precision of the MPN results, as observed in our experiments (Figs. 3 and 4), can be improved either by increasing the number of replicates per dilution (Fig. 3) or by narrowing the dilution ratios (Fig. 4). We do not recommend the implementation of an MPN with only 3 replicates per dilution because of the large 95% confidence limits (Fig. 3), although it fulfills its purpose when only a rough estimate of the heterotrophic bacteria count is needed.

The higher bacteria count for 22°C than for 36°C (Fig. 3) is a phenomenon which was observed regularly when analyzing groundwater samples and is quite plausible considering that groundwater habitats usually have a

Table 2 Comparison of direct cell count and MPN with spread plates as a reference

Organism	Cells/ml	MPN/ml		Spread plate/ml	
		36°C	22°C	36°C	22°C
Alcaligenes ssp.	2.5×10^7	3.6×10^6	3.1×10^6	3.3×10^6	4.2×10^6
Bacillus ssp.	4.9×10^8	2.1×10^7	1.7×10^7	2.8×10^7	2.2×10^7

constant but rather low temperature [14, 17] and groundwater microbes are well adjusted to these conditions [14]. This fact also accounts for the better results of the spread plate technique for groundwater samples compared to the pour plates because isolated cultures of these microorganisms often showed no growth at all at 36°C incubation temperature (data not shown) and are probably killed instantaneously when covered with molten agar of more than 40°C [24, 25].

The results obtained from direct cell counts of pure cultures of *Alcaligenes* ssp. and *Bacillus* ssp. are significantly higher than the results obtained by the MPN and the plating method (Table 2). A possible reason could be that many microorganisms had already entered death phase. However, this phenomenon was also observed by others [30], and multiple repetition of the experiment always resulted in similar numbers (data not shown).

As not only drinking water samples, but also groundwater and surface water samples, need to be analyzed, a wide detection range with many dilution steps analyzed at a time is a prerequisite clearly fulfilled by our method but which would not be possible using the IDEXX kit [24] or Walser's approach [26]. Additionally, photometric measurements eliminate user-dependent interpretation concerning the perception of turbidity. Unbiased and reproducible estimations of total cultivable bacteria counts are achieved without the need of certain enzymes or chromophores as markers [24, 26].

The use of 24-well plates instead of 96-well plates allows analyses of 1-ml sample aliquots. This permits direct comparability to the pour plate method and thus to the stipulations of the drinking water directive [12]. The very low sample volumes used for analyses in 96-well plates, which can be as small as 1 nl per well or even less [6] cannot be representative for low contaminated water samples containing just a few CFU/ml and thus must inevitably lead to biased results [30]. Furthermore, handling of 96-well plates may prove difficult without the use of pipetting robots or other automated devices [6]. Visual evaluation of microbial growth is nearly impossible for 96-well plates whereas it is still feasible when using 24-well plates, which was also described by Brown and Braddock [5] for enumeration of oil degrading microorganisms.

Our MPN method in 24-well plates requires less laboratory training than the ordinary MPN or plating techniques; it is less labor intensive and produces operator-independent results. Based on observations in our laboratory, we conclude that the detection of bacterial growth via OD measurement is much faster and more reliable than visual assessment of turbidity or counting methods. However, visual evaluation of microbial growth is still practicable if performed carefully and against a dark background (Table 1). For the past year, the MPN in 24-well titer plates with photometric detection and computation with the BAM MPN program [4] has been used for routine analysis of water samples of various origins in our laboratory. Adaptation of this method for analysis of Coliforms, other primary indicators or pathogens is feasible using appropriate media and incubation conditions.

Acknowledgments This work was co-financed by the Federal Country Lower Austria and by the European Regional Development Fund (ERDF) of the European Union.

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