COMPARISON OF D.I. WATER BACTERIA COUNTS BY CULTURE AND FLUORESCENCE MICROSCOPY

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ABSTRACT

The traditional Culture technique for bacterial enumeration requires prolonged incubation times and only measures viable bacterial densities. In comparison, the technique of Fluorescence Microscopy offers a method for obtaining total bacterial determinations in a much shorter time. This paper is concerned with a preliminary comparison of these two methods.

INTRODUCTION

High quality ultrapure deionized water is extensively used in the Microelectronics industry. A finished IC may require up to 150 gallons of high purity water for the complete processing cycle. Since DI water purity has a direct relationship to device yield every component in water that may affect process yield is being studied, analyzed and where possible removed before it can destroy devices. References 1, 2, and 3 are a general overview of D.I. water systems.

Several parameters are used to define ultrapure water quality. These include bacterial count, particle content, metallic ions, silica, and total organic carbons. Although all of these parameters are important to product quality, bacterial contamination represents one of the most serious threats to ultrapure water quality. Part of the reason for the concern is that bacteria is in essence a self generating particulate. Although an ultrapure water system may be capable of removing bacteria down to the level of 1 cell per 100ml, bacteria can reproduce by binary fission. Thus one cell that makes it through can multiply to 8000 cells per 100ml in just 8 hours.

Bacteria have been found in ultrapure water systems that have only trace levels of nutrients present. This is due to the fact that the bacterial organism is capable of adapting its metabolic processes in order to survive in extreme nutrient starved environments. Thus an ultrapure water system may produce 18 megaohm water and have total organic carbon levels as low as 20 ppB but contain 10

bacteria per 100ml. Because bacteria are nonionizable they cannot be detected by standard resistivity measurements. In addition both viable and nonviable bacteria can cause conductance between adjoining circuit paths, so although most bacteria is killed by UV-treatment within the final polishing loop, it still must be filtered out of the water prior to dispensing. This can cause further problems since cells range in size from 5um to .2um, not to mention fractionated nonviable cells.

For all the above mentioned reasons an accurate monitoring technique for bacteria is an essential component of any microelectronic grade water system's preventive maintenance program. Bacterial determinations in purified DI water systems have traditionally been done using Membrane Filtration followed by incubation in order to culture bacteria colonies. This culture technique is relatively easy to preform and is capable of detecting low levels of viable bacteria. However, there are four distinct disadvantages associated with the culturing technique; (1) they tend to underestimate the total number of bacteria; (2) they require 48 to 72 hour incubation times; (3) they do not count bacteria present in clumps or microcolonies; (4) they do not detect nonviable bacteria. Because of these drawbacks there has been a need for a technique that will provide more accurate estimates of viable and nonviable bacteria in a shorter time period, thus allowing more immediate process control.

Epifluorescence Microscopy [4] has been extensively used by Environmental Microbiologists to count populations of marine and freshwater bacteria. This method, if performed right, offers the qualities of both speed and accuracy. Epifluorescence is based on a microscopic system that uses a mercury vapor source to transmit shortwave radiation to a membrane filter that has been fluorochrome stained with an acridene orange dye. The stained bacteria receive the shortwave radiation and emit longwave radiation which is collected for observation by the microscope. The bacteria cells, both viable and nonviable, present on the filter membrane will fluorese and can be viewed and counted under 1000x magnification. The number of cells present in several microscopic fields can then be used to determine the total number of bacteria in a water sample.

This paper is an effort to compare and correlate the technique of Epifluorescence Microscopy with a standard bacterial culture technique, with respect to their ability to detect and enumerate bacteria in RIT's high purity DI water system.

EXPERIMENT

The sampling apparatus for the Epifluorescence technique consists of a 25mm glass filter holder that is connected to a 500ml vacuum flask as shown in Figure 1.

Figure 1
Sampling Appartus

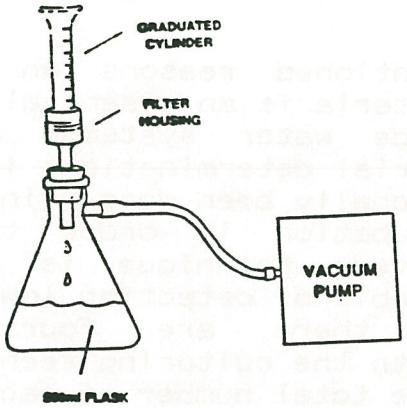
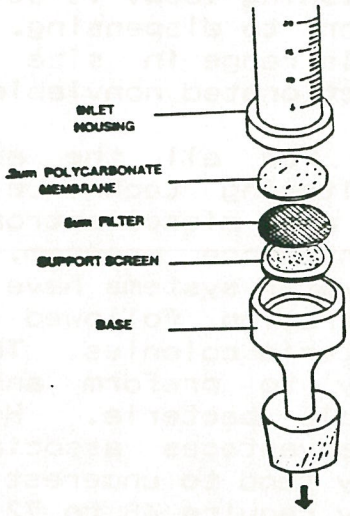


Figure 2
Filter Housing



After the apparatus has been flushed with ethanol to remove any previous contamination, a 25mm support filter is placed in the bottom section of the holder. On top of this is placed a 25mm, 5um cellulose acetate membrane filter, followed by the 25mm, .2um polycarbonate filter as shown in Figure 2. Once the filters are in place the filter housing is sealed. Sample volumes from 100ml to 1L were passed through the sampling apparatus with the aid of a vacuum created in the underlying flask. After the membrane is given one minute to dry the top of the filter housing is removed and the acridene orange dye is applied to the surface of the polycarbonate filter. The dye is allowed to sit for two minutes and then the excess dye is removed by vacuum application. The polycarbonate filter is then carefully removed using a pair of clean tweezers and allowed to dry suspended in the air for two minutes. The membrane is now placed between two drops of immersion oil on a clean glass microscope slide and covered with a 22mm coverslip.

The slide is then observed under a microscope specially fitted with a mercury vapor source and a combination of filters appropriate for acridene orange activation. The cells are observed under 1000x magnification and the bacterial count determined.

The Culturing technique uses filter membranes contained in sterile prepackaged Millopore grid monitors, with .22um porosity and 37mm diameter. Using a sterile sampling tube and a two-way vacuum suringe, 100ml of water was passed

through the gridded membrane. A light vacuum was then applied to remove any excess water contained in the monitor. Next the sampling tube is removed and a growth medium is applied to the membrane surface followed by vacuum removal of the excess medium. The grid monitors were then sealed and placed in the lab incubator maintained at 30 C for 72 hours. After incubation the membrane is removed from the monitor and the colonies are counted under low power magnification.

Using both techniques, bacterial determinations were performed at two locations within RIT's D.I. water system. Samples were taken at point of use, a wet etch area, and at the D.I. storage tank in the basement. The storage tank location was chosen because there is no sampling point beyond the final polishing loop prior to dispensement.

RESULTS/DISCUSSION

Varying volumes of water were passed thru the filter membrane until a volume was found that gave a population of bacterial cells suitable for counting visually under 500x magnification. A sample volume of 100ml proved to be the appropriate sample volume. This volume also proved to give good results using the culturing technique. At this point it should be mentioned that there is an easier method for bacterial enumeration that does not require this preliminary optimum sample volume determination. The sample determination takes time which can be avoided if one has a delineated optical grid present within the eyepiece of the viewing microscope. With the use of a counting grid it is not necessary to count every cell present on the membrane thus the sampling volume is not critical. One can obtain a statistically accurate determination by counting 20-50 fields present on the counting grid and converting this data into total bacterial cells present per 100ml by the following equation.

$$N = \frac{a \times n \times (.01)}{m \times v}$$

Where:

N = total number of bacteria per 100ml.
a = area of the filter membrane (mm**2).
n = mean count of bacteria present per field delineated on counting grid.
m = total area of counting grid (mm**2).
v = volume of water sampled.

Since we do not have a counting grid such as this available for use with our microscope I had to proceed with the method of sample volume determination. Five samples were taken using each method at each of the locations. The results of the five samples for each method and at each location were then averaged. The averaged data appears in Table 1 below.

BACTERIAL COUNT (CELLS per 100ml)		
SAMPLING LOCATION	CULTURING TECHNIQUE	FLUORESCENCE TECHNIQUE
POINT OF USE	9	29
SOURCE	21	56

Table 1 : Bacterial Counts Obtained.

The results showed as expected that the Fluorescence technique gave consistently higher cell counts. Another fact that is apparent from the data is there appears to be some correlation between the two techniques. The Fluorescence technique gave approximately three times the count of the Culture technique, however this factor can not be confirmed until a larger number of samples are taken, also more locations need to be looked at. The instillation of a sampling port downstream of the polishing loop is a matter that needs to be addressed. Although the level of bacteria seen at point of use is well within the appropriate levels for a system such as ours.

In regard to testing time, once the sample volume was determined the Fluorescence method could produce results within 25 minutes. This time included obtaining a sample and counting the cells under the microscope. On the other hand the Culture technique requires a minimum time of 48 hours and in some cases 72 hours before the colonies were of appropriate size for visual counting. The shorter time in obtaining results is a big advantage of the Fluorescence technique. This allows for more immediate attention to D.I. system maintenance before a sudden bacterial increase is able to cause significant reduction in product yield.

When it comes to ease of use both sampling techniques are relatively easy to perform, however the direct- count technique of Fluorescence requires more experience when it comes to differentiating bacteria cells from other foreign matter present upon inspection under the microscope. Due to this fact more operator training would be required for the Fluorescence method than when using the Culture technique where the colonies present on the membrane are much more obvious.

SUMMARY / FUTURE CONSIDERATIONS

Both the Culture and Epifluorescence techniques gave relative counts of bacterial contamination, with the Epifluoresence counts higher as expected. Accurate quantitative counts by Epifluorescence will require the acquisition of a 100x objective and a mercury source specifically designed use with this technique. I also recomend obtaining a statistically accurate sampling grid for use with the scope. Finally I believe some practice in bacterial identification will be necessary.

ACKNOWLEDGEMENTS

Scott Blondell for his greatly appreciated assistance in the laboratory, Anne Moschella of Nuclepore corp. for her technical assistance, Mike Jackson for his ideas on where to go next, and Dr. Fuller for his idea of exploring this technique and help in obtaining necessary equipment.

REFERENCES

[1] Attwell, Caldwell, Gough, Hardy. "Microbial Contamination in Ultra-Pure Water." Solid State Technology. February 1986, pp. 139-142.

[2] Frith, C. F. "Future Requirements of Pure Water Analysis". Semiconductor International. February 1986, pp. 93-95.

[3] Iscoff R. "The Challenge for Ultra-Pure Water" Semiconductor International. February 1986, pp. 74-82.

[4] Geesey, Hite, Mittelman. "Epifluorescence Microscopy" Microcontamination. August/September 1983.