Use of Membrane Filters for the Enumeration of Autotrophic Thiobacilli

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Abstract. A new membrane filter technique for field use was developed for the enumeration of either aerobic or anaerobic, autotrophic, sulfur-oxidizing bacteria in waters and soils. Immediately after collection, samples were filtered through sulfur-coated filters and incubated in selective media. Acidification or gas evolution was used as a growth indicator of aerobic and anaerobic thiobacilli, respectively, and related to the initial number of cells deposited on the filter.

Introduction

Several groups of bacteria are involved in the oxidation of reduced inorganic sulfur componds in waters and soils. The relative importance of chemical oxidation and of different sulfur-oxidizing microorganisms, including the *Chromatiaceae*, colorless sulfur bacteria, and heterotrophs, has been discussed by various workers (13,20). It is generally agreed that the dissimilatory sulfur oxidizers are of greater importance in nature (8,21,27). Various dilution techniques and agar plate counts have been used for the enumeration of thiobacilli (9,14,17), but none is easily used in the field due to the quantity of material necessary and the requirement for aseptic working conditions. Moreover, no data have been published on the quantitative relationship between microscopic counts of thiobacilli and plate counts on thiosulfate agar.

In this paper a new technique for the enumeration of autotrophic aerobic or anaerobic thiobacilli is described. This technique was developed to evaluate the contribution of sulfur bacteria to the corrosion of the concrete of a dam in the Ivory Coast (15) and standardized for water samples in a survey of the activity of the sulfur cycle in rivers of Cameroun and Gabon (16).

Materials and Methods

Sulfur Coated Filters. Membrane filters coated with colloidal sulfur were used to support growth of both aerobic and anaerobic sulfur oxidizers. Edge-hydrophobic filters (Millipore Corp., Bedford, Mass., USA ; Ref. HAEG 047A0) were used in order to center successive filtrations of the sulfur coating and the sample. Sulfur-coated filters were prepared as follows: in a 1 liter flask, 2 ml of concentrated HCl were added to 100 ml of a 10% (w/v) solution of $Na_2S_2O_3$. After 10 min, 1000 ml of demineralized water were added, and the liquid allowed to set for 20 min. Then 150 ml of the

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suspension were filtered through each filter, using "Sterifil" apparatus (Millipore Corp; Ref XX 11 4700). The filters were immediately washed by passing through 1000 ml demineralized water and allowed to dry. Approximately 40 mg of sulfur were fixed on each filter. Microscopic examination of the sulfur coating showed that the range in size of the sulfur granules was from 30 μm to 0.3 μm (5). Sulfur-coated filters were stored as long as necessary without modification of their properties. Just before use, the filters were soaked in a 1% solution (v/v) of Tween 80, a wetting agent that has been shown to improve the oxidation rate of elemental sulfur by thiobacilli (7,12,23).

Water and Soil Samples. Samples were collected from tropical rivers, dammed waters, and from waters and muds of estuary environments where thiobacilli are known to be especially abundant (3,9). Immediately after collection, water samples, usually 50 to 100 ml, were passed through the sulfur filters using "Sterifil" filter holders (Millipore Corp. Ref. XX 1104710) and a manifold (Millipore Corp. Ref. XX 2604735) connected to a vacuum reservoir (Millipore Corp. Ref. XX 6700001). Vacuum was created in the field by means of a manual pump (Arthur Thomas, Ref. 1015). Samples of 10 gm of sediments or soils were ground in a mortar and suspended in sterile 0.5% (w/v) $MgCl_2 \cdot 6H_20$ solution to a final volume of 100 ml. A 2.5-ml aliquot of the suspension was diluted to 500 ml with the same $MgCl_2$ solution, and 100 ml of the final suspension were filtered through the sulfur filters, corresponding to 50 mg of soil on each filter. Water and soil samples were run in triplicate.

Test for Aerobic Thiobacilli. While at the site, filters supporting the samples were placed in plastic Petri dishes (Millipore Corp., Ref PD 1004700) containing a paper pad (Millipore Corp., Ref AP 1004700) saturated with 2 ml of medium S0. This medium was prepared as described in Table 1, and stored in sterile screw-cap tubes before distribution at the field. In the laboratory, membrane filters were transfered to S0 medium solidified by silica gel, prepared as follows: 120 ml of sodium silicate solution (d. 1.33, Prolabo France, Ref 28084) were diluted to 1:1 with glass distilled water and passed across an acidic column (5 × 40 cm) of an ion-exchange resin, Amberlite IR 120 (Prolabo, Ref 27362). The acidic eluate was collected and could be stored at 4°C for 2 weeks. The silic acid and the mineral solution were mixed just before distribution, as described in Table 1, and 3 ml of the mixture were distributed in each Petri dish. Sulfur filters were placed on the gel surface, sulfur side up, prior to solidification. The Petri dishes were incubated at 30°C and examined daily for a change in the color of methylorange from yellow to red; positive growth was indicated by the red color of the pH indicator. As the color faded when incubation exceeded 1 week, a small drop of 2% (w/v) solution of methylorange was added, when necessary, at the edge of the filter.

Test for Anaerobic Thiobacilli. Anaerobic growth was conducted in screw-cap test tubes (16 × 150 mm) filled with medium AN prepared as described in Table 2. Tubes containing 50 mg FeS and 1 ml distilled water were autoclaved and then filled with sterile AN medium at the laboratory. Samples were passed through sulfur-coated filters as described, and the filters introduced into the test tubes. A sterile Durham tube (5 × 50 mm) was lowered into each tube, so that it remained full of air, and the tubes were sealed without trapping air, the atmosphere of the Durham tube being the only compressible volume. The diameter of the aperture of the Durham tube was reduced by flaming to prevent entrance of gas evolving during anaerobic growth. The anaerobic *Thiobacillus denitrificans* reduces nitrate and evolves nitrogen, which is poorly soluble in water, causing the medium to rise in the Durham tube during growth. The number of days of incubation necessary for the medium to fill half of the Durham tube was used to characterize positive growth of anaerobic thiobacilli.

Standardization for Aerobic Thiobacilli in Water Samples. Filters from samples producing the best growth in the conditions described for the test were used as inoculum for enrichment cultures in 250-ml Erlenmeyer flasks containing 80 ml Starkey's sulfur medium (18). The mixture was shaken on a rotary shaker. Turbidity and acidification were measured during incubation, and growth was continued until the pH reached 1.5. At this stage microscopic examination showed that morphology of the microorganism was homogenous and corresponded to the description of *Thiobacillus thiooxidans* (6). Cell concentration was determined using a microscope, and 100 ml aliquots of serial dilutions were prepared in sterile culture medium. The various dilutions of cells were passed through the

Table 1 *Media for Enumeration of Aerobic Thiobacilli*[a]

I.	*Liquid Medium SO Used with Paper Pads*
A.	$(NH_4)_2SO_4$, 2gm; KH_2PO_4, 2gm, $MgSO_4 \cdot 7H_2O$, 0.5 gm; $CaCl_2 \cdot 2H_2O$, 0.25 gm; $FeSO_4 \cdot 7H_2O$, 0.01 gm; trace metal solution[b] 1 ml, distilled water 950 ml.
B.	NaOH N 2.6 ml; 2% (w/v) solution of methylorange 2 ml; distilled water 45.5 ml.
	A was autoclaved 20 min at 120°C, B was sterilized by filtration. Mix A and B, distribute aseptically in sterile screw-cap test tubes. Final pH = 6. Sulfur was supplied on membrane filters as described in text.
II.	*Silica gel medium SO.*
A.	As above, but only 200 ml of distilled water.
B.	NaOH N 5.6 ml; 2% methylorange 2 ml; distilled water 42.4 ml.
C.	Acidic eluate from Amberlite, as described in text, 750 ml.
	A was autoclaved 20 min at 120°C, B was sterilized by filtration. Solution C could be stored at 4°C for 2 weeks. Just before distribution in Petri dishes, mix (A + B) to C.
	Final pH = 6. Sulfur was supplied on membrane filters as described in text.

[a] Adapted from Starkey (18).
[b] Augier's modified solution (supplemented with $MnSO_4$ 0.05%) (1).

Table 2 *Medium for Anaerobic Thiobacilli*[a]

A.	NH_4Cl 0.5 gm; KH_2PO_4, 2 gm; $MgCl_2 \cdot 6H_2O$, 0.5 gm; KNO_3, 2 gm; trace metal solution[b] 1 ml; distilled water 750 ml.
B.	$NaHCO_3$, 2.5 gm; distilled water, 200 ml
C.	$FeSO_4 \cdot 7H_2O$ 0.01 gm; distilled water, 50 ml
	A was autoclaved 20 min at 120°C, B and C were sterilized by filtration, successively through the same filter. Mix A to B + C and distribute aseptically in sterile screw-cap tubes autoclaved with 50 mg FeS and 1 ml of water. Final pH = 6.8. Sulfur was supplied on membrane filters as described in text.

[a] Adapted from Baalsrud and Baalsrud (2).
[b] Augier's modified solution (supplemented with $MnSO_4$ 0.05%) (1).

Table 3. *Characteristics of the two Soils from Balingore (Sénégal)*

Sample Description	1 Mangrove soil	2 Rice field
ph *in situ*	6.6	6.4
pH after drying (1/1 paste)	3.1	4.0
Moisture (% d.w.)	140	120
Clay (% d.w.)	80	60
C (% d.w.)	13	0.9
N (% d.w.)	0.15	0.03
Totals (% d.w.)	3	2

sulfur-coated filters, and the filters incubated in Petri dishes as described. The number of days of incubation necessary for the indicator to turn red was plotted against the log of the number of cells initially deposited on the filter. The same procedure was used for comparison with a strain of *T. thiooxidans* isolated from a mud sample.

Standardization for Anaerobic Thiobacilli in Water Samples. Enriched cultures of *T. denitrificans* were obtained in Baalsrud's medium (2) from positive tubes and used as inocula for serial dilutions in the anaerobic medium, as previously described. A pure strain of *T. denitrificans* isolated from mud (4) was tested in the same conditions. The number of days necessary for positive growth, detected when N_2 evolution increased the pressure in the culture tube so that the medium filled half of the Durham tube, was plotted against the log of the number of cells initially present on the filter.

Standardization for Soil Samples. To test the influence of the presence of 50 mg of soil on calibration curves, various dilutions of a known number of cells were filtered through the sulfur filters in the presence of freshly collected or sterilized soil, and autoclaved for 1 hr at 100°C for 3 successive days. The dilutions were prepared in 0.5% (w/v) $MgCl_2 \cdot 6H_2O$. Sufficient freshly collected or sterilized soil (Soil N° 2 in Table 3) in suspension was added so that the final concentration of the soil equalled 50 mg/100 ml. This mixture was gently stirred for 15 min prior to filtration.

Influence of Clay Minerals on the Growth of Thiobacilli on Sulfur Filters. Kaolinite and montmorillonite, prepared as homoionic to Mg^{++} ion from two soils of Sénégal, were provided through the courtesy of Dr. C. Tobias. Clay suspensions were prepared in 0.5% (w/v) $MgCl_2 \cdot 6H_20$, with a final quantity of 50 mg (dry weight) of clay for each different dilution of sulfur-oxidizing bacteria. Filters supporting clay and inoculum were incubated as described earlier.

Toxicity of Membrane Filters to Acidophilic Thiobacilli. Growth of *T. thiooxidans* was conducted in 80 ml of Starkey's sulfur medium shaken on a gyratory shaker at 30°C in 250-ml Erlenmeyer flasks. Five sulfur-coated filters, prepared as described, were added to each flask after inoculation and the cultures held stationary for 1 day to allow the cells to attach to the sulfur before stirring (200 rpm). Growth was measured as the decrease in pH and compared to control flasks that did not contain membrane filters.

Results

Results of the application of this method in the enumeration of thiobacilli in different biotypes, i.e. seawaters, freshwaters, soils, and sediments, are given below.

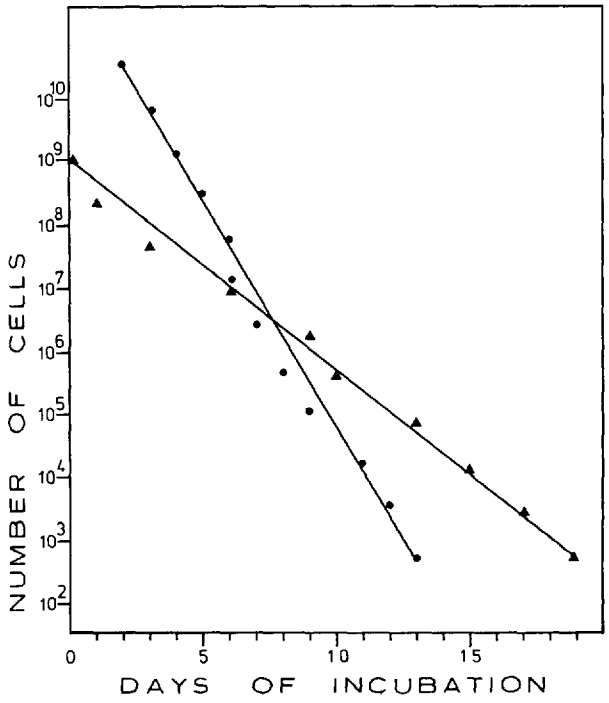

Fig. 1. Incubation time necessary for positive reaction as a function of the initial number of cells, water samples. ▲——▲ aerobic thiobacilli (*Thiobacillus thioocidans*) ●——● anaerobic thiobacilli (*Thiobacillus denitrificans*)

Standardization for Water Samples. A relation was found to exist between the log of the number of cells deposited on the filter and the incubation time necessary for the appearance of a positive reaction (Fig. 1). The number of aerobic thiobacilli that could be evaluated ranged from 10^9 to 10^2 in the sample, as more than 10^9 bacteria produce an acidic reaction within an hour, and less than 10^2 cells will not acidify the methylorange even after 19 days of incubation. Similarily, 2 days were necessary to obtain a positive anaerobic test with 10^{10} or more cells on the filter, and less than 10^2 bacteria did not cause a positive reaction after 13 days of incubation. Identical results were obtained using either pure strains of *Thiobacillus thiooxidans* and *T. denitrificans* or enriched cultures from positive samples as inoculum.

Influence of 50 mg of Soil on the Enumeration Test. The standardization curves obtained for aerobic and anaerobic tests when 50 mg of soil were added on each filter are shown in Figs. 2A and B. If sterilized soil was used, a positive effect was observed for both calibration curves. When freshly collected soil was used, the initial number of sulfur oxidizers present on the filter was the sum of the

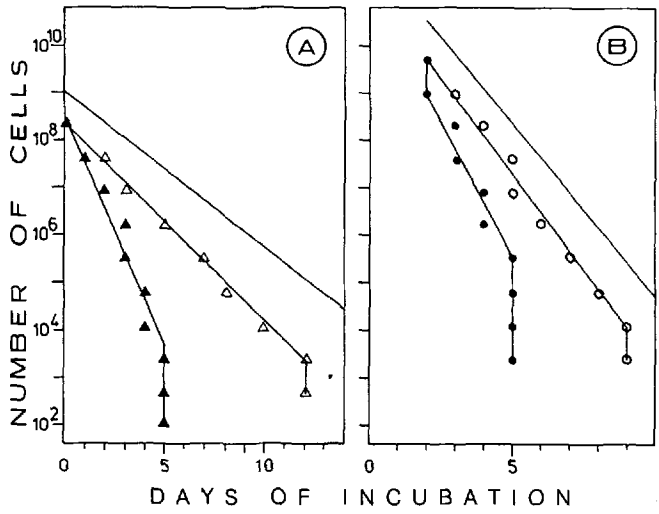

Fig. 2A and 2B. Influence of 50 mg of soil in the enumeration test. A. aerobic thiobacilli: Δ—Δ sterilized soil; ▲—▲ freshly collected soil. B. anaerobic thiobacilli: ○—○ sterilized soil; ●—● freshly collected soil. Nude line: control without soil

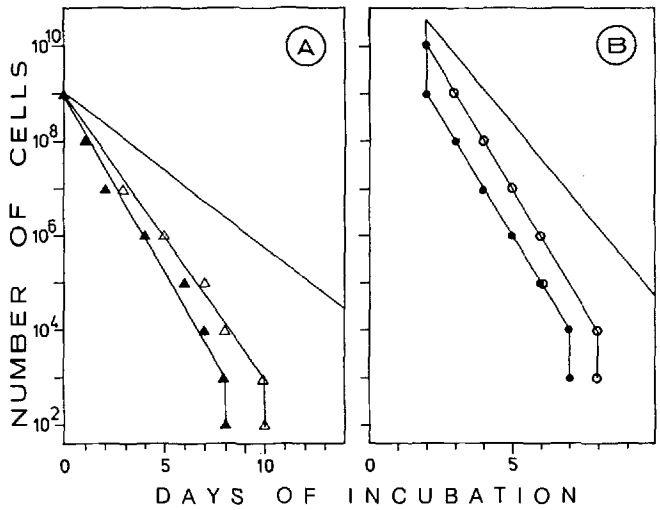

Fig. 3A and 3B. Influence of clay minerals on the enumeration test. A. aerobic thiobacilli: Δ—Δ 50 mg kaolinite; ▲—▲ 50 mg montmorillonite. B. anaerobic thiobacilli: ○—○ 50 mg kaolinite; ●—● 50 mg montmorillonite. Nude line: control without clay

number of cells in 50 mg of soil and the number of bacteria prepared as dilution. In Fig. 2A, with $5 \cdot 10^6$ aerobic thiobacilli present in 50 mg of soil, all dilutions were positive after 5 days of incubation. With 10^7 anaerobic thiobacilli in the soil, all dilutions were positive after 5 days (Fig. 2B).

Influence of Clay on the Growth of Thiobacilli on Sulfur Filters. The influence of sterile clay minerals filtered with the inoculum is shown in Figs. 3A and B. Montmorillonite had a more positive effect on growth than kaolinite.

Growth of Acidophilic Thiobacilli and Membrane Filters. As can be seen in Table 4, growth of *T. thiooxidans* was not affected by the presence of five sulfur-coated Millipore filters in the culture flask.

Examples of Field Results.

1. *Seawater*: A profile 100 miles long was sampled, 10 miles from the shore along the coast of Sénégal, in order to evaluate the abundance of aerobic thiobacilli in surface seawater and their distribution in relation to the arrival of continental waters from two small rivers. Every 5 miles, surface water was collected, and 1500 ml were immediately filtered. The sulfur filters were incubated at 30°C on medium S0 supplemented with 3% (w/v) NaCl. A significant increase in the number of acidophilic thiobacilli was evidenced, from 10 cells/ml (open ocean sample) to $5 \cdot 10^3$ cells/ml (off the mouth of the River Sine).
2. *Fresh water*: 50 samples were collected each month at different sites and depths during a 1-year survey of sulfur-oxidizing activity in Ayame dam (Ivory Coast). From all enumerations it was concluded that (a) In the River Bia feeding the dam, the mean densities of aerobic and anaerobic thiobacilli were respectively 10^6 and 10 bacteria/ml. The high density of aerobic oxidizers may be explained by the fact that the Bia River flows through a dense tropical forest, the activity of the sulfur cycle being increased by the forest's contribution of organic sulfur. (b) In the dam itself, aerobic thiobacilli were equally distributed at all depths from the surface to 18 m deep, with a mean density of 10^6 bacteria/ml. On the contrary the density of anaerobic thiobacilli increased from 10 bacteria/ml in the 5 upper meters to 10^3 cells/ml from 6 to 18 m deep. (c) In a second dam situated 2 km down from the hydroelectric dam, the densities were respectively $5 \cdot 10^7$ for aerobic and $5 \cdot 10^6$ cells/ml for anaerobic thiobacilli, showing that the increased aeration of the water that has passed through the turbine had a positive effect on the activity of the sulfur cycle.
3. *Soils*: Densities of thiobacilli were compared during a seasonal cycle of 1 year in a mangrove soil, supporting a vegetation of *Rhizophora mangle* and submersed daily by the tide, and in a paddy soil supporting one annual flooded rice cultivation. Both soils had a high total sulfur content, as indicated in Table 3. In the mangrove soil, densities of thiobacilli remained at a high level throughout the year, without a significant difference between the dry and

rainy season. The mean densities were 10^6 bacteria/gm for aerobic and 10^5gm for anaerobic thiobacilli. On the contrary, in the rice soil the densities increased from 10^4 to $5 \cdot 10^8$ sulfur oxidizers/gm after the flooding, and fluctuated during the rice cultivation, with a minimum at the heading stage.

4. *Sediments*: Core samples of mud sediment were collected by means of an hydrolic drill in a sedimental valley in Sénégal. A depth of 15 m of fossil mangrove soil was sampled every 1 m before the granitic rock was reached. Aerobic thiobacilli were only detected between 0.5 and 3 m (10^5 cells/gm) and at the bottom of the sediment at the contact of the weathered rock. Similarily anaerobic thiobacilli were found between 1 and 4 m deep (10^6 cells/gm) and at the bottom (10^6 cells/gm).

Discussion

During previous ecologic studies, dilution procedures, using plate count techniques, were found to be inconvenient for field use. The delay between sampling and inoculation of the dilutions could cause considerable alteration in the microflora and lead to inaccurate results in the enumeration of sulfur oxidizers, even when the samples were cooled during transit. Using the methods described, samples were collected and placed immediately in the different culture media with relative ease.

The validity of plate counts of aerobic thiobacilli on thiosulfate agar plates has been discussed by Postgate (18). For the author, the general question of plate-counting of thiobacilli needs further refinements, as plate counts could be misleading due to the sensitivity of the isolated cells to aeration.

Membrane filters were used as a modification of the agar plate counting method, by Tilton *et al.* (24), to enumerate marine thiobacilli, and by Tuovinen and Kelly (25) to determine numbers of viable *Thiobacillus ferrooxidans*.

Filtration of liquid samples was used to obtain adequate numbers of colonies to be counted on the membrane surface. This modification undergoes errors associated with the plate-count method discussed by Postgate (18) for thiobacilli.

On the contrary, the purpose of this work was to evaluate the number of viable cells by the detection of metabolites, similar to Spurny *et al.* (19), using the evolution of H_2S to enumerate sulfate reducers.

In this perspective, it was desirable to initiate growth with the largest number of cells in order to minimize the lag phase and to obtain a positive response, i.e. appearance of acid for aerobes and gas for anaerobes, in the minimum time. This was accomplished by filtering the largest sample possible without clogging the membrane. With waters from tropical rivers or dams, which have relatively large amounts of colloids, only 50 to 100 ml could be filtered

through the sulfur-coated membranes, but up to 2000 ml of seawater could be filtered before clogging. For soil samples, the weight of soil that could be deposited on the filter was related to the soil texture; samples of 50 mg could generally be used.

We have found a relation between the log of the initial number of cells deposited on the filter and the time after which a definite quantity of metabolite is produced from elemental sulfur. As the substrate is poorly soluble in water, proportionality between the number of cells and the production of a metabolite is limited to a higher number of cells corresponding to the saturation of the surface of sulfur granules by bacteria. From scanning electron microscopy (5), this number can be estimated to be 10^{10} for each sulfur filter, and corresponds to the limit of validity experimentally determined for anaerobic thiobacilli.

We have shown that this same number of 10^{10} is necessary for the saturation of a sulfur filter by *Thiobacillus thiooxidans*, if the decrease in pH is measured in silica gel medium S0 (Table 1) with a pH paper (Acilit, Merk Ref. 9531) placed in the Petri dishes. With $5 \cdot 10^{10}$, $3.5 \cdot 10^{10}$, and $2 \cdot 10^{10}$ cells deposited on the filter, pH 2.5 was reached in 10 min; however 60, 180, and 720 min were necessary to reach pH 2.5 with $1 \cdot 5.\ 10^{10}$, $5 \cdot 10^{9}$, and $2 \cdot 10^{9}$ cells, respectively.

Then the limit of 10^9 cells determined with methylorange (Fig. 1) does not correspond to the saturation of sulfur granules by bacteria, but to the selection of the pH indicator methylorange (change of color between pH 4.4 and 3.2). The validity of this choice is discussed below.

Therefore, we may assume that in the conditions of our calibration curves, the surface of the sulfur granules was not saturated by bacteria.

Assuming that exponential growth occurs immediately after the filters are placed in the appropriate media, the mathematic expression of the relation between the initial number of cells, n_0, and the time, t, necessary to observe the production of a definite quantity of metabolite, m, can be deduced from the following equations:

(1) $\frac{dm}{dt} = A.n,$

where n is the number of cells present at time "t".

(2) $n = n_0.\ 2^{kt}$

where k is the exponential growth constant of the organism. Then:

(3) $m = A.\ n_0 \int^t 2^{kt}.\ dt,$

if zero time is the starting time for exponential growth. As m, the definite quantity of metabolite for a positive test, is constant, then if $K = k_m$:

(4) $\log_2 n_0 = \log_2 K - \log_2 (2^{kt} - 1)$

(5) $\log_2 n_0 = A' - \log_2 (2^{kt} - 1).$

From equation (5) it can be seen that the graphic expression of $\log_2 n_0$ as a function of time is close to a straight line, with a negative slope if $2^{kt} >> 1$.

All our data from calibration experiments and sample determinations were in agreement with this theoretical expectation.

Therefore, detection of metabolites (acid for aerobes, gas for anaerobes) produced in a selective medium is a reliable test for estimating the number of microorganisms present.

From the principle of the test and from equation (5), it is obvious that calibration curves are related to k, the growth constant of the organism enumerated, without the possibility of differentiating *a posteriori* between a large number of slow growers or a small number of fast growers. As the test described is selective for enumerating either acidophilic or anaerobic autotrophic thiobacilli, it can be assumed that the variability of k differed slightly from one strain to another. From all calibration experiments, we obtained smilar results either with enriched cultures from various environments or with pure strains subcultured for 2 years in the laboratory. However, in order to obtain more reliable results, it is desirable to calibrate the membrane filter technique in each different biotope, using enriched subcultures of the organism present in positive tubes or Petri dishes.

The stimulatory effect of clay on the growth of *Escherichia coli* was studied by Stotzky and Rem (22), who found that montmorillonite exerted the greatest stimulation during all stages of development, but especially in shortening the lag phase. Interaction between bacterial cells and clay particles was recently reviewed by Hattori (10). Formation of a bacterium-clay complex was evidenced, and montmorillonite was always found to enhance bacterial growth and activities to a greater extent than kaolinite. In the conditions of the enumeration tests, we have also found that growth of *Thiobacillus thiooxidans* and *T. denitrificans* was stimulated more by montmorillonite than by kaolinite, as indicated by the observation that for an identical inoculum a positive reaction was noticed after a shorter incubation time (Figs. 3A and B). It was then necessary to consider the amount and, if known, the mineral nature of the clay deposited on the filter by soil samples for correct enumeration of sulfur-oxidizing bacteria. Figures 3A and B give standardization curves with 50 mg of montmorillonite or kaolinite; we may assume that the curves obtained with sterile soil (Figs. 2A and B, open triangles and open circles) correspond to the action of 20 mg of kaolinite, as the soil used contained 40% clay, determined as kaolinite (Table 3). It is likely that colloids suspended in water samples, such as clay minerals and iron hydroxides, could also enhance bacterial growth. Indeed we found generally that in water samples from tropical rivers the number of cells per milliliter enumerated by our test increased with the volume of the sample filtered.

A possibility exists that the final result of the enumeration tests described, and especially the test for acidophilic thiobacilli in which growth is detected by a decrease of pH, could be markedly influenced by the buffering capacity of soil particles in the sample. This was not observed for all soils with a pH range of from 6.6 to 3.5 tested. With more acidic soils, the presence of 50 mg of soil did not change the initial pH of 3 ml of S0 medium by more than 0.2 units. However, if the presence of buffering particles (i.e. limestone in alkaline soils) is suspected, it is possible to calibrate the enumeration curves in the presence of sterilized soil.

The toxicity of membrane filters to *thiobacillus ferrooxidans* was discussed by Tuovinen *et al.* (26) and by Tuovinen and Kelly (25). Unsoaked Millipore membranes were found by the authors to be suitable for growth of the organism on thiosulfate agar. In our experiments growth of *Thiobacillus thiooxidans* was not affected by the presence of sulfur-coated filters. Use of this type of membrane was convenient for enumeration of aerobic thiobacilli.

All thiobacilli, except certain strains of *T. novellus* and *T. trautweinii*, are able to oxidize elemental sulfur (11), but soluble sulfur compounds (thiosulfate, sulfite, sulfide) are generally preferred in physiologic studies. During aerobic growth of *T. thiooxidans*, the reduction in pH was significantly greater with elemental sulfur than with thiosulfate. Thus, elemental sulfur was a better substrate for detecting growth of acidophilic thiobacilli by the production of sulfuric acid.

The change in color of methylorange occurs between pH 4.4 and 3.2. After Hutchinson *et al.* (11), the final pH, with sulfur as the electron donor, is higher for *Thiobacillus thioparus*. Therefore, only the acidophilic aerobic species *T. thiooxidans*, *T. ferrooxidans*, *T. neapolitanus*, and *T. intermedius* were taken into account in the method described; the anaerobe *T. denitrificans* being enumerated by the anaerobic test. All attempts to use a pH indicator with a higher pH color change in order to take into account *T. thioparus* were unsuccessful, as no relation could be evidenced in the conditions of standardization with sterilized soil. This result may be ascribed to the lack of specificity of a weak acidification in the Petri dishes when some organic matter was supplied as a soil sample. It was then necessary to use methylorange, as a decrease of more than 2 units of pH must be due to aerobic oxidation of the elemental sulfur on the filter.

Interpretation of the color change of methylorange has to be specified with experience by the experimenter; the changing point being empirically stated. However, this interpretation was sometimes difficult if aerobic incubation of soil samples exceeded 1 week, and it was then necessary to add an additional small drop of indicator at the edge of the Petri dishes. The fading of methylorange may be due to progressive adsorption on the clay complex, or to bleaching by the SO_2 evolved during incubation.

Under anaerobic conditions, sulfur was oxidized more slowly than thiosulfate. But in the conditions of the enumeration test at the site, with fresh samples from natural environments, it was evidenced that the evolution of gas was generally detected earlier with elemental sulfur than with thiosulfate. Elemental sulfur was then preferred as the sulfur source for enumeration of *Thiobacillus denitrificans*. Furthermore it was noticed that when FeS was added to the culture tube, the sensitivity of the enumeration was considerably increased: for three series of 52,48, and 46 samples; 47,38, and 44, respectively, were positive (produced N_2) with 50 mg FeS, while only 9,26, and 23 were positive without FeS. The role of FeS remains unknown; it acts perhaps as a reducing agent in lowering the redox potential. But the replacement of FeS by 20 mg/liter of $Na_2S.\ 9H_20$ had a little depressive effect on the number of positive samples.

Tween 80 is commonly used as wetting agent to improve contact of thiobacilli to elemental sulfur. In the enumeration method described, sulfur coated membranes were soaked in a 1% (v/v) solution of Tween 80 before filtration of the sample, placing the bacteria in direct contact with the substrate. Attachment of sulfur oxidizers to the sulfur granules has been evidenced by scanning electron microscopy (5). When incubation of aerobic thiobacilli was conducted on paper pads soaked with the mineral solution described in Table 1, development of cellulolytic microorganisms may occur after some days and prevent final evaluation of the test. It was then necessary to use an inert support for incubation, and silica gel was preferred to agar. Since they are more convenient for field use, paper pads can still be used temporarily if the membranes are transferred onto silica gel medium, as described in the text, as soon as possible in the laboratory.

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References

1. Augier, J. 1956. A propos de la numération des Azotobacter en milieu liquide, *Ann. Inst. Pasteur* **91**, 759-765.

2. Baalsrud, K. and Baalsrud, K.S. 1954 Studies on *Thiobacillus Denitrificans*, *Arch. Mikrobiol.* **20**, 34-62.

3. Baas-Becking, L.G.M. and Wood, F.E.J. 1955. Biological processes in estuarine environment. I : Ecology of the sulphur cycle, *Proc. K. Ned. Akad. Wet.* **58** : 160-181.

4. Baldensperger, J. and Garcia, J.L. 1975. Reduction of oxidized inorganic nitrogen componds by a new strain of *Thiobacillus denitrificans*, *Arch. Microbiol.* **103**: 31-36.

5. Baldensperger, J. Guarraia, L.J. and Humphreys, W.J. 1974. Scanning electron microscopy of thiobacilli grown on colloidal sulfur, *Arch. Microbiol.* **99**:323-329.

6. Buchanan, R.E. and Gibbons, N.E. 1974. *Bergey's Manual of Determinative Bacteriology* (eighth ed) Williams and Wilkins, Baltimore.

7. Cook, T.M. 1964. Growth of *Thiobacillus thiooxidans* in shaken cultures, *J. Bacteriol.* **88**: 620-623.

8. Freney, J.R. 1967. Oxidation of sulphur in soils, *Miner. Deposita* **2**: 181-187.

9. Hart, M.G.R. 1959. Sulphur oxidation in tidal mangrove soils of Sierra Leone, *Plant Soil* **11**: 215-236.

10. Hattori, T. 1974. *Microbial Life in the Soil, An Introduction*. Marcel Dekker, New York.

11. Hutchinson, M., Johnstone, K.I. and White, D. 1969. Taxonomy of the genus *Thiobacillus* : the outcome of numerical taxonomy applied to the group as a whole, *J. Gen. Microbiol.* **57**: 397-410.

12. Kodama, A. and Mori, T. 1968. Studies on the metabolism of a sulfur oxidizing bacterium. V. Comparative studies on sulfur and sulfite oxidizing systems of *Thiobacillus thiooxidans*, *Plant Cell Physiol.* **9**: 725-734.

13. LaRivière, J.W.M. 1966. The microbial sulfur cycle and some of its implications for the geochemistry of sulfur isotopes, *Geol. Rundschau* **55**: 568-582.

14. Moser, U.S. and Olson, R.V. 1953. Sulfur oxidation in four soils as influenced by soil moisture tension and sulfur bacteria, *Soil Sci.* **76**: 251-257.

15. Mouraret, M. 1971. Etude biologique des eaux du barrage d'Ayame I en Côte d'Ivoire. Convention report ORSTOM-EECI, ORSTOM, Paris.

16. Mouraret, M. 1972. Etude biologique des eaux dans quelques cours d'eau du Cameroun et du Gabon. Convention report ORSTOM-S.N.E.C.-S.E.E.G. ORSTOM, Paris.

17. Pochon, J. and Tardieux, P. 1962. *Techniques d'analyse en microbiologie du sol.* Editions La Tourelle, Saint-Mondé France.

18. Postgate, J.R. 1966. Media for sulphur bacteria, *Lab. Pract.* **15**: 1239-1244.

19. Spurny, M., Dostalek, M. and Ulehla, J. 1957. A method of quantitative determination of sulphate reducing bacteria, *Folia Biol. (Praha)* **3**: 202-211.

20. Starkey, R.L. 1950. Relations of microorganisms to transformations of sulfur in soils, *Soil Sci.* **70**: 55-65.

21. Starkey, R.L. 1966. Oxidation and reduction of sulfur compounds in soils, *Soil Sc.* **101**: 297-306.

22. Stotzky, G. and Rem, L.T. 1966. Influence of clay minerals on microorganisms. I. Montmorillonite and kaolinite on bacteria, *Can. J. Microbiol.* **12**: 547-563.

23. Taylor, B.F. 1968. Oxidation of elemental sulfur by an enzyme system from *Thiobacillus neapolitanus, Biochim. Biophys. Acta* **170**: 112-122.

24. Tilton, R.C., Cobet, A.B. and Jones, G.E. 1967. Marine Thiobacilli. I. Isolation and distribution, *Can. J. Microbiol* **13**: 1521-1528.

25. Tuovinen,O.H. and Kelly, D.P. 1973. Studies on the growth of *Thiobacillus ferrooxidans*. I. Use of Membrane filters and ferrous iron agar to determine viable numbers, and comparison with $^{14}C0_2$ fixation and iron oxidation as measures of growth, *Arch. Mikrobiol.* **88**: 285-298.

26. Tuovinen, O.H., Niemelä, S.I. and Gyllenberg, H.G. 1971. Tolerance of *Thiobacillus ferrooxidans* to some metals. *Antonie van Leeuwenhoek* **37**: 489-496.

27. Vishniac, W. and Santer, M. 1957. The Thiobacilli. *Bacteriol. Rev.* **21**: 195-213.