Technology for Real-Time Detection of Microbes in Water

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Water is widely used in the production of cosmetics, foodstuffs, and pharmaceutical and other products as an ingredient, for washing, and for other purposes. The number of microbes in water used for such purposes must be kept below the level prescribed by a quality standard. Since existing testing methods require several days before results are obtained, there is a compelling need for technology that can detect microbes more rapidly. This article describes the development of equipment for real-time detection of microbes in water, and good results obtained in terms of detecting microbes and distinguishing them from particles.

1. Introduction

1.1. Methods for detecting microbes in water and associated challenges

Water is widely used in the production of cosmetics, foodstuffs, and pharmaceutical and other products as an ingredient, for washing, and for other purposes. Quality requirements for water put to such uses include the proper maintenance of equipment in order to keep the number of microbes in the water below a prescribed level, and the periodic monitoring of the number of microbes.[1]

The size of a microbe varies with the species, but generally speaking microbes look like particles with a diameter of around 1 μm (Figure 1). Identifying them requires a method such as observation with a high-power microscope. However, since what water quality standards actually require is a low concentration of microbes, such as 0.1 CFU/ml (colony-forming units per ml) for water used in injections, and 100 CFU/ml for purified water, monitoring microbes with a microscope is not practical. As an alternative, therefore, culturing is widely employed for detecting microbes. Water potentially containing microbes is applied to a growth medium to encourage the formation of colonies, clusters of microbes that are visible after several days of growth. The number of colonies is counted visually or by image identification to estimate the number of microbes contained in the water (Figure 2).

The time-tested culturing method has high specificity and other advantages. Unfortunately, however, since this kind of test takes several days to determine the number of microbes, if equipment for producing purified water is contaminated, for example, there are risks associated

with the fact that several days will pass before the contamination is detected and can be mitigated. Moreover, since some microbes do not readily form colonies, there is a risk that the contamination will not be detected. In addition, culturing raises operating costs because each periodic inspection incurs personnel costs for water sampling, culturing, counting, and related tasks.

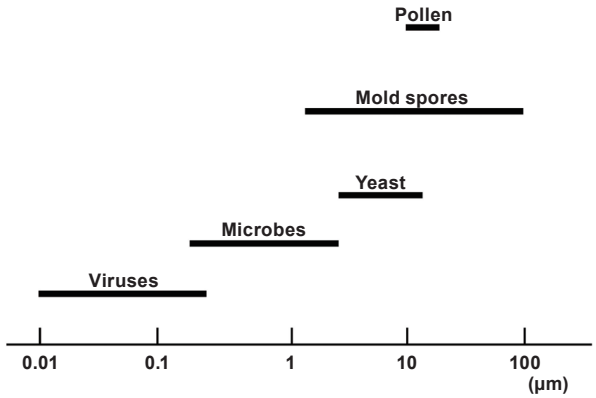

Figure 1. Size of microbes and other biotic particles

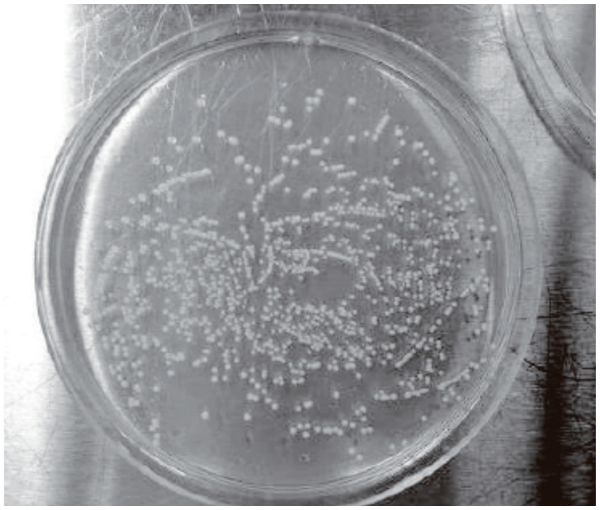

Figure 2. Visible colonies after culturing

Figure 3. Appearance of the IMD-W

For the foregoing reasons, there is a compelling need for a new technology that can detect water bioburden more quickly.[2]

In response to this need, the IMD-W™ Online Water Bioburden Analyzer was developed (Figure 3). This article describes the measurement principle and design concept of the IMD-W, and the results of evaluating its basic performance.

2. Real-time detection technology for microbes in water

2.1 Measurement principle

Water samples that pass through the measurement section of the IMD-W are interrogated with an excitation light. The resulting optical signals generated by particles in the water consist of scattered light and two different wavelength bands of fluorescent light. The intensity of each type of light is measured respectively by a photo diode (PD) and a photomultiplier tube (PMT). Figure 4 schematically illustrates this optical system.

Any particle in the water that is irradiated with the excitation light generates scattered light, the intensity of which depends primarily on the diameter of the particle and the difference between its refractive index and that of the surrounding medium, which makes it possible to estimate the intensity of light scattered from microbes in the water.

However, if the water contains not only microbes, but also inert particles of various sizes, it is impossible to differentiate the microbes solely on the basis of the intensity of scattered light. This is why the intensity of fluorescent light from the particles is also measured for the identification of microbes.

Physiologically active substances and proteins in microbes, notably riboflavin and NADH, emit their own fluorescent light in a particular wavelength band when microbes are irradiated with excitation light.[3, 4]

Detection of this unique autofluorescence makes it possible to differentiate microbes from a variety of other particles.

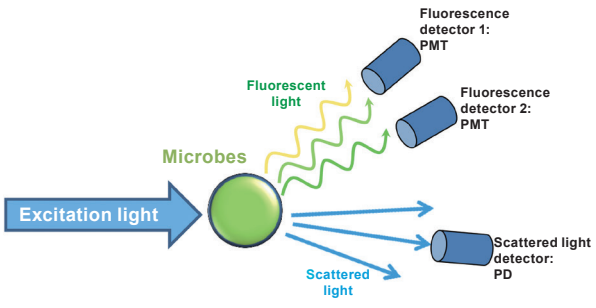

Figure 4. Schematic illustration of the optical system of the IMD-W

This basic principle of detecting microbes with scattered light and self-generated fluorescent light from microbial substances has already been applied in our commercially available products, such as IMD-A™, a real-time detector for bioaerosols.

Unlike airborne particles, however, particles in water produce very weak fluorescent signals. Development of the IMD-W therefore required the design of a new optical system with enhanced sensitivity.

2.2 Discrimination of microbes based on the characteristics of fluorescent light

Field tests using a prototype revealed that water used at manufacturing sites contains nonbiological particles, some of which also generate fluorescent light.

These particles are generated during the production process for purified water, and are thought to be metal-oxide particles from the corrosion of pipes, resin particles from gaskets and moving parts of pumps, etc. These nonbiological particles can be confused with microbes in a measurement that simply checks for the presence of fluorescence.

The fluorescence spectra from microbes and inert particles were analyzed using a spectrofluorometer in order to define a method for distinguishing between them. Figure 5 shows the fluorescence spectra of major microbes and those of resin particles that autofluoresce. The two green lines in Figure 5 represent the fluorescent spectra of microbes, and the four other lines are the spectra of resinous materials. Examination revealed that, although the peaks of the fluorescent spectra all but overlap, microbes and resinous materials have different intensity distribution profiles.

Since the measurement time of the IMD-W is limited in order to achieve real-time measurement, obtaining a detailed intensity distribution of the fluorescence spectra is difficult. Fortunately, it proved to be possible to differentiate between the fluorescent spectra of microbes and resin particles by dividing the fluorescent light into two wavelength bands and obtaining the ratio of the light intensity in these bands.

Another design factor in analyzing fluorescent light from particles in water is the presence of Raman scattering by the water, which forms a peak at a wavelength of about 50–70 nm longer than that of the excitation light.[5] In order to detect the weak fluorescent light from particles, overlap of the Raman scattering light band and the wavelength detection bands for fluorescent light must be avoided.

Determining the wavelength of the IMD-W's excitation light involves finding a wavelength that brings the Raman scattering peak between the two fluorescent light wavelength bands used for differentiating the fluorescent spectra of microbes and resin particles, while maintaining sufficiently sensitive detection and the ability to discern each microbes' unique autofluorescence. There are multiple effective combinations of wavelength bands for detecting fluorescence and excitation light wavelength. The right combination can be selected depending on the characteristics of the fluorescent light generated by the target substance. Figure 6 shows a distribution of two wavelength bands for fluorescence detection and the Raman scattering peak wavelength band.

3. Evaluation of microbial detection performance

3.1 Testing equipment

To evaluate the performance of the IMD-W, a simplified pure water loop was constructed to simulate the water purification process. The pure water loop is schematically illustrated in Figure 7. Pure water circulates at a constant rate through the loop, and the connected IMD-W monitors the water quality in real time.

A test sample in any desired concentration can be introduced into the pure water loop. In our performance evaluation, water containing microbes at concentra-

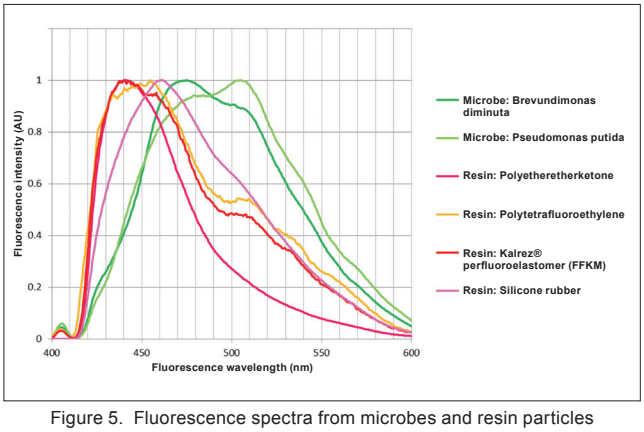

Figure 5. Fluorescence spectra from microbes and resin particles

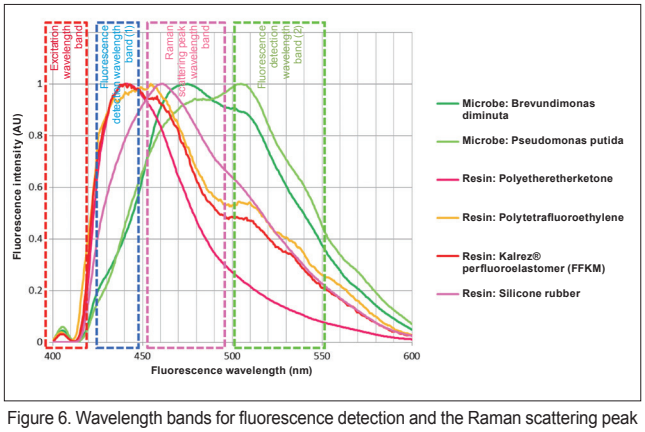

Figure 6. Wavelength bands for fluorescence detection and the Raman scattering peak

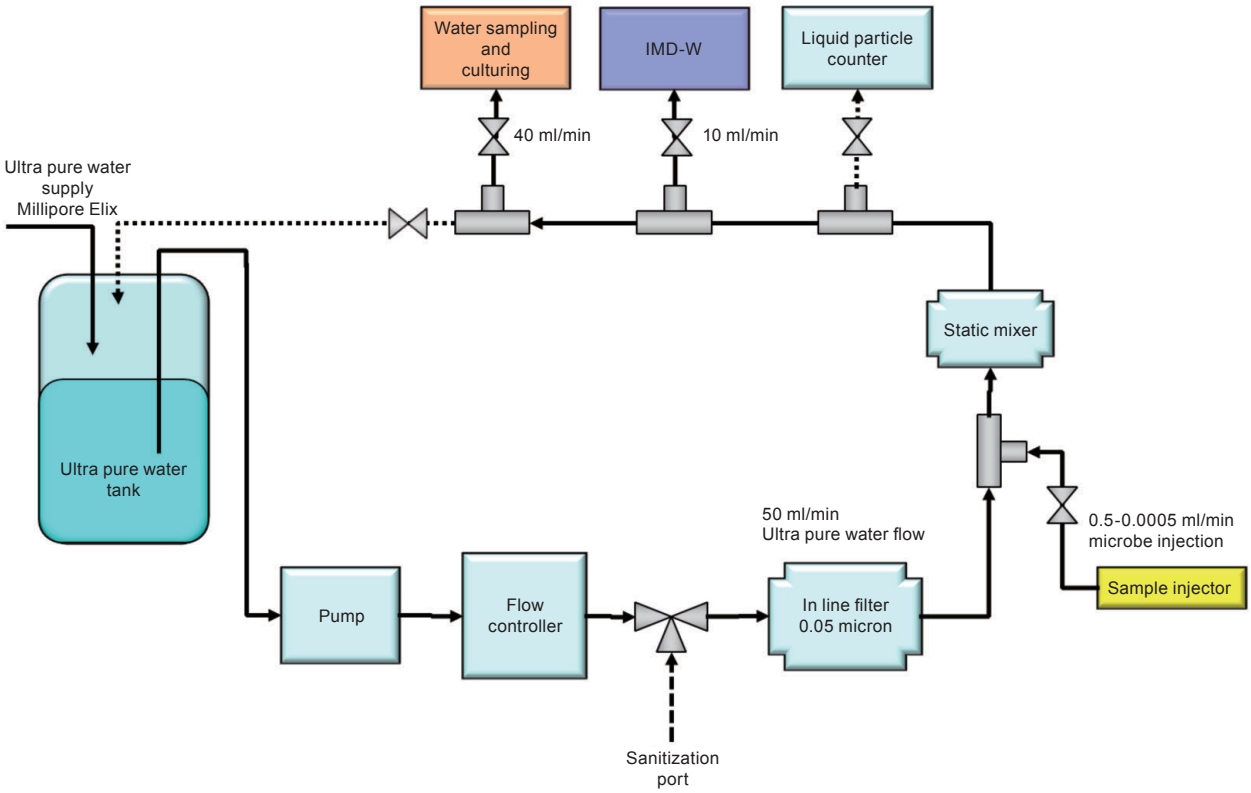

Figure 7. Pure water loop

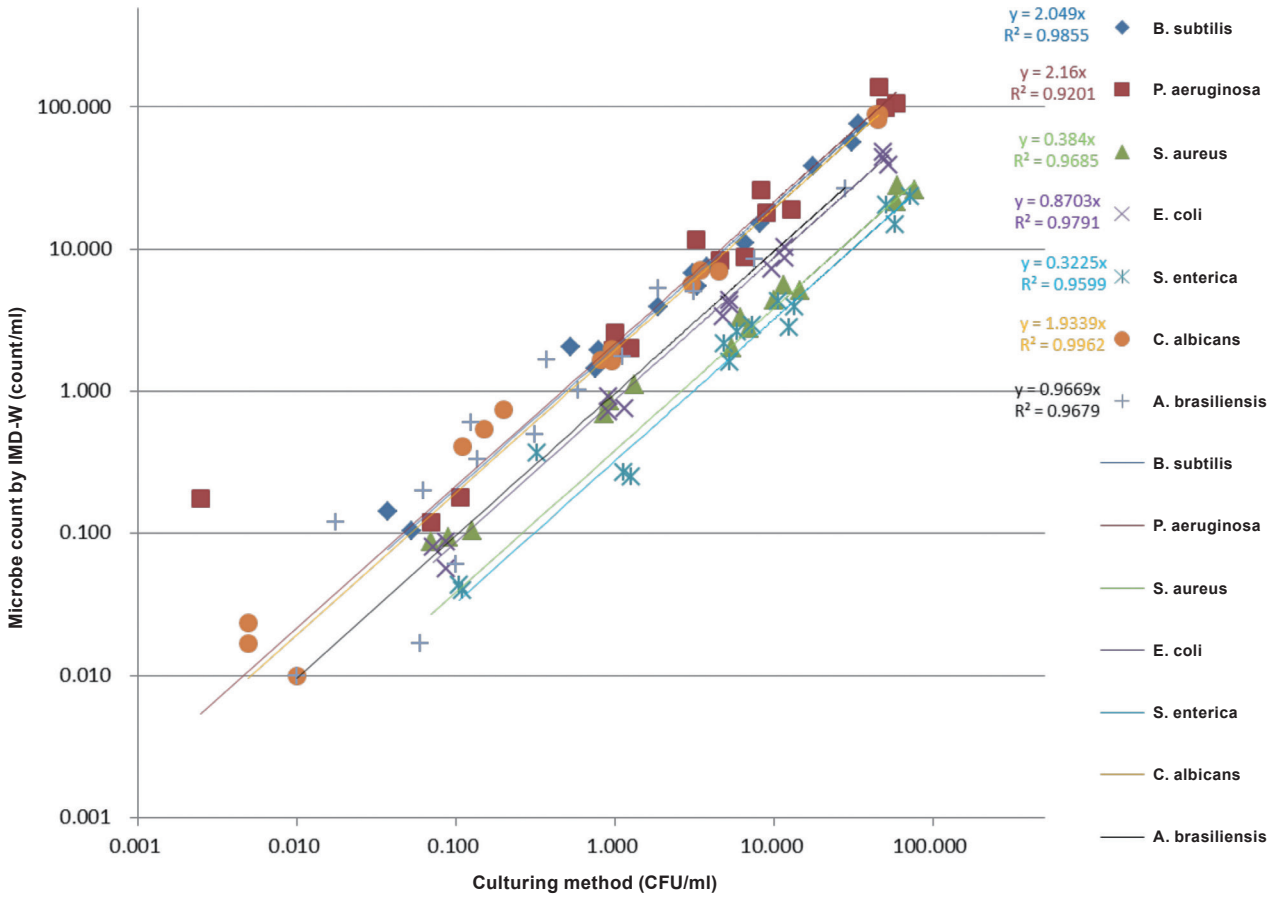

Figure 8. Scatter diagram showing microbe detection performance

tions ranging from 0.1 to 1,000 CFU/ml was introduced to see if the IMD-W could detect microbes at the different concentration levels.

At the same time, the widely employed culturing method of microbe detection was also used in order to compare its results with those of real-time detection. Seven strains of microbes were used in the tests: *Aspergillus brasiliensis* (ATCC 16404), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* (NCTC 6017), and *Staphylococcus aureus* (ATCC 6538). In order to verify reproducibility, measurements were repeated three times for each strain at four different concentration levels.

3.2 Results of the experiment

The results of multiple measurements at different microbe concentration levels are shown as a scatter diagram in Figure 8. The vertical axis shows the number of detected microbes during real-time measurement with the IMD-W, and the horizontal axis shows the number of microbes found after several days of culturing water sampled at the same time. The linear approximation and correlation coefficient are given for each strain.

A linear relationship was demonstrated between the two methods. With the exception of one strain, the correlation coefficient was 0.95 or greater.

In terms of the ratio of detected microbes, compared to the culturing method, the IMD-W was found to have detected a smaller number of microbes in the series, with a coefficient for linear approximation of 1 or less. The IMD-W detected a smaller or greater percentage of microbes depending on the strain. The difference possibly owes to the fact that the IMD-W's sensitivity tends to depend on the optical properties of particles, whereas the sensitivity of the culturing method depends on the adaptability of the microbes to the culturing environment.

This experiment demonstrated that the IMD-W, for each of the tested microbial strains, displayed a response to contamination by microbes at low concentrations of around 0.1 CFU/ml, and that it can monitor changes in the concentration of microbes in water even at a low level.

4. Distinguishing microbes and abiotic particles

4.1 IMD-W's function for distinguishing particles

The IMD-W measures the scattered light and fluorescent light in two wavelength bands from particles. The intensity of these three types of light is analyzed to distinguish microbes from other particles. Figure 9 is a sample scatter diagram of the intensity of three types of light during particle measurement. Red dots show signals of microbial origin, and blue dots signals from resin particles.

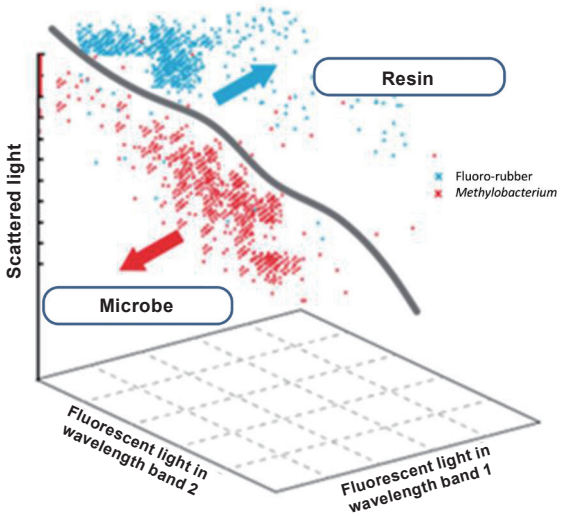

Figure 9. Microbe identification using the discrimination border

The IMD-W can train separately on known microbes and abiotic particles to analyze their three-dimensional distribution and define the best border for efficiently differentiating them.

In the testing conducted for this article, measurements were done with three strains of microbes, particles from two types of resin gaskets, and the product of stainless steel corrosion.

The results in terms of identification are shown in Table 1. For the three strains of microbes, the device identified microbes correctly with 98% or greater accuracy and could correctly determine that particles from resin gaskets and stainless steel corrosion were abiotic with around 90% accuracy.

Good identification results were also obtained with abiotic particles that emit their own fluorescent light, but performance sometimes varies depending on the types and conditions of the particles, since the IMD-W identifies them based on the characteristics of scattered light and fluorescence. Further relevant data needs to be collected.

Table 1. Accuracy rate in discrimination of resin particles and microbes

Target	Microbe exclusion rate	Remarks
Kalrez® (DuPont product)	89%	Resin: fluorinated gaskets
Silicone rubber	92%	Resin: gaskets
Products of stainless steel corrosion	91%	Main component of rouge

Target	Microbe ID rate	Remarks
Bd (*Brevundimonas diminuta*)	99%	Aquatic microbe
Pp (*Pseudomonas putida*)	99%	Aquatic microbe
Me (*Methylobacterium extorquens*)	98%	Aquatic microbe

5. Conclusion

This article presents the measurement principle of the IMD-W and the results of evaluating its basic performance. A field demonstration is being carried out with an eye to the applications below.[6]

- Improving quality control for microbial contamination in the production process of purified water by real-time and continuous measurement of microbe count to supplement the officially required periodic culturing method
- Early understanding of the extent of contamination risk by means of prompt response when contamination of water occurs
- Understanding of equipment integrity by tracking trends in the amount of particles that appear in water

By means of this technology, microbes can be detected using a principle that is entirely different from the culturing method that has been widely used to date. Although application requires careful attention to the properties of the target microbes, the authors hope to provide further benefit to users by making the most of the possibilities of real-time and continuous measurement and control of microbes in the water purification process.

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