Epifluorescence Microscopy

ASTM D4455 - 85 Standard Test Method for Enumeration of Aquatic Bacteria
by Epifluorescence Microscopy Counting Procedure (2)
(acridine-orange epifluorescence direct-microscopic counting)

Sample Preparation for Source and Treated Waters:

1. Assemble a filter apparatus with a 25 mm diameter neutron-track-etched polycarbonate membrane having a pore size of 0.2 µm. Since bacteria are commonly in the 1 µm size range, this will retain bacterial cells plus larger particles. For the examination of algal cells, a membrane pore size of 3 µm is commonly used. This will allow most of the planktonic bacterial cells to pass through.

2. Pipette the water sample (~1 ml lake water to 10 ml filtered water) into the column of the assembled membrane filter apparatus and, if necessary, dilute to 10 ml with particle-free water (water that has recently been membrane-filtered to remove particles).

3. Apply gentle vacuum until about 1 ml of liquid remains in the column. Then, add two drops of acridine orange fluorescent stain and allow 1 minute for the stain to be taken up by the RNA and DNA of the cells.

4. Draw the liquid through the membrane and, then, add 5 ml of particle-free rinse water to remove the excess acridine orange. Draw the rinse water completely though the column.

5. Disassemble the column and carefully remove the membrane. Allow the membrane to air dry for a minute.

6. Place a drop of immersion oil on a microscope slide and place the membrane on top of the drop observing the ‘clearing’ of the membrane surface as the pores fill with oil. Then, place another drop of oil on the top of the membrane before applying a cover slip.

Microscopic Observation and Enumeration of Cells

Observe the slide under low magnification (100x) to determine that the particles have been deposited uniformly on the surface of the membrane. Then, under oil immersion (1200x) and under ultraviolet illumination, count the number of cells inside the 10 x 10 optical grid. Repeat in 10 grid fields chosen at random.

For this Nikon microscope, the total number of bacterial cells in the sample equals the average number of cells counted per field times 25,000, the wet area of the membrane filter divided by the area of the grid. In a lake water, this number will often be in the range of 1 to 10 million cells per milliliter. In settled and filtered water, the total number of cells should be one to two orders of magnitude less.

A 35 mm film camera back, a digital camera or a digital camcorder can be used to photograph or record the images obtained for purposes of archiving the results.

Conventional and Direct Microscopic Methods for Evaluation of Water Treatment Plant Performance

The conventional measures used for assessing the microbiological efficiency of water treatment plants are the regulated parameters, turbidity and total coliform. Although not required, some water utilities have also compiled supplementary data on HPC organisms. Recently, a number of water treatment plants have started to utilize electronic particle counters in an effort to quantify the particle removals achieved as a function of particle size. Alternately, the studies reported here have employed direct microscopic techniques (1) to observe and compare the removals of various groups of particles and to observe seasonal variations in water treatment plant particle removal efficiency.

New federal regulations, recent waterborne disease outbreaks and advancing technology are now forcing a reappraisal of the methods by which water treatment plant microbiological efficiency is evaluated. The following discussion is directed toward an assessment of the appropriate technology for engineering evaluation and control of water treatment processes. Appropriate technology would permit evaluation of the two–barrier protection (physical removal; disinfection) on which regulation of drinking water quality for the protection of public health is predicated. Such technology would assist regulatory authorities in evaluating compliance with the filtration/disinfection rule and provide a basis for allowing additional "credit" for treatment which goes beyond conventional treatment (e.g., two–stage treatment and lime softening). More scientific methods of process evaluation should also facilitate a reevaluation of filtration theory and make it possible to optimize water treatment plant performance for particles most directly related to health concerns.

Appropriate technology should also lead to improved plant design with respect to flocculation energy input, sedimentation requirements and filter rate and media selection. This is especially critical at a time when the engineering profession is attempting to reduce treatment plant construction and operational costs by eliminating large sedimentation tanks, reducing energy inputs and increasing filter flow rates. Finally, appropriate technology should enable plant operators to monitor plant performance and maintain daily operational control.

Validity of Turbidity as a Primary Microbiological Drinking Water Standard

There has been considerable discussion of the use of turbidity as one of only two primary microbiological standards for drinking water. The USEPA Turbidity Criteria Document was developed in 1985 to provide the justification for continued use of the measurement both as a standard and for the evaluation of water treatment plant performance (9). Even while USEPA held workshops and hearings to develop a consensus for its continued use, scientific evidence continued to accumulate which undermined the rationale for using the turbidity measurement as a primary drinking water standard.

As previously noted, it had become increasingly clear that turbidity levels in raw and finished waters did not reflect the numbers of organisms present (3-8). Nor did treatment provide parallel removals of turbidity and microbial indicators, such as total coliform, HPC, Giardia cysts, virus or total bacteria.

Perhaps the major flaw in the rationale for the use of turbidity as a microbiological surrogate lies in the widely held concept that bacteria and virus in natural waters are generally attached or adsorbed to the surface of suspended solids. Consequently, the near-complete removal of the suspended solids is believed to ensure an equally–complete reduction in the naturally-occurring population of microorganisms.

Total Coliform for Evaluation of Water Treatment Plant Performance

In studies conducted to assess treatment plant bacterial removal efficiency and define microbial changes in the distribution system, there are several very simple and obvious reasons for questioning the use of the coliform group. To begin with, coliform organisms comprise only an extremely small fraction of bacteria in raw or treated drinking waters. They are often one-millionth or less of the total bacterial population. Improvement in coliform detection by 1000 times would still result in a lack of sensitivity for assessing bacterial removal efficiencies during water treatment. It seems likely that, if the coliform was not a regulated public health parameter, it would not be considered for evaluation of treatment plant performance.

Monitoring of coliform organisms does play a major role in the protection of drinking water supplies from wastewater discharges. However, because coliform organisms are readily inactivated so that coliform are generally absent in treated waters, they have no value for the monitoring of water treatment plant particle removal performance. No coliform were found in the finished water at Jefferson City, Missouri. For all practical purposes, coliform was undetectable in treated and distributed water.

HPC for Plant Performance Evaluation: Removal of Single Cells vs. Aggregations

The observed effectiveness of filtration in reducing HPC highlights several important differences between HPC and the total bacterial cell count. The first difference is that the HPC enumerates, as colonies, both clumps of organisms and selected single cells capable of growing on the medium under the prescribed conditions, e.g., pour plate or spread plate. The reduction in HPC seen on filtration may reflect the selective removal of the clumps of organisms over the individual cells. This results in a decrease in the ratio of HPC to total bacterial cell count following filtration (7).

In addition, observed plant reductions in HPC (>99.9 percent) were considerably higher than total cell removals because physical removal is confounded with chemical inactivation which may kill or merely injure the organisms so that they do not develop into HPC colonies.

Alternate Parameters for Evaluation of Water Treatment Plant Performance with Respect to the Removal of Microbial Particles of Potential Health Significance

Total Bacterial Cells: Bacteria are generally the most numerous particles larger than 0.2 μm in water supply sources, often ranging from 10^6 to 10^7 particles per milliliter. Direct enumeration gives a measure of the removal of all particles larger than 0.2 μm. While coagulated, settled and filtered waters may contain 10^5 to 10^6 cells per milliliter, comparatively few particles larger than 3 μm are found (10^3 per milliliter). Therefore, the effective removal of total bacteria is a strong indication of the effective removal of all particles larger than 0.2 μm. However, achieving a goal of 99 percent bacterial removal might require significant treatment modifications during winter months.

Planktonic and Particle–Associated Bacteria: Of the total bacterial population in drinking water sources, a fraction are attached to particles and a fraction are planktonic. The fraction attached to particles appears to vary with season in the Missouri River, averaging 39 percent in the winter and 71 percent in the summer. The fraction of bacteria attached to larger particles is as well removed as the larger solids, validating the long–held contention that surface attachment to particles in natural water provides a major advantage for the removal of bacteria. What remains unknown are the reasons for seasonal variations in the degree of microbial attachment.

Alternately, the planktonic bacteria tend to remain highly dispersed. They appear to be less readily coagulated than silt and clay particles which appear to have a tendency to aggregate spontaneously. As a result, planktonic bacteria offer an outstanding challenge to the coagulation process. If such bacteria are not entrained in precipitates or coagulant floc during pretreatment of water prior to filtration, they appear to penetrate filters readily. Because of the comparatively poor efficiency of physical removal of planktonic bacteria, particularly during periods of low temperature and high hydraulic loads, this measure may be taken as the most critical test presently available of the removal of sub–micrometer and larger particles during water treatment.

Seasonal (Temperature) effects on Treatment Performance

Direct microscopic particle counting, while requiring the human ability to recognize particles, is very precise and offers unparalleled scientific information. In a Missouri water treatment study, since microscopic examination had shown that most particles smaller than 3 μm were, indeed, planktonic microbial cells, treated water samples were passed through a 3 µm membrane filter prior to direct particle counting. The number of particles recovered on the 3 µm membrane averaged 1116 per ml in the winter and 293 per ml in the summer. This seasonal difference again sharply delineated the increased effectiveness of treatment under warm water temperature conditions.

More important, the direct microscopic count allowed the observer to identify the major particles penetrating the water treatment plant. In this instance, about one–third of the particles present were long, almost needle–like, bacterial rods. This percentage was the same, both summer and winter.

Overall, algal cells and colonies were second in abundance, ranging from 49 percent in the winter to 9 percent of the total in the summer. Both these results contrast with the consistently effective removal of the most abundant silt and clay particles, as indicated by turbidity reduction, from the raw water.

Electronic Particle Count for Evaluation of Water Treatment Plant Performance

In a study of the performance of high–rate filtration plants, Cleasby, et al., reported a relationship between turbidity and particle count (as measured using a 60 µm HIAC PC320 sensor) in both raw and filtered water (10). The "total particle count" reported in the filter effluents of 21 water treatment plants ranged from 41 to 2200 per ml. From data presented, the particle counter failed to detect the majority of particles less than 5 µm.

The data reported by Cleasby confirms the importance of pretreatment to overall filtration plant particle removal performance. Conventional water treatment plants which employ sedimentation and filtration were found to average 99 percent overall particle removal. However, the four conventional plants which achieved over an order of magnitude particle removal by pretreatment averaged 99.6 percent reduction in particle counts. The eight other conventional plants where pretreatment was less effective averaged 98.8 percent. Where sedimentation was not provided, average particle count reductions declined to 97.7 percent.

All four of the direct filtration plants failed to achieve 99 percent removal of influent particles and averaged 96.2 percent overall reduction.

Lime softening plants serve as notable exceptions to plant performance evaluation schemes relying on turbidity or electronic particle counting. The softening plant studied utilizes coagulation during pretreatment plus two–stage clarification in addition to dual media filtration. The apparent low particle reduction (52 percent) during pretreatment is misleading since softening plants produce a far greater number of particles within the plant than are present in the source water. The reported 97 percent overall particle removal may have been orders of magnitude greater if the calcium carbonate crystals formed during treatment had been redissolved. Removals of particle–associated bacteria in a similar softening plant were routinely found to exceed 99.9 percent (7). Moreover, particles larger than 3 µm having 5 or more bacteria attached (potentially health–significant particles) were found to be reduced by 99.997 percent in the winter and 99.99994 percent in the summer. In virtually all water treatment plants where particles are generated or modified during treatment, the inability of the electronic particle counter to discriminate native from plant–generated particles may result in a gross underestimation of water treatment plant efficiency.

The data of Cleasby, et al., clearly demonstrate that it will be to the disadvantage of most water utilities which generate particles during treatment (e.g., by coagulation or precipitation) to utilize either turbidity or particle counting to demonstrate the efficiency with which microorganisms are removed by physical treatment processes. Eleven of the 21 plants evaluated failed to achieve 99 percent reductions of particles as determined by electronic particle counter. Evidence based on direct microscopic count indicates the actual removal of "native" particles 3 µm and larger by surface water treatment plants markedly exceed these values.

Another major difference between electronic particle counting and microscopic counting is that electronic particle counts indicate a near–uniform reduction in particles irrespective of particle size. Direct microscopic counts clearly reveal a far greater removal of large particles and far smaller removals of small particles. Electronic particle count data for the City of Corvallis, Oregon (Table 3) illustrate the uniformity of apparent particle reduction. The results would imply that particles ranging in size from 1 to 50 µm are removed with equal efficiency.

Implications of Particle Morphology for the Theory of Filtration

Whereas the particles used to establish theoretical filtration models were uniformly charged, rigid latex spheres, the particles observed in natural water sources are incredibly varied. Some scatter light well, others are translucent. Some are dense and settle readily, others approach the density of water. Many biotic particles are flexible and deformable, able to squeeze through pores and narrow passages. Still others are motile. Particle surfaces may have a high charge density or virtually none. In the case of microorganisms, surface properties and their tendency to attach may even vary with metabolic activity. In the series of Missouri River water treatment studies, particle attachment was found to vary markedly with seasonal temperature changes.

The most obvious difference in waterborne particles to the observer is their shape. The long, thin flexible (needle–like) rods commonly observed in the finished water in the present study would clearly be expected to have different filtration properties than their equivalent spheres.

A willingness to observe and characterize the particles present in natural waters, with a special emphasis on the biotic particles of potential health significance, is a prime prerequisite to the establishment of a rational filtration theory. This is particularly important if reliance for particle removal is to be shifted from conventional coagulation and sedimentation to polymer–assisted direct filtration. Since turbidity and electronic particle counting are inadequate for quantitating or characterizing the particles in treated water, it is especially important that direct observations be made when traditional particle removal technologies are not utilized.

SELECTED REFERENCES

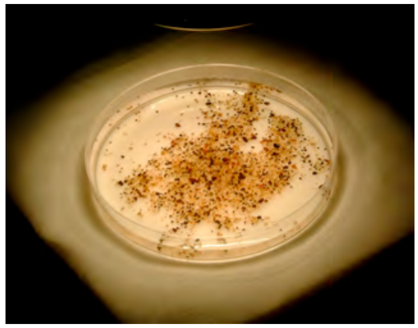
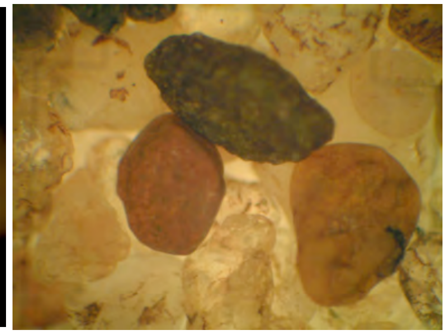
1. Hobbie, J. E., Daley, R. J. and Jasper, S. "Use of Nuclepore Filters for Counting Bacteria by Fluorescence Microscopy", Applied Environmental Microbiology 33:1225–1228 (1977).
2. Standard Test Method for "Enumeration of Aquatic Bacteria by Epifluorescence Microscopy Counting Procedure", D.4455–85, American Society for Testing and Materials (1985).
3. O'Connor, J. T., et al., “Chemical & Microbiological Evaluations of Drinking Water Systems”, AWWA WQTC, 1984.
4. O'Connor, J. T., et al., “Chemical and Microbiological Evaluations of Drinking Water Systems in Missouri: Summer Conditions”, Proc. AWWA Ann. Conf., Washington, D.C. (1985).
5. Brazos, B. J., O'Connor, J. T. and Lenau, C. W., “Seasonal Effects on Total Bacterial Removals in a Rapid Sand Filtration Plant”, Proc. AWWA WQTC, Portland, Oregon (1986).
6. Brazos, B. J. and O'Connor, J. T., "Relative Contributions of Regrowth and Aftergrowth to the Number of Bacteria in a Drinking Water Distribution System", Proc. AWWA WQTC, Baltimore, Maryland, (1987).
7. Brazos, B. J. and O'Connor, J. T., "Seasonal Effects on Removal of Particle–Associated Bacteria in a Rapid Sand Filtration Plant", Proc. AWWA WQTC, St. Louis, Missouri (1988).
8. Brazos, B. J. and O'Connor J. T., "Seasonal Effects on the Generation of Particle–Associated Bacteria During Distribution, AWWA WQTC, Philadelphia, PA (1989); published in Proc. AWWA WQTC San Diego, CA (1990).
9. USEPA (1986) Drinking Water Research Division, MERL, ORD and Office of Drinking Water,Turbidity Criteria Document, Washington, D.C.
10. Cleasby, J. L., A. H. Dharmarajah, G. L. Sindt and E. R. Baumann (1989), Design and Operation Guidelines for Optimization of the High-Rate Filtration Process: Plant Survey Results, AWWA Research Foundation, Denver, Colorado.

Questions related to bacterial cell observation and enumeration:

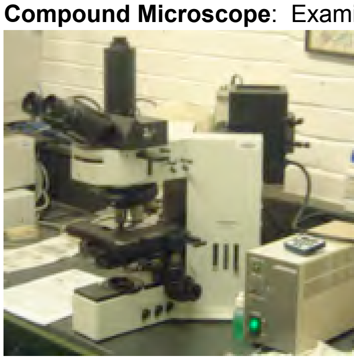
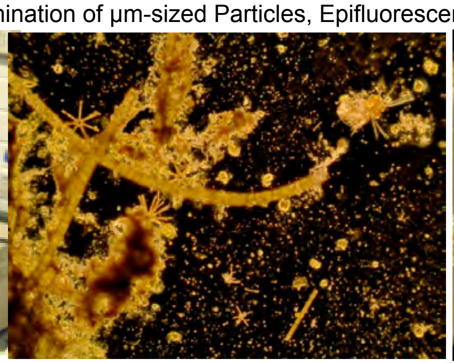
1. How many *total bacterial cells per ml* might be present in a surface water? ...a ground water?
2. Why is a 0.2 μm membrane used to recover bacterial cells? Will this membrane retain virus particles?
3. How can one determine the effectiveness of entrainment of microorganisms in coagulant floc?
4. Why is turbidity often poorly related to *total bacterial cell count*? ...to *particle count*?
5. The most popular theory of filtration predicts that 1 μm particles will penetrate filters most efficiently; smaller and larger particles will become attached to the media and retained? If so, what particles should be used to evaluate filter efficiency?
6. What is the relative abundance of members of the coliform group versus the total number of bacteria in a surface water?
7. What is the distinction between physical removal of organisms and chemical inactivation? What does HPC reduction during treatment measure? What does total bacterial cell count measure?
8. Why would bacterial cell removal be expected to decrease markedly during periods of low water temperature?
9. Distinguish between planktonic and periphytic bacteria?
10. Why is the microscope not currently widely used in the waterworks profession?
11. Do you believe you could learn to effectively use a microscope to evaluate treatment process performance?

Microscope Types and Techniques

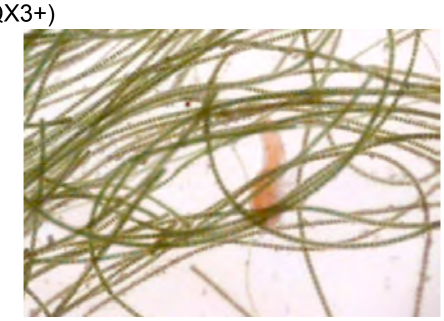
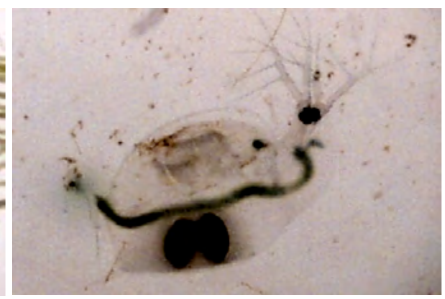
Stereo Microscope: Examination of Filter Media - Sand, GAC

Compound Microscope: Examination of μm-sized Particles, Epifluorescence Microscopy


Computer Microscope (Intel QX3+)

Treatment Process Evaluation and Distribution System Water Quality

- Water Treatment Process Performance Evaluation:
  Coagulation, Sedimentation, Filtration, Backwash
- Evaluation of Microbial Regrowth and Post-Precipitation
  in Water Distribution Systems and Household Plumbing
- Microbially-Mediated Corrosion and Water Quality Deterioration
  During Distribution

Particle Counters

- Evaluation of Particle Count Data
- Relation of Particle Count and Cell Count to Turbidity