

**DEVELOPMENT OF A MODIFIED  
MPN PROCEDURE TO  
ENUMERATE IRON OXIDIZING  
BACTERIA**

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**DEVELOPMENT OF A MODIFIED MPN PROCEDURE TO  
ENUMERATE IRON OXIDIZING BACTERIA**

**Final Report by**

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### ABSTRACT

Iron oxidizing bacteria are microorganisms difficult to enumerate using conventional bacteriological methods. A modification of the most probable number (MPN) procedure was developed to improve their enumeration. Assays were conducted with *Thiobacillus ferrooxidans* strains which all showed comparable results. In the dilution range tested, a good correlation was observed between direct microscopic counts and the modified MPN method. The detection limit of the proposed procedure is less than 100 bacterial cells per ml.

### RESUME

Il est relativement difficile d'énumérer les microorganismes ferroxidants en utilisant les techniques bactériologiques conventionnelles. Nous avons mis au point une méthodologie basée sur une modification de la technique normale de la détermination du nombre le plus probable (NPP). Toutes les souches de *Thiobacillus ferrooxidans* employées lors des essais ont démontré des comportements physiologiques comparables. Nous avons observé une excellente corrélation entre l'énumération visuelle microscopique et l'énumération obtenue en utilisant la méthode NPP modifiée. Nous proposons donc une méthode où le seuil de détection est inférieur à 100 bactéries par ml.

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## Introduction

*Thiobacillus ferrooxidans* is an acidophilic, chemolithotrophic bacterium which is well known because of its role in production of acidic mine drainage (1). The autotrophy of this organism has prevented the use of standard bacteriological methods, such as the viable plate counts for direct enumeration. This limitation has been overcome in the past by the use of non specific counting methods, such as biomass estimation. Recent advances in standard plate count techniques have permitted the measurement of viable counts of this organism and complemented the previously used procedures.

Until now, direct counting methods have included agar plating, floating filters, the Petroff-Hauser counting chamber and immunological assays. Agar plate colony counts have been used in several studies (2-10); this method requires 1 to 2 weeks of incubation. Original media were only 50%-80% effective when compared with liquid counts (Petroff-Hauser counting chamber) (1). The variability in results can be explained by the toxicity of gel components and subsequent methods were modified accordingly. In order to minimize the problem of agar gel toxicity, de Bruyn *et al* (11) suggested the use of floating filters. Microscopic enumeration, using a Petroff-Hauser counting chamber, does not distinguish between live and dead bacteria and accurate counts are difficult to obtain when the suspension contains fine particles. Fluorescent antibody staining and dot-immunobinding assays have been developed to detect *T. ferrooxidans*, as well as many microorganisms different from thiobacilli that may be present (12-14).

Indirect quantitative methods used for *T. ferrooxidans* include different types of measurements such as nitrogen, protein, ATP, etc, meant to evaluate the bacterial biomass (15-18). None of these methods is specific for *T. ferrooxidans* and most of these are more or less correlated to cell viability. The conventional most probable number (MPN) method requires at least a three week incubation period and underestimates the overall population. For example, only 0 to 21% of the total population evaluated by epifluorescent microscopy could be recovered by MPN (13-14).

The present study reports on a modified method for obtaining viable cell counts for *T. ferrooxidans*, which associates the presence of one or several bacteria with the oxidation of ferrous iron. The modified method uses the same basic principle as the conventional MPN assays in which the ferrous iron concentration of the 9K media is reduced from 161 mM to 80  $\mu$ M. The detection of ferrous iron is carried out using 1,10 phenanthroline indicator. The main advantage of this method is the significant decrease of the incubation period, also accomplished by reducing the growth medium volume from 10 to 0.1 ml. The method was tested with 3 strains of *T. ferrooxidans* and the collected data were compared with cell counts obtained using solid media and a Petroff-Hauser counting chamber.

## Materials and methods

### Chemicals

All chemicals, including the 1,10 phenanthroline indicator, were A.C.S. reagent grade. Tryptone soya broth was from Difco (Detroit, MI). Type 1 low EEO agar (Sigma Chemical Co., St. Louis, MO) was used as gelling agent for solid cultures.

## Culture

The *T. ferrooxidans* strains used were: MULB 1001, MULB 1002 and MULB 1003. Pure cultures of each strain were obtained from isolated colonies after two successive platings on FeTSB media (4). The pure strains were routinely maintained in 9K liquid medium (19) at pH 2.0. One hundred ml of sterile medium were distributed into 500-ml Erlenmeyer flasks, inoculated with 1 ml of culture and incubated at 30°C on a gyratory incubator shaker at 200 rpm. Strains were re-inoculated after complete oxidation of the media in order to maintain them in exponential phase of growth. Bacterial counts were carried out on 25 ml of original culture centrifuged for 5 min at 1000 x g, in order to eliminate ferric iron precipitates.

## Counting methods

Three counting methods were conducted in parallel: 1. direct microscopic observation using a Petroff-Hauser counting chamber, 2. agar plate counts and 3. MPN counts in liquid medium.

Direct counts with the Petroff-Hauser counting chamber were done following the manufacturer's instructions with cells suspended in liquid 9K medium.

The modified MPN method was performed in 96 well microtitration plates. Original cultures of *T. ferrooxidans* were diluted to obtain cell suspensions of 18, 16, 14, 12, 10, 8, 4 and  $2 \times 10^4$  cells ml<sup>-1</sup>. The cell suspension density was determined from direct microscopic counts. Dilutions were made in a salt solution, pH 2.0, corresponding to the formulation of the TSM media (3), to which FeSO<sub>4</sub> was added at a final concentration of 80 μM (Fe<sup>2+</sup>). Cell suspensions were serially diluted 7 times (ten fold). Using a multichannel micropipette, 0.1 ml replicates of each dilution were pipetted into a row of the microplate. Each microplate therefore contained 12 replicates of 8 dilutions. The experiment was carried out in three sets of triplicates at 30°C during 7, 10 and 14 days respectively in a static incubator. Complexation of ferrous iron with the indicator produces an orange color at the concentration used (80 μM). Therefore, the oxidation of ferrous iron to ferric form, in a positive reaction, is visually indicated by the absence of color upon addition of 50 μL of 1,10 phenanthroline (1 g L<sup>-1</sup>). As each microplate well was considered as an individual tube, MPN was determined from published tables (20).

Validation of microplate counts was done in parallel using agar plate counts with FeTSB and TSM media. The TSM medium was modified by increasing the amount of agar used from 5 to 7 g L<sup>-1</sup>. A 10 μL aliquot of the appropriate dilution was spread onto the plate with a glass rod. Colonies were counted visually after 7, 10 and 14 days of static incubation at 30°C.

## Results

Direct visual counts in a Petroff-Hauser counting chamber on the bulk liquid culture medium gave the following results for the *T. ferrooxidans* MULB 1001, 1002 and 1003 strains:  $1.77 \pm 0.67 \times 10^8$  cells ml<sup>-1</sup>,  $9.68 \pm 2.92 \times 10^7$  cells ml<sup>-1</sup> and  $2.58 \pm 0.86 \times 10^8$  cells ml<sup>-1</sup>. The data obtained, using the modified MPN method, were in good agreement with those derived from direct microscopic counts for MULB 1001 and 1002 strains while underestimated numbers (3 times) were observed with the MULB 1003 strain (Tables 1 to 3).

Table 1: Comparison between microscopic and MPN counts for the *Thiobacillus ferrooxidans* MULB 1001 strain

Direct count <sup>a</sup> (x10 <sup>4</sup> cells ml <sup>-1</sup> )	MPN counts (x10 <sup>4</sup> cells ml <sup>-1</sup> ) <sup>b</sup>		
	7 d	10 d	14 d
18.0 ± 6.78	6.22 ± 0.00	23.55 ± 3.95	15.97 ± 5.23
16.0 ± 6.03	5.48 ± 1.05	12.95 ± 2.05	13.17 ± 4.16
14.0 ± 5.28	6.22 ± 0.00	13.20 ± 1.39	16.30 ± 3.30
12.0 ± 4.52	3.66 ± 0.93	9.07 ± 1.46	10.59 ± 2.41
10.0 ± 3.77	4.50 ± 1.25	9.64 ± 5.45	8.30 ± 2.41
8.0 ± 3.02	3.99 ± 0.00	6.88 ± 1.55	7.12 ± 4.49
6.0 ± 2.26	3.63 ± 0.95	4.32 ± 1.44	8.27 ± 3.34
4.0 ± 1.50	1.21 ± 0.14	4.41 ± 1.66	3.30 ± 0.56
2.0 ± 0.75	0.76 ± 0.34	2.79 ± 1.58	1.52 ± 0.02
	$r^2 = 0.84^c$	$r^2 = 0.84$	$r^2 = 0.89$

<sup>a</sup> Mean from at least 20 unit areas of the counting chamber.

<sup>b</sup> Mean of triplicate assays.

<sup>c</sup> Regression coefficient of the mean MPN results vs direct count data.

Table 2: Comparison between microscopic and MPN counts for the *Thiobacillus ferrooxidans* MULB 1002 strain

Direct count <sup>a</sup> (x10 <sup>4</sup> cells ml <sup>-1</sup> )	MPN counts (x10 <sup>4</sup> cells ml <sup>-1</sup> ) <sup>b</sup>		
	7 d	10 d	14 d
18.0 ± 5.44	20.25 ± 14.36	33.70 ± 6.20	22.95 ± 3.35
16.0 ± 4.84	12.91 ± 4.19	17.50 ± 2.20	21.65 ± 10.05
14.0 ± 4.23	12.46 ± 4.54	21.00 ± 3.90	15.00 ± 2.00
12.0 ± 3.63	8.65 ± 4.66	14.15 ± 0.85	14.00 ± 1.00
10.0 ± 3.03	12.32 ± 7.39	16.07 ± 6.74	23.60 ± 3.90
8.0 ± 2.42	7.05 ± 3.06	9.51 ± 2.09	11.55 ± 1.45
6.0 ± 1.81	5.11 ± 1.12	9.76 ± 1.84	7.47 ± 1.25
4.0 ± 1.21	3.14 ± 0.86	4.74 ± 0.87	8.27 ± 3.34
2.0 ± 0.60	6.91 ± 1.02	2.96 ± 0.33	4.11 ± 0.82
	$r^2 = 0.76^c$	$r^2 = 0.85$	$r^2 = 0.72$

<sup>a</sup> Mean from at least 20 unit areas of the counting chamber.

<sup>b</sup> Mean of triplicate assays.

<sup>c</sup> Regression coefficient of the mean MPN results vs direct count data.

Table 3: Comparison between microscopic and MPN counts for the *Thiobacillus ferrooxidans* MULB 1003 strain

(Direct count <sup>a</sup> (x10 <sup>4</sup> cells ml <sup>-1</sup> ))	MPN counts (x10 <sup>4</sup> cells ml <sup>-1</sup> ) <sup>b</sup>		
	7 d	10 d	14 d
18.0 ± 6.03	5.48 ± 0.74	6.54 ± 2.49	6.34 ± 2.69
16.0 ± 5.36	6.17 ± 1.25	4.62 ± 0.90	6.74 ± 2.23
14.0 ± 4.69	3.88 ± 0.00	4.50 ± 1.25	4.78 ± 0.66
12.0 ± 4.02	4.20 ± 0.74	4.70 ± 1.07	4.01 ± 0.59
10.0 ± 3.35	3.31 ± 0.68	3.74 ± 1.08	2.80 ± 1.37
8.0 ± 2.68	2.09 ± 0.12	3.16 ± 0.58	3.52 ± 0.33
6.0 ± 2.01	1.91 ± 0.38	1.79 ± 0.12	2.79 ± 1.58
4.0 ± 1.34	0.90 ± 0.40	1.26 ± 0.25	1.63 ± 0.71
2.0 ± 0.67	0.61 ± 0.02	0.62 ± 0.10	0.56 ± 0.06
	r <sup>2</sup> = 0.94 <sup>c</sup>	r <sup>2</sup> = 0.94	r <sup>2</sup> = 0.92

<sup>a</sup> Mean from at least 20 unit areas of the counting chamber.

<sup>b</sup> Mean of triplicate assays.

<sup>c</sup> Regression coefficient of the mean MPN results vs direct count data.

The underestimation of strain MULB 1003 cell density, as compared to microscopic count, was also seen in plates counts expressed as colony forming units (CFU) (Table 4). The two other thiobacilli strains (MULB 1001 and 1002) showed consistent results irrespectively of the method used. Plate count figures obtained using TSM medium were slightly lower than those from FeTSB medium. Almost all of the colonies appeared within 10 days when growth was done on FeTSB medium. This was not observed for the TSM medium so an insufficient incubation period cannot be excluded to explain the lowest counts.

Table 4: Comparison between microscopic, MPN and plate counts for *Thiobacillus ferrooxidans* MULB 1001, MULB 1002 and MULB 1003 strains

<i>Thiobacillus ferrooxidans</i> MULB Strain	Method			
	Counting chamber	Plate count (TSM)	Plate count (FeTSB)	Modified MPN
	Direct count <sup>a</sup> x10 <sup>2</sup> cells ml <sup>-1</sup>	CFU <sup>b</sup> x10 <sup>2</sup> cells ml <sup>-1</sup>	CFU x10 <sup>2</sup> cells ml <sup>-1</sup>	MPN <sup>c</sup> x10 <sup>2</sup> cells ml <sup>-1</sup>
1001	200 ± 75	169 ± 56	198 ± 35	228 ± 137
1002	200 ± 60	233 ± 61	267 ± 33	345 ± 104
1003	200 ± 67	86 ± 46	165 ± 80	59 ± 8

<sup>a</sup> Mean from at least 20 unit areas of the counting chamber.

<sup>b</sup> Mean of 9 plates after a 14 days incubation period.

<sup>c</sup> Mean of at least six assays.

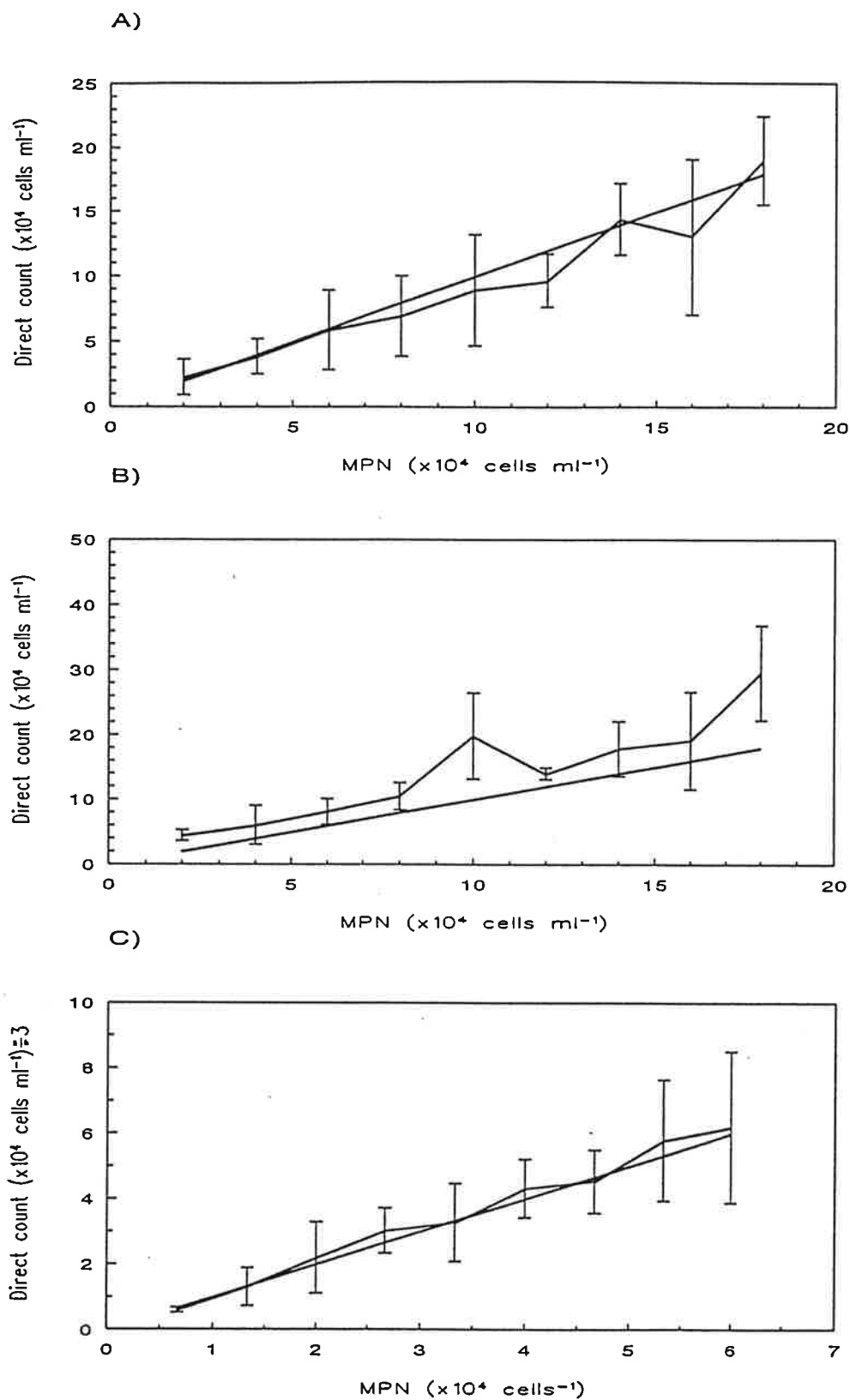


Figure 1: Correlation between *T. ferrooxidans* cellular counts obtained by microscopic evaluation and using the modified MPN methodology after an incubation period of 10 days; (A) strain MULB 1001, (B) strain MULB 1002 and (C) strain MULB 1003. Plotted data represent a mean of 9 determinations. The straight line on each graph is a theoretical curve corresponding to direct microscopic counts.



Data obtained using the modified MPN method for the *T. ferrooxidans* MULB 1001, 1002 and 1003 strains were plotted against expected values provided by microscopic counts (Figure 1) in order to verify the method linearity over one log range. The reference values were divided by a factor of 3 in the case of MULB 1003. It is clearly shown from these results that the modification of the MPN method can provide accurate evaluation of the cell number within one log range.

### Discussion

A method for counting iron-oxidizing bacteria, based on the MPN technique, has been developed. The aim of this study was to verify the method with 3 strains of *Thiobacillus ferrooxidans*.

According to Koch (20), the MPN procedure offers many advantages over the use of solid media for the determination of viable cell counts. It is particularly useful when there is no solid medium for growth, when existing media are unreliable or poorly selective and when bacterial numbers are distributed over a large range. However, the MPN method is not statistically robust and it requires many dilution series when accuracy is needed. Quite a few solid media have been designed to test the growth of *Thiobacillus ferrooxidans* and yet their reliability has not been firmly established. All of them have only been tested with a limited number of strains, most of which were specifically adapted to growth on the medium prior to testing. These media should be used cautiously for the numeration of bacterial populations in the environment. We effectively noted that the growth of iron oxidizing bacteria is often limited by the presence of heterotrophic microorganisms (especially molds). This can be overcome by the use of a liquid medium free of organic substrate that will not support the growth of heterotrophs. In this view, the modified MPN method thus provides the double advantage of eliminating the problems of potential inhibitors in solid media and of reducing nutritional competition amongst other microorganisms.

There was, in general, a good agreement between the three numeration techniques used in this study: direct microscopic observation, agar plate counts and the modified MPN method. The only discrepancy was observed between microscopic and both viable counting methods tested in the case of the MULB 1003 strain. Three possible causes may explain this result: 1. the direct count was in fact overestimated, 2. about 60% of the cells were unable to grow on MPN or gelled media and 3. about 60% of the cells were non-viable. TSM medium was also slightly less efficient than FeTSB medium with regards to growth performance. It should be noted that we used pure strains selected from single colonies on FeTSB agar. Bacterial growth may have been stimulated by TSB, and it may explain the comparatively reduced growth observed on TSM media. Another possibility is that TSM is a more restrictive medium for *T. ferrooxidans*. However, this observation confirms the fact that while different media may support adequate growth, *T. ferrooxidans* does not have the same capacity to form colonies when grown on different media. Growth in liquid culture, as described here, is not without its disadvantages; samples with a high ferrous ion concentration may give a false negative result because of the extended incubation period. Rinsing of the sample may then become necessary, which may eventually change the results. It is also not possible to follow the reaction during the incubation period. The phenanthroline indicator interacts both with the substrate and with the microorganisms, it is then detrimental to the procedure. The risk of an insufficient incubation period is

therefore possible, as is the case with agar plate counts. However, the results of this study show that there was no significant difference in cell counts measured from 10 to 14 days. Finally, the detection limit has been found to lie between 10 to 20 bacteria ml<sup>-1</sup>. This is the minimum bacterial density needed to perform the method with a maximum of accuracy, ie 80% positive reactions in one triplet needed to calculate the MPN using existing tables (21).

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**ACIDOPHILES:**

microorganisms showing a preference for growth at low pH, e.g., bacteria that grow only at very low pH values, ca. 2.0.

**ADENOSINE TRIPHOSPHATE (ATP):**

a major carrier of phosphate and energy in biological systems, composed of adenosine and three phosphate groups; the free energy released from the hydrolysis of ATP is used to drive many energy-requiring reactions in biological systems.

**AUTOTROPHS:**

organisms whose growth and reproduction are independent of external sources of organic compounds, the requirement for cellular carbon being accomplished by the reduction of carbon dioxide and the need for cellular energy being met by the conversion of light energy to ATP or the oxidation of inorganic compounds to supply the free energy for the formation of ATP.

**CHEMOAUTOTROPHS:**

bacteria that derive their energy source from the oxidation of inorganic compounds; organisms that obtain energy through chemical oxidation and use inorganic compounds as electron donors, also known as chemolithotrophs.

**CHEMOLITHOTROPHIC BACTERIA:**

see chemoautotrophs.

**IMMUNOFLUORESCENCE:**

any of a variety of techniques used to detect a specific antigen or antibody by means of homologous antibodies or antigens that have been conjugated with a fluorescent dye.

**MOST PROBABLE NUMBER (MPN):**

the statistical estimate of a bacterial population through the use of dilution and multiple tube inoculations.

**PLATE COUNTS:**

method of estimating numbers of microorganisms by diluting samples, culturing on solid media, and counting the colonies that develop to estimate viable microorganisms in the sample.

**THIOBACILLUS:**

bacterial genus - small rod-shaped cells; motile by means of a single polar flagellum; no resting stages known; Gram negative; energy derived from the oxidation of one or more reduced or partially reduced sulfur compounds; final oxidation product is sulfate; obligate aerobes; optimal growth 28-30°C; G + C 50-68 mole%.