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COMPARISON OF EPIFLUORESCENT VIABLE BACTERIAL COUNT METHODS

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13. ABSTRACT *(Maximum 200 words)*

Two methods, the 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) method and the direct viable count method (DVC), were tested and compared for their efficacy for the determination of the viability of bacterial populations. Use of the INT method results in the formation of a dark spot within each respiring cell. The DVC method results in elongation or swelling of growing cells that are rendered incapable of cell division. Although both methods are subjective and can result in false positive results, the DVC method is best suited to analysis of waters in which the number of different types of organisms present in the sample is assumed to be small, such as processed waters. The advantages and disadvantages of each method are discussed.

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COMPARISON OF EPIFLUORESCENT VIABLE BACTERIAL COUNT METHODS

INTRODUCTION

Epifluorescent microscopy is a useful tool in the rapid determination of total bacterial populations in aqueous environments. An acridine orange (AO) dye that binds Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA) enables rapid bacterial enumeration under a microscope adapted for fluorescent illumination. Among the dye's limitations, however, is the inability to distinguish viable from nonviable cells. The Marshall Space Flight Center (MSFC) is currently conducting a water recovery test to determine the suitability of processing wastewater for crew reuse. The epifluorescent technique is one method being used to assess the microbial content of the water. The ability to further define the viable population present would be advantageous.

The purpose of this study was to test and compare two methods for the determination of viable populations using epifluorescent microscopy. These are the 2–(4–Iodophenyl)–3–(4–nitrophenyl)–5–phenyltetrazolium chloride (INT) and the direct viable count (DVC) methods. In the INT method, respiring cells reduce a tetrazolium dye, forming a dark spot within each cell. The DVC method consists of a reaction mixture containing two major components, a nutrient and naladixic acid. Cells that respond to the nutrient grow, but are unable to divide due to the naladixic acid. This results in elongation of the cell or, in the case of gram-positive cocci, swelling.

MATERIALS AND METHODS

Water Sources/Cultures

Two wastewater (condensate and hygiene) and five processed water samples collected from the MSFC water recovery test (WRT) were examined. Samples were taken directly from the collected water. Two bacteria isolated from earlier stages of the WRT, *Enterobacter agglomerans*, a gram-negative rod, and an unidentified gram-positive coccus were also chosen for this study. These bacteria were plated on brain heart infusion (BHI) agar and incubated for 24 h at 35 °C. Isolated colonies were placed in 99 mL of sterile phosphate buffer, which then served as the sample source for evaluation of the INT and DVC methods.

INT Method

A 1-mL water sample was placed in a sterile test tube, and 0.1 mL of INT reagent (2 percent) was added to the tube. The tube was incubated in the dark at room temperature for 30 min. The bacteria were then inactivated by the addition of formaldehyde (4-percent final concentration), and the sample was filtered across a 0.2-μm black polycarbonate filter. The INT-treated/preserved sample was stained for 2 min with AO (0.01 percent). The filter was then rinsed with sterile buffer and examined under the microscope for the presence of dark spots (indicative of respiring bacteria) within the green to orange fluorescing cells.

DVC Method

Several DVC methods are available in the literature, differing primarily in the proportion of each reagent used in the reaction mixture. Table 1 shows the reagent ratios for the three DVC methods tested for this study. Method 2 was found most effective in a preliminary analysis of the types of samples studied here and was used for all subsequent analyses. A 3-mL volume of the reaction mixture was added to 1 mL of sample in a sterile tube which was incubated at 35 °C for 6 h. The bacteria were then inactivated by the addition of formaldehyde (4-percent final concentration), and the sample was filtered across a 0.2-μm black polycarbonate filter and was stained for 2 min with AO (0.01 percent). The filter was then rinsed with sterile buffer and examined under the microscope for green to orange fluorescing bacteria that were elongated to at least twice their original length or, in the case of cocci, swollen.

RESULTS

Samples from the WRT were scored as positive or negative based on the presence of any bacteria fitting the criteria described above for the two viable count methods (table 2).

Wastewater Samples

Some bacteria from the condensate wastewater initially appeared positive for the INT method as indicated by the presence of one or more dark spots within the cells (fig. 1). Hygiene wastewater gave similar results. These samples were also positive by the DVC method (fig. 2). In no instance was a sample positive for only one method. During analysis of DVC samples, it was observed that both responsive (elongated) and nonresponsive cells appeared very similar to cells that would have been scored as positive by the INT method due to the presence of dark spots (fig. 2). The spots were more readily apparent in the DVC responsive cells for which the increased size allowed better observation of cell structure. These samples were then tested using AO alone and again were found to contain dark spots (fig. 3).

Processed Water Samples

Of the five processed waters examined, two were positive for the INT method (fig. 4). These two samples were also positive using the DVC method, although the response was much less pronounced than that seen in wastewater samples (fig. 5). As was true of the wastewater sources, no sample was found to be positive for only one method alone. In addition, black spots were again seen in DVC-treated samples. Four of the samples tested were found to contain populations of ultramicrobes (microbes that will pass through a 0.2-μm filter), which is not surprising considering the low-nutrient environment of the processed waters. Because these cells were reduced in size, they had insufficient detail to reveal the presence of any INT-positive bacteria.

Laboratory Isolates

The unidentified gram-positive isolate was negative for INT, but some bacteria appeared to swell using the DVC method (fig. 6). There was no evidence of the false INT response that had been seen in WRT samples analyzed by the DVC method. The gram-negative bacterium (*Enterobacter agglomerans*) appeared positive for both the INT and DVC methods. A few of the cells mimicked the INT response in the DVC procedure (fig. 7). After being exposed to starvation conditions for 5 days, numerous *E. agglomerans* cells mimicked the INT response using the DVC method. Elongation of these cells was much less pronounced than in 24-h cultures.

DISCUSSION

Both methods evaluated for determining viable populations of bacteria using epifluorescent microscopy appear largely unusable. Determination of positive and negative responses was found to be quite subjective. Because both methods are based upon the ability of the organism to respond to certain conditions such as nutrient type, temperature, and/or pH, there exists the possibility that some organisms are viable but nonresponsive to the conditions employed.

The DVC method appeared to work for both gram-negative and gram-positive laboratory isolates, although it was much easier to distinguish the gram-negative bacterium. A major disadvantage to the DVC method is the problem that may be encountered in a mixed population of bacteria. Because this method is based on the ability of a viable cell to elongate two to three times the size of a nonviable cell, one would have to assume that all cells in the mixed population were roughly the same size prior to analysis, which is often not the case. For instance, a nonviable *Bacillus* sp. may actually be as long as a viable *E. coli*. Because species cannot be distinguished using this method, both cells would be scored incorrectly as positive. Another disadvantage of the DVC method is that an incubation period of at least 6 h is required. Previous studies have indicated that, in general, gram-negative bacteria require a shorter incubation time (3 to 4 h) in the presence of the reaction mixture than gram-positive bacteria. Also, the DVC reaction mixture itself is not standardized. One combination of the reaction mixture components used provided much better results for the samples tested. However, samples containing other types of organisms might not respond as favorably. Singh et al.,[1] using pure cultures of organisms, found that changes in the concentration of nalidixic acid in the reaction mixture improves the response of certain species of bacteria over others. It is possible that the black spots originally seen in wastewater samples analyzed by the DVC method represent cell damage due to the effects of nalidixic acid, which at higher concentrations results in cell destruction and death.

The INT method requires much less time to perform (30 to 40 min total) than the DVC method. The major problem with this method is the inability to distinguish the black spot, indicative of respiration, from unrelated nonuniformities observed within the cell. This was especially apparent with stressed organisms. The resolution of the microscope (× 1,000) was not sufficient to detect black spots, if present, in stressed organisms that had reverted to an ultramicrobial state. Nonstressed organisms analyzed by the use of AO alone were also found to occasionally contain these cell nonuniformities. In addition, this method was unable to reveal viable gram-positive cocci.

1. Singh, A., Yu, F.-P., and McFeters, G.A.: "Rapid Detection of Chlorine-Induced Bacterial Injury by the Direct Viable Count Method Using Image Analysis." Applied and Environmental Microbiology, vol. 56, No. 2, 1990, pp. 389–394.

CONCLUSIONS

The two viable count methods examined appear unsuitable for use in the WRT. Both are quite subjective and could readily result in false positive results. Reading of results is tedious, especially for the INT method, and requires training of laboratory technicians. Each method uses epifluorescent microscopy for enumeration of bacteria, and is, therefore, subject to the same constraints of that technique, including insufficient sensitivity to meet current WRT specifications for number of bacteria allowed in reclaimed water.

If a viable method is necessary for the WRT, the DVC method would appear to be the best suited to analysis of waters in which the number of different types of organisms present in the sample is assumed to be small, such as processed waters. Even so, this study suggested that elongation of starved gram-negative laboratory isolates is much less dramatic following starvation conditions, which would presumably be those of processed water samples.

The reduced size of bacteria in processed WRT samples and starved laboratory isolates made scoring of these samples using the INT method difficult. Wastewater samples from the WRT were better suited to this method, because of nutrient conditions that promote cell uniformity (i.e., less cell degradation). However, nonuniformities were occasionally found in both these samples and the 24-h laboratory isolates without the addition of INT. Thus, analysis of processed and wastewater sources using this method is subject to inaccuracies.

Table 1. Ratio of reagents used in direct viable count methods.

Reagent	Method[a] 1	2[b]	3[c]
Yeast extract (mg)	0.25	—	—
Yeast extract (gm)	—	0.5	0.3
Naladixic acid (mg)	0.02	0.128	0.025
HEPES (0.1 m) (mL)	—	3.0	—
BHI (mL)	—	50.0	—
BHI (gm)	—	—	3.0
Phosphate buffer (mL)	—	—	10.0

a. All methods use 1-mL sample volume.
b. Mix yeast extract in BHI and add 1 mL to sample.
c. Mix all reagents and add 1 mL to sample.

Table 2. Analysis of water recovery test samples using the INT and DVC viable count methods.

Sample No.	Source	Results INT[1]	DVC
WRT-4*5-22-2-METM-020-M-TNK	Hygiene wastewater	+	+
WRT-4*5-23-1-METM-021-M-TNK	Condensate wastewater	+	+
WRT-4*5-10-1-METM-008-M-PU1	Processed potable water	–	–
WRT-4*5-39-1-MEEH-006-M-PT1	Processed potable water	–	–
WRT-4*5-23-1-METM-006-M-PT2	Processed potable water	+	+
WRT-4*5-39-1-MEEH-002-M-HT3	Processed hygiene water	+	+
WRT-4*5-22-1-MEEH-002-M-HT2	Processed hygiene water	–	–

1. Samples were judged positive or negative based on criteria described in Materials and Methods section. Refer to Discussion section for interpretation of results.

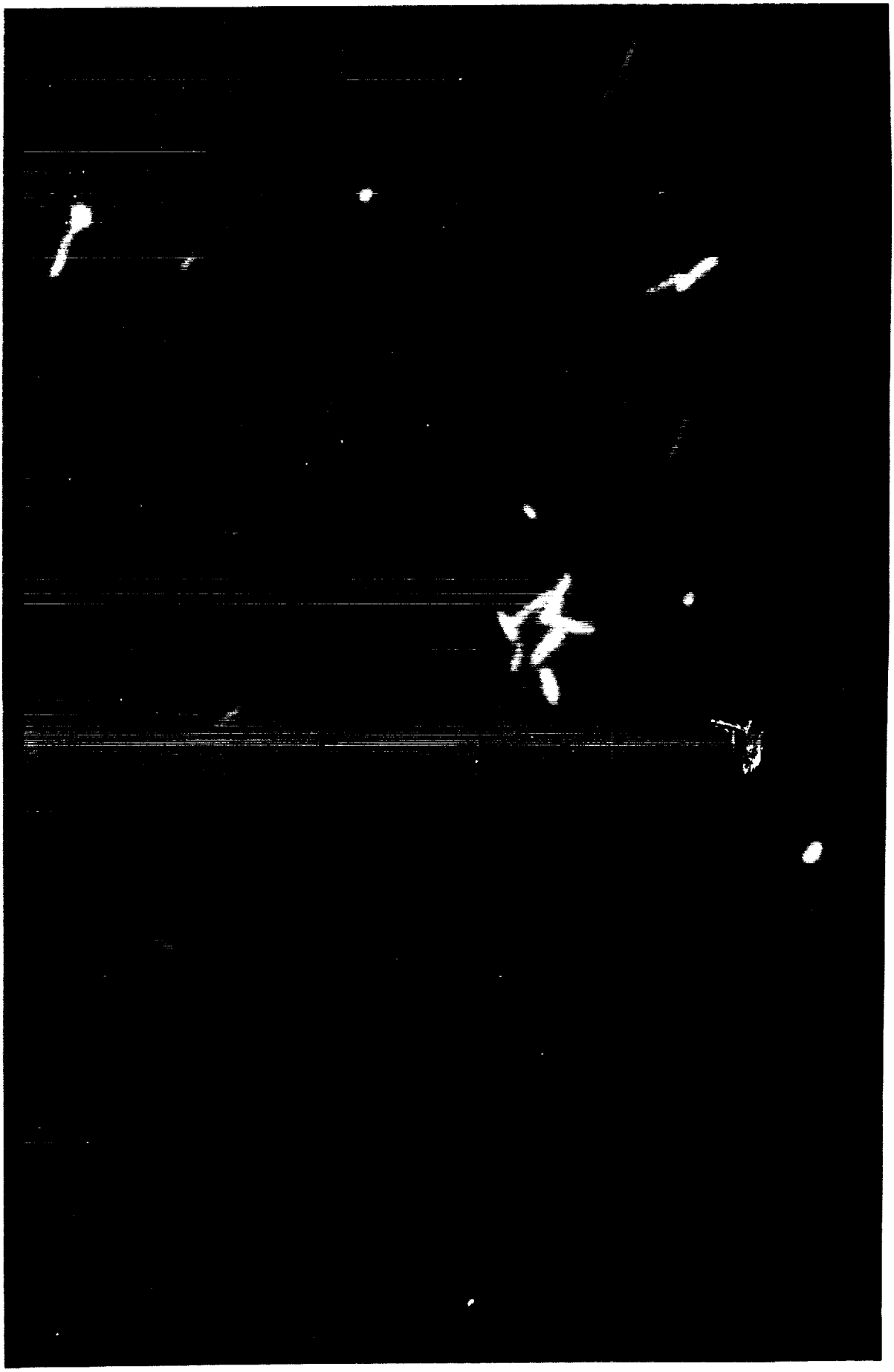

Figure 1. INT positive cell (wastewater).

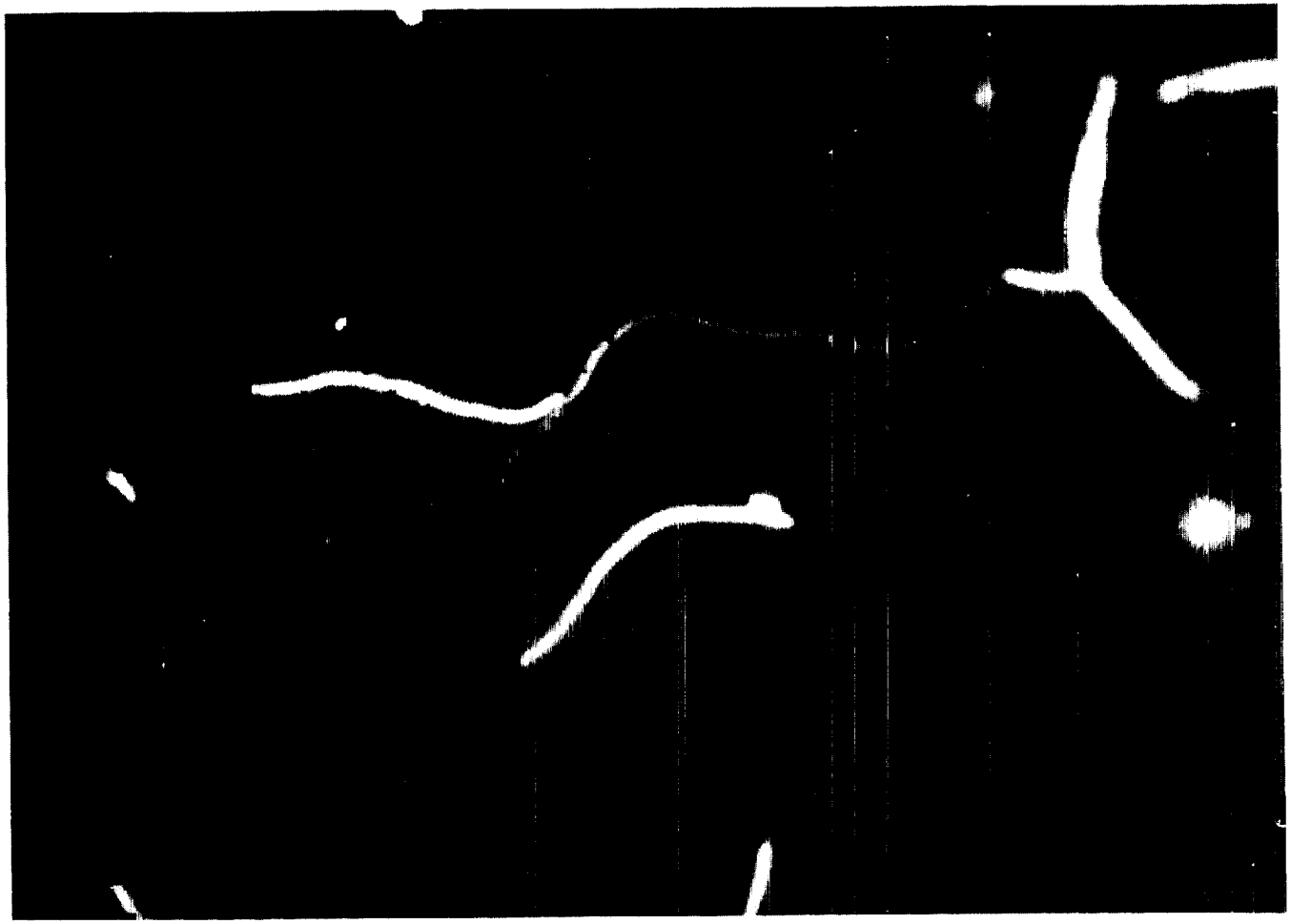

Figure 2. DVC assay.

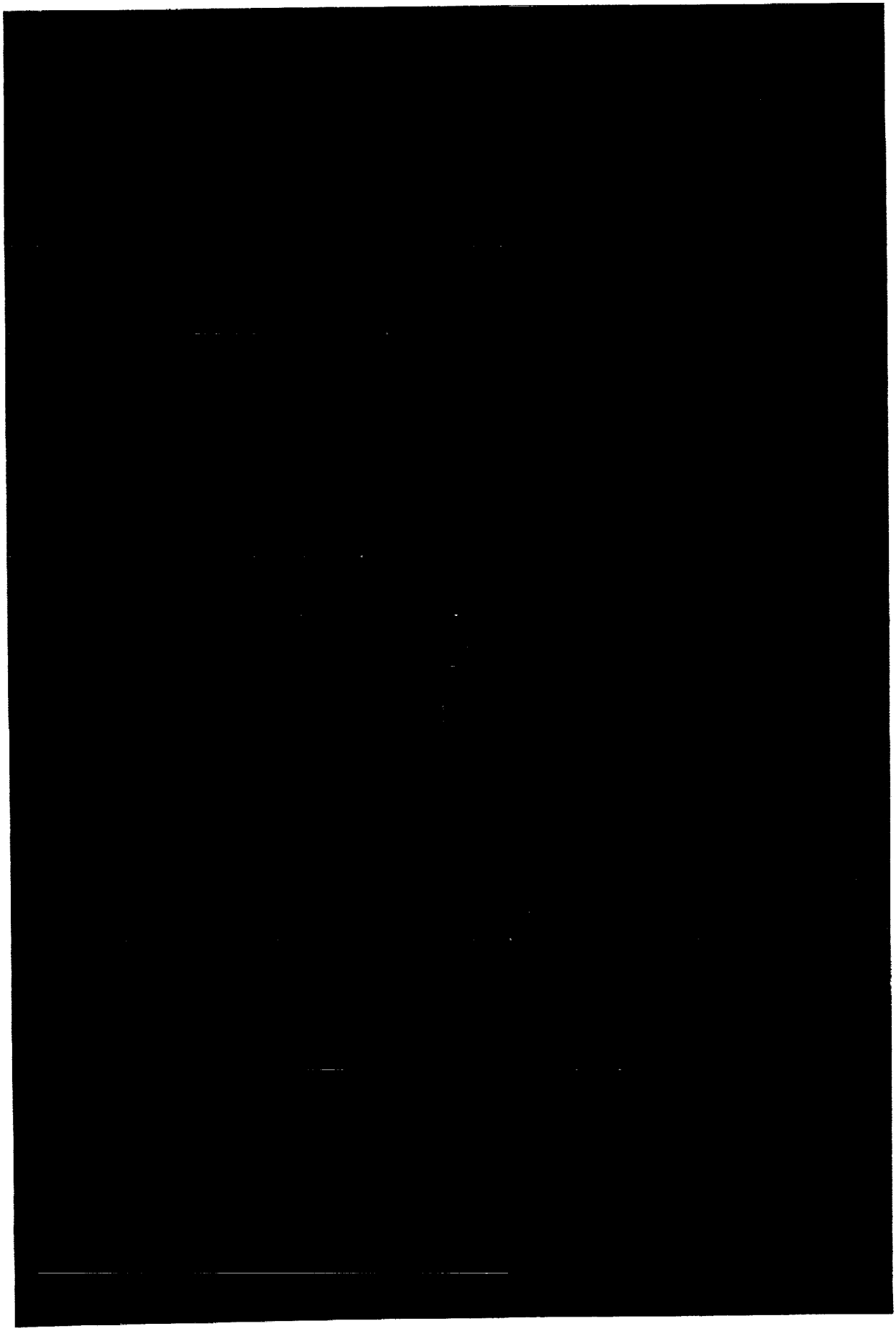

Figure 3. Acridine orange stain (wastewater).

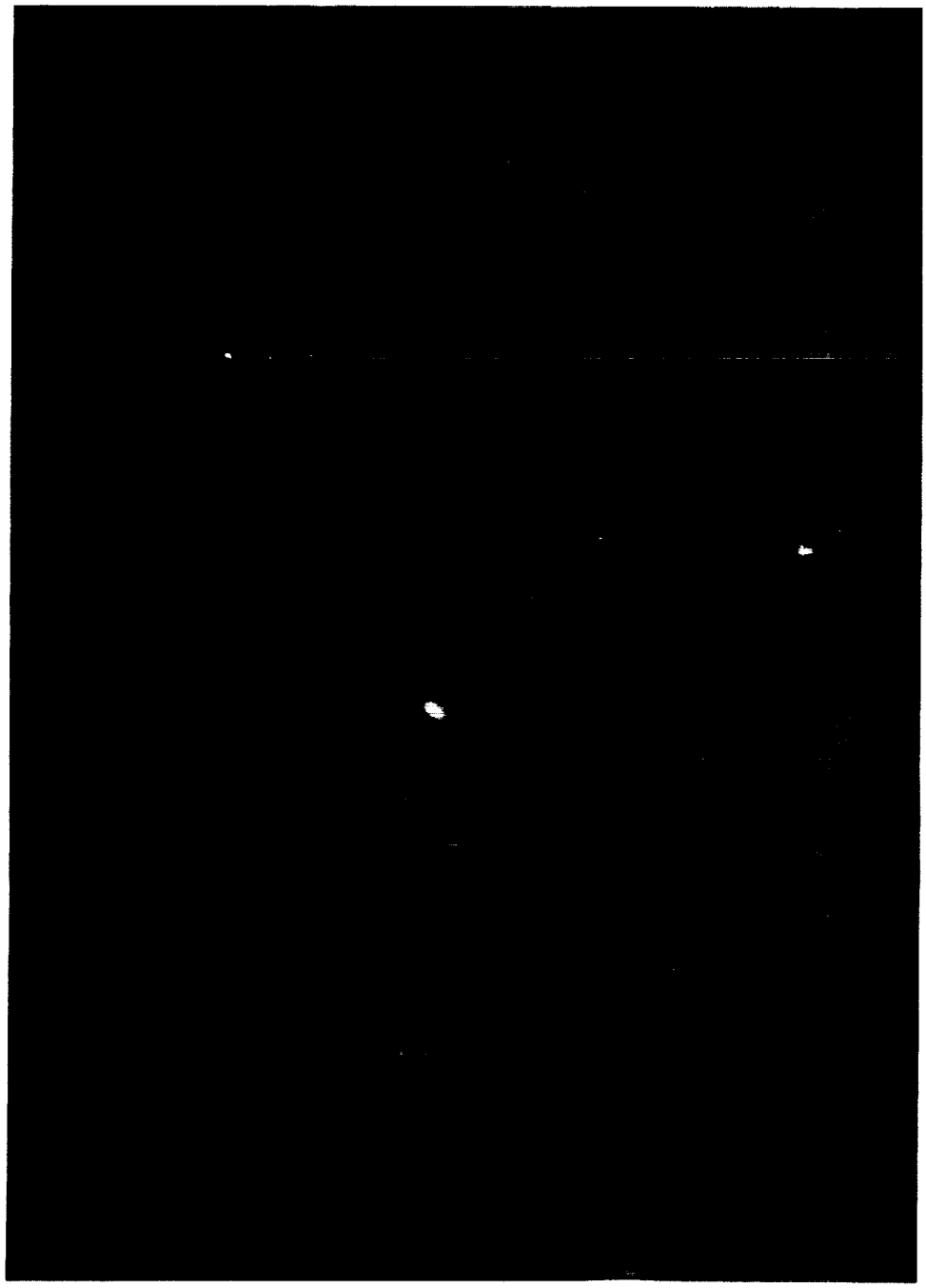

Figure 4. INT positive cell (processed water).

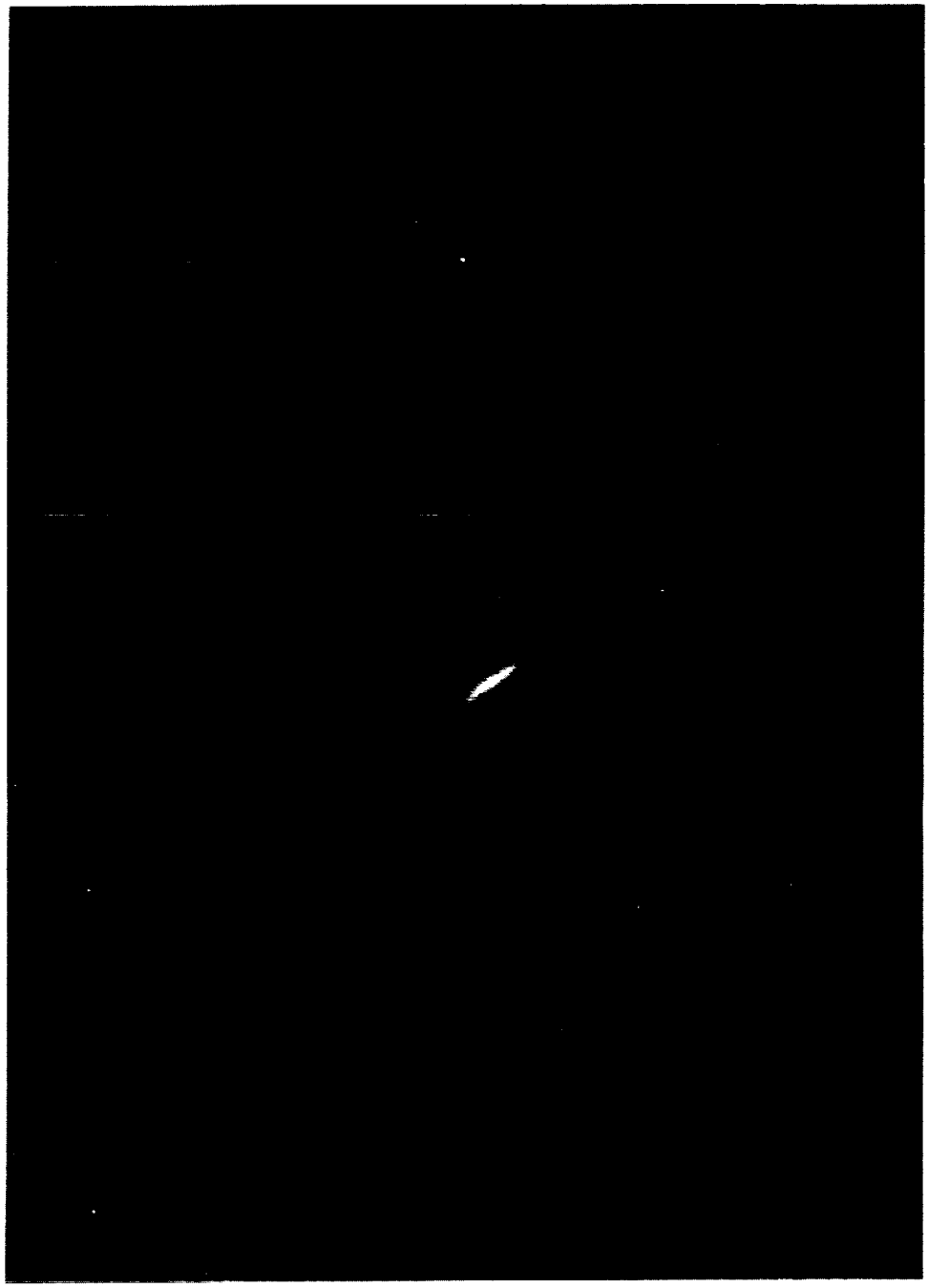

Figure 5. DVC positive cell (processed water).

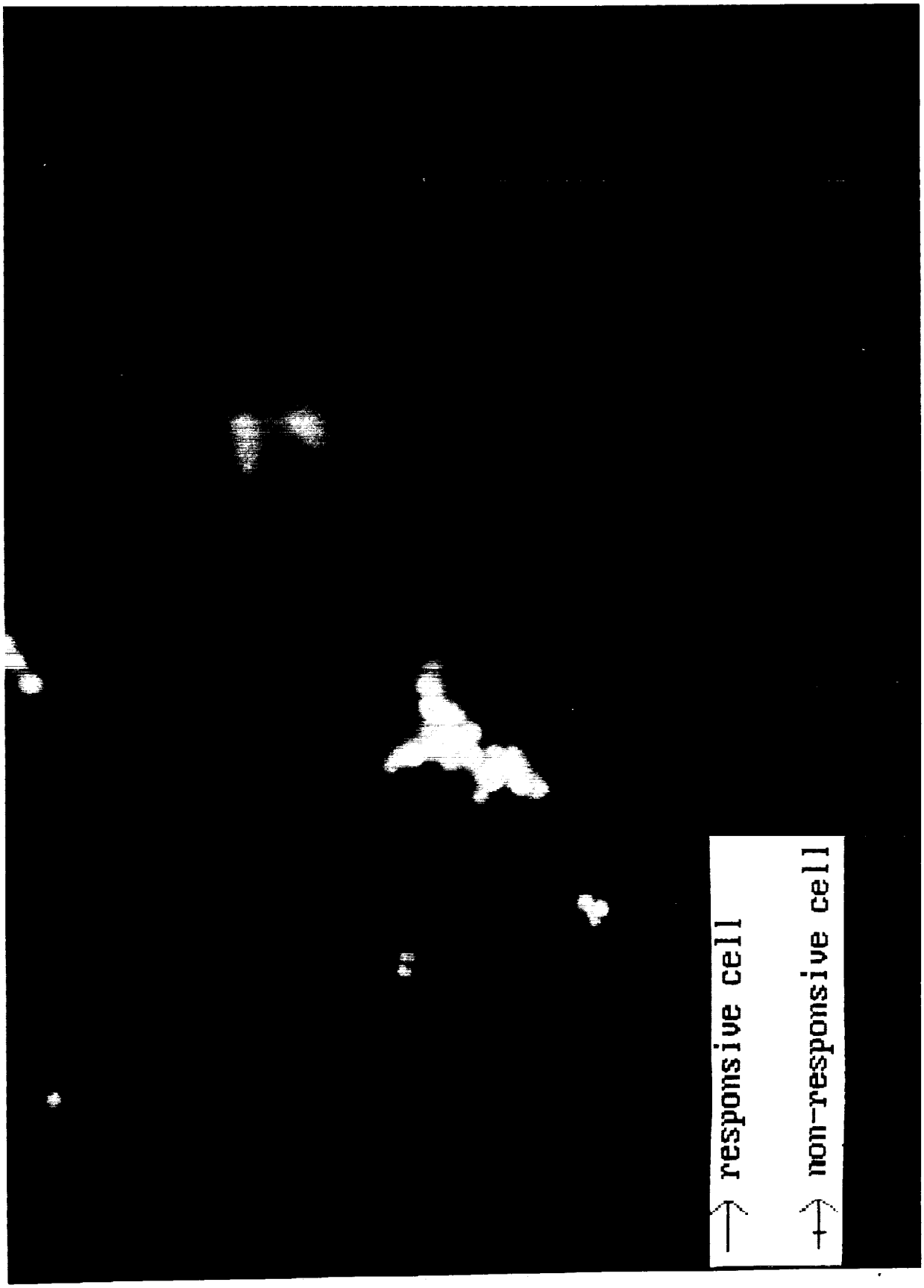

Figure 6. DVC assay (gram-positive laboratory isolates).

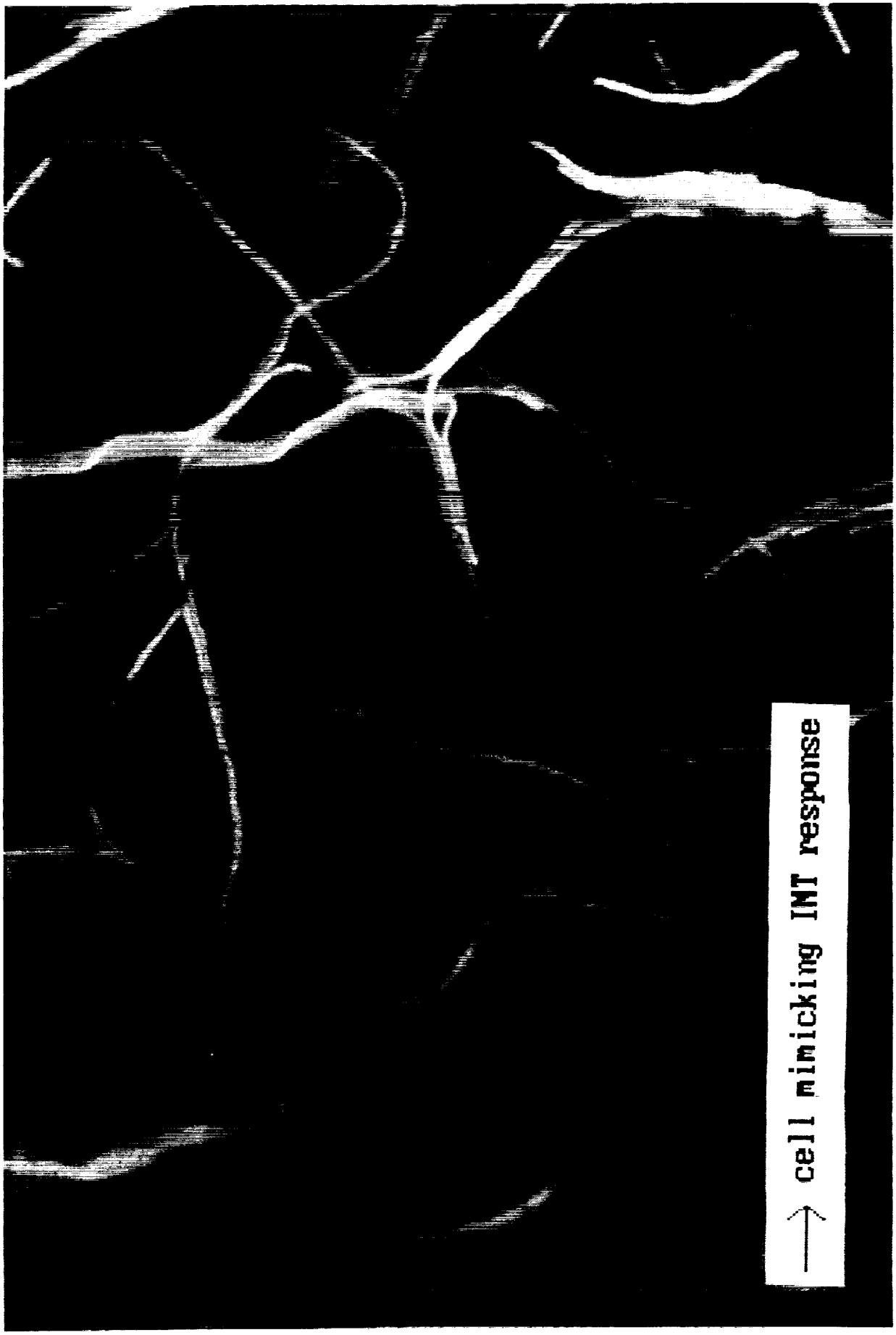

Figure 7. DVC assay (gram-negative laboratory isolate).

APPROVAL

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The information in this report has been reviewed for technical content. Review of any information concerning Department of Defense or nuclear energy activities or programs has been made by the MSFC Security Classification Officer. This report, in its entirety, has been determined to be unclassified.

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