Research Article

Monitoring the Microbial Quality of Water

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Monitoring the microbial quality of water is essential due to the global impact of waterborne infections such as E. coli, Salmonella, Vibrio cholerae, Giardia, and others. This brief review outlines key physicochemical and microbiological standards and describes methods for detecting fecal and non-fecal pathogens, including culturing, optical density measurements, and molecular techniques like 16S rRNA sequencing, qPCR, and FISH. Emerging tools such as flow cytometry, microfluidics, and stable isotope probing are also discussed. Disinfection practices, especially chlorination, are evaluated alongside environmental, procedural, and usage factors critical to accurate microbial risk assessment.

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Contaminated water is responsible for numerous waterborne infections usually acquired through drinking water, swimming in pools and natural resources (lakes, rivers), and bathing in hot tubs and on beaches. The most common infections are *Escherichia coli (E. coli), Salmonella, Vibrio cholerae* (cholera), *Hepatitis A, Rotavirus, Giardia, Naegleria fowleri* (brain-eating amoeba), and *Cryptosporidium*[1]. Estimates are that about 2 billion people use contaminated drinking water, and about 502,000 die due to waterborne diarrheal infections yearly[2]. Besides, schistosomiasis, an acute and chronic disease caused by parasitic worms contracted through exposure to infested water, has burdened the health authorities with over 251.4 million people requiring preventative treatment for the infection[2]. There are almost 1.7 billion cases of childhood diarrhoeal disease every year, and contaminated water or no access to safe drinking water is the primary cause[3]. Diarrhoea stands 2nd in killing children after pneumonia, and it alone kills more than those killed by malaria, AIDS, and measles combined[1]. Diarrhoeal disease ranks in third place, causing deaths in children below five years of age, killing about 443,832 children below five and 50,851 more children five to nine years of age[3]. Therefore, the assurance of the microbial quality of water is of utmost importance for safe drinking water supply agencies. Physicochemical standards of

water, including *p*H (acceptable range is 6.5-8.5), turbidity (acceptable limit is 1 Nephelometric Turbidity Unit, but the permissible limit is 5 NTU), total dissolved solids (TDS, acceptable limit is 500 mg/L, permissible limit is 2000 mg/L), temperature (10-22^{o}C), biological oxygen demand (BOD, limit is <5mg/L), dissolved oxygen (DO, should be between 6-8 mg/ L), chemical oxygen demand (COD, <10 mg/L), and heavy metals (as lead <15 ppb, arsenic 10 ppb, cadmium 5 ppb, copper 1.5 ppm, iron 0.3 ppm, etc.) are regulated by various national and international agencies[4][5]. Like physicochemical standards, microbiological standards are also available for some pathogenic and indicator (sentinel) organisms[2]. However, the emergence of new pathogens and improvement in detection limits of microbial contaminants in water is still an emerging area of research and warrants continuous monitoring of microbial water quality. Though fecal intrusion is considered the biggest risk in water, there are several pathogenic bacteria which are of non-fecal origin and transmitted through water, including *Aeromonas* (causes gastroenteritis, soft tissue, and skin infections), *Legionella* (legionnaires' disease), *Serratia* (causing septicaemia, pneumonia, and soft tissue infections), and *Mycobacterium* (causing tuberculosis, leprosy, nontuberculous soft tissue, and skin infections). In a recent study on drinking water supplies in India through samples collected from public taps[6], several potentially pathogenic bacteria like *Aeromonas salmonicida, Pseudomonas aeruginosa* and *P. pseudoalcaligenes* were detected in drinking water. A waterborne outbreak of A. hydrophila in mares at a thoroughbred stable led to infertility and abortions in mares[7]

Monitoring of microbes, specifically bacteria in the drinking water system, can be done using various techniques, including culturing methods, optical density measurements, and molecular techniques like 16S rRNA amplicon sequencing and metagenomic approaches for emerging pathogens. Culturing means facilitating microbes by providing a nutrient solution and growing them in the appropriate environment at an optimal temperature. Optical density is a measure of the turbidity of the solution or the growth medium; it increases with an increase in bacterial cells. Molecular techniques facilitate the detection of culturable and non-culturable microbes and provide insights into diversity, elucidating the whole spectrum of microbial communities or microbiomes. In brief;

1. Culturing methods: Dr. Mark Sobsey, of the University of North Carolina at Chapel Hill, outlined the ideal characteristics of a microbial test for water quality monitoring on a limited budget:
It should be portable, low-skill, self-contained, lab-free, and electricity-free. It should be available globally at a cost of less than $0.10 (USD) per test, and it should be easy to interface with data reporting and communications technologies. The test should also be good enough to be integrated into education

programs to mobilize stakeholders. The test should preferably result in (semi) quantitative data available quickly, without an incubation period. This "**holy grail**" test does not yet exist. The culture test may be

- **Simple Tests:** Samples are tested in tubes, bags, or plates with a nutrient solution, and positive growth is indicated by a color change of the indicator. The common indicators used are; TTC (2,3,5-triphenyltetrazolium chloride), is reduced by metabolically active (growing) bacteria to form a red formazan product, visibly detected; INT (2-[4-iodophenyl]-3-[4-dinitrophenyl]-5-phenyltetrazolium chloride), it is also reduced to a red-orange formazan product by growing microbial cells, XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt), it is also a tetrazolium salt, growing bacteria reduce it to an orange-colored product by active microbes, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is reduced to a purple formazan product by multiplying bacteria, resazurin, it is a colorless dye in its oxidized form and turns blue-pink when reduced by metabolically active microbes, and CTC (5-Cyano-2,3,-ditolyl tetrazolium chloride).
- **Enumeration:** This test involves counting the number of colonies growing on a solidified culture medium, providing an estimate of bacterial cell counts. But it can count only those that can grow in the specified medium.
- **Differential microbial analysis:** This method is used to indicate the diversity of microbes present using several different growth media and incubation conditions. It can give an indication about faecal contamination of water. The count for Enterococci is used to detect a relatively recent past, and the *Escherichia coli* and coliform count for the recent faecal intrusion into water. Besides, sulphite-reducing *Clostridia* spore count using the roll tubes, heterotrophic bacterial count as an indicator of total bacterial load, is done through spread agar methods. In drinking water, no *Giardia lamblia, Cryptosporidium, Legionella,* and enteric viruses are detectable in a 100 mL water sample. Further, no more than 5.0% of samples of water should be total coliform-positive (TC-positive) in a month for water supplies collecting water samples at multiple times regularly every day. For waterworks systems collecting <40 samples per month at regular intervals, no more than one sample can be tolerated if positive for total coliforms per month. Every sample that has total coliform (bacteria that ferment lactose with acid and gas) must be analyzed for fecal coliforms (ferment lactose to produce acid and gas at 44.5 °C (include *E. coli, Klebsiella pneumoniae, Citrobacter freundii, Enterobacter aerogenes, Hafnia, Serratia, Yersinia*) and *E. coli.* If two consecutive samples are TC-positive, and one also contains *E. coli,* fecal coliforms, the system is considered an acute MCL violator.

- **Detection of Enteric viruses** may be used for past contamination. Somatic coli (MS2) phages are sometimes monitored for detecting coliform load, either using plaque assay or quantitative polymerase chain reaction (Q-PCR).

2. Optical density (turbidity) measurements: As bacteria grow, the cell number increases, turning a transparent solution cloudy or turbid. The turbidity can be measured using a spectrophotometer to measure the refraction of light at a 600nm wavelength (OD_{600}). A standard curve is required to be created relating colony-forming units (CFU) to optical density. The curve can be used to approximate the number of bacterial cells in the solution. Along with turbidity, the electrical conductivity (EC) of the solution can also be used to further improve the efficacy of the method.

3. Molecular Techniques: Usually, 100-200 mL of sampled water is processed using dead-end ultrafiltration (DEU) through a 0.22 µm pore size membrane filter; most of the microbes, except viruses (enteric viruses may escape without possible detection), are retained on the filter membranes. The filter membranes are used for the extraction of DNA. On the extracted DNA, several tests can be used to understand the diversity of microbes present in the test water. The common methods include:

a. **16S rRNA amplicon sequencing:** This technique analyses the genetic makeup of microbial communities, revealing species composition and diversity in groundwater. The common method is sequencing the PCR product using primers Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC), which target the V3–V4 variable region of the 16S rRNA gene. This method can be specialized to detect faecal intrusion into drinking water through specific detection of Bacteroidetes and *Bacteroide*s. Bacteroidetes (including genera of *Bacteroides*, *Parabacteroides*, *Prevotella*, and *Alistipes*), and *Bacteroides* (*B. vulgatus, B. thetaiotaomicron, B. distasonis,* and *B. fragilis*). Both groups of bacteria are anaerobic, non-spore-forming gram-negative bacteria, constituting about 25% of the intestinal gut microbiome, with many beneficial functions for human health.

b. **Fluorescence in Situ Hybridization (FISH):** Allows for in-situ identification and visualization of specific bacterial species employing fluorescent conjugated specific DNA sequences which hybridize with homologous regions in the target bacterial genome.

c. **Quantitative PCR (qPCR):** Provides quantitative information on the abundance of specific microbial genes or organisms using specific primers.

4. Other Techniques for monitoring microbial contamination in water:

a. **Time-lapse microscopy:** Capturing microscopic images at regular intervals and analyzing images of micro-colonies. It allows monitoring bacterial growth and replication rates of the microbe at the single-cell level using microcolonies.
b. **Microfluidics and nanofluidics**: Microfluidics and nanofluidics study the mass (including molecular and colloidal) and momentum transfer, heat transfer, and reactive processes, coupled with transport in microscale and nanoscale systems using microfluidic chips enclosed in a leak-free microfluidic circuit. This method uses a controlled growth environment for bacterial growth. This method largely improves the temporal and spatial resolution of microbial growth dynamics and kinetics.
c. **Flow cytometry:** This method offers rapid and accurate counting of bacteria using a fluorescent dye to stain bacteria in real time, and it also differentiates between live and dead bacteria. It provides high-frequency measurements of microbial dynamics, revealing temporal patterns and fluctuations.
d. **Stable isotope probing:** This technique can be used to determine the bacterial count and growth of specific functional groups in a microbial community. In the technique, an isotopically labelled substrate (e.g., 13C introducing) is incorporated into the microbial community, and then isotope accumulation identifies organisms from the most active to the least active. It can also measure the fraction of degradation directly caused by microbial activity during bioremediation. Determining dual isotope ratios (2H/1H and 18O/16O) is a useful indicator technique in this method.

More about bacterial culture methods: In waterworks organizations, the most popular method is culture, and for the enumeration of bacteria, some specific media are used. Common media used include Luria Bertani (LB) and 2XYT broth media, but none of these media support the growth of waterborne viruses, protozoa, and helminths (worms) that can be harmful to human health. Further, testing for all or a large number of different pathogens is infeasible. Thus, a practical solution is to test for an "indicator organism," which may act as a sign of fecal contamination, like *E. coli*. In water, *E. coli* should not be detectable in a 100 mL water sample to ensure water safety. Bacteria by culture methods may be detected qualitatively, quantitatively, or semi-quantitatively by different methods.

a. **Presence-absence (PA)**: PA tests don't provide quantitative information about microbial water quality. These tests employ some dyes reduced by metabolically active or multiplying bacteria. The changes in color of the media due to the reduction of the dye indicate the presence of microbes, indicating microbial contamination. The PA tests are comparatively inexpensive; they involve

adding a sterilized powdered nutrient mixture to the test water and a 24-hour incubation period for observing the growth. The PA test is suitable for screening in situations where the chances of microbial contamination are minimal, such as deep groundwater. However, the presence of slow-growing bacteria or those growing at a different incubation temperature (than used in the test), requiring a specific growth medium, and a growth environment may lead to false negative observations.

b. **Most probable number (MPN)**: All MPN tests are semi-quantitative. In the process of testing, several sample quantities of the same water are tested in multiple tubes, plastic bags, or small plastic plates with multiple wells. Single strength (for quantities less than 1% of the sample volume to media) and double strength culture media are added and incubated at the desired temperature (30-32^{o}C for total coliforms and at 44.5^{o}C for faecal coliforms) for 12-48 hours for organisms to grow before counting the number of positive tubes for different dilutions of the samples. The positives are indicated by a color change in the media. You can convert the number of positives to a statistical estimate of bacterial concentration using MPN tables[8].

c. **Membrane filtration**: Membrane-based tests are quantitative and the most accurate for counting live bacteria. For the test, a 100 mL water sample is forced or vacuumed through a small, round filter membrane (of about 45 mm diameter) having a pore size of 0.22 µm. The membrane retains almost all the bacteria in the sample as the water passes through it. The filter membrane is then carefully removed and incubated in appropriate culture media plates. Each bacterium caught on the filter membrane is supposed to multiply and form a little colony. After the incubation for 24-48 hours at an appropriate temperature, the colonies are counted using a colony counter or using a magnifying hand lens to determine “colony-forming-units” per membrane (present in the 100 mL of the sample). However, these tests are a bit labour-intensive and time-consuming.

d. **Counting sulphate reducing bacteria in water:** Sulphate-reducing bacteria (SRB) belong to a diverse and heterogeneous group of heterotrophic anaerobic bacteria. They are important microorganisms in natural ecosystems and in deep well waters. They are often used for removing heavy metals and sulphate from acid rock drainage and other wastewater. Sulphur-reducing bacteria live in anoxic or oxygen-deficient (reducing) water, such as from deep wells, stagnant plumbing systems, stagnant distribution systems, biofilms in water softeners, biofilms in water heaters, on the hot-water side of a water distribution system, and on the side of hot springs. The presence of sulphate-reducing bacteria can be smelled by a rotten egg odour (due to the production of hydrogen sulphide, H_2S,

instead of oxygen they use sulphur molecules as electron receptors in their metabolic pathways), a metallic sheen, slimy coatings that appear yellow, brown, red, and or black, and brown foam on the surface or water colour. The human nose can detect H_2S if it is in the air at as low as 0.5 ppm levels, but gradually we adapt to the odour and stop smelling it. Water having <1 ppm H_2S smells "musty" or "swampy," and with 1-2 ppm H_2S smells like a "rotten egg." Water with a rotten egg smell is very corrosive and harms plumbing pipelines. The SRBs are counted using the MPN method, inoculating in Starkey's medium (A or B) and layered with paraffin to create anaerobic conditions. A litre of Starkey's medium contains 10.0 g tryptone, 1.0 g sodium sulphite, and 10.0 ml of 5% ferric citrate. They can also be detected using qPCR employing *dsrA* and *apsA* gene primers.

e. **Detection of bacteriophages**: It is not possible to detect all phages, but MS2 phages, an indicator of *E. coli* and enteric viral contamination in drinking water, are often counted (detected) and have an easy system to detect and count them. The MS2 phage must be absent in 1 litre of water. It can be detected using *E. coli* strain C-3000 (ATCC 15597). The MS2 phage detection is considered a more accurate indicator of viral pathogens and fecal pollution, while enterococci are a more traditional bacterial indicator. Enterococci are widely used as indicators of fecal contamination, especially in recreational water. Enterococci are more persistent than *E. coli*, but MS2 phages can be more persistent and abundant than either of the two bacteria. Coliphage MS2 survives for 12 days, *Vibrio cholerae* for >27 days (however, it goes undetectable after 3 days and after 5-6 days of eclipse, it reappears again on the 9^{th} day of contamination), *E. faecalis* survives for 9 days in water. In sediment, MS2 phages, *V. cholerae*, and *E. faecalis* are detectable up to 4 days, >27 days, and 18 days, respectively. The presence of zooplanktons like algae, zooplanktons, flagellates, parasites, toxin-producing organisms, Bryozoans (*Plumatella*), molluscs (*Dreissena*), and crustaceans (*Asellus*) in water affects the survival of MS2 phages, *V. cholerae*, and *E. faecalis* and protects them from the detrimental effect of chlorination/ disinfection.

Factors necessary to be considered while assessing water quality:

a. **Environmental Factors:** Altitude, vegetation, temperature, *p*H, nutrient availability, air flow, and other components of the environment influence microbial growth and their persistence in the water system.

b. **Sampling Methods:** Proper sampling procedures and selection of sampling sites are crucial to avoid contamination and ensure accurate assessment of microbial quality of water.

c. **Scale:** Monitoring scales for water quality (frequency of sampling, single sample vs. continuous sampling, regular and irregular sampling) can affect the outcome of the testing. The continuous real-time sample analysis is the best, but highly expensive, way to understand different aspects of water microbial dynamics.

d. **Expected use of the water to be tested:** There are different standards for natural, potable, drinking, and irrigation waters, and thus, the tests are adjusted and chosen as per the need.

Curing of water

Chlorination is the most commonly used method in most of the water works units supplying water for human consumption. Chlorination deactivates microorganisms through damaging the cell wall, altering the permeability of the cell, altering the cell protoplasm, inhibiting enzyme activity so it'sunable to use its food to produce energy, and inhibiting cell reproduction due to its strong oxidizing potential. However, at the dosages normally employed in waterworks, it is ineffective against certain spore-forming bacteria, parasites, and their cysts. To handle the persisting microbes, either filtration or higher chlorine doses without subsequent dechlorination are needed. To ensure freedom from causal agents of amoebiasis, giardiasis, and cryptosporidiosis, microscopic examination after concentration is recommended. The cyclops carrying embryos of *Dracunculus medinensis* (cause of dracunculiasis or Guinea-worm disease) are common in open wells, harvested rain water, and water from water reservoirs used as the source of water for humans and animals in most of the tropical areas.

Further, small, non-enveloped viruses (Poliovirus, Enterovirus, Rhinovirus) are more difficult to inactivate using chlorination; they are often more resistant to chlorination than MS2 phage for chlorination than canine parvovirus, and human poliovirus to chlorination. Large, non-enveloped viruses (Adenovirus, rotavirus, or papillomavirus) are moderately difficult to inactivate, while enveloped viruses (Influenza, herpes virus, or hepatitis virus) are easy to inactivate with most water treatment methods.

Chlorination can be done at several stages of the water treatment process. Pre-chlorination is done almost immediately after the water enters the treatment facility, where the chlorine is added directly to the raw water to eliminate aquatic flora and fauna. It removes off-tastes and odors and oxidizes any iron, manganese, or hydrogen sulfide if present in the water. Chlorination done after sedimentation and before filtration aims at controlling microbial growth, removing iron and manganese, removing off-tastes and odors, and also removing the coloration of the water without affecting the growth of the biota in the sediments. Chlorination at the final step in the water treatment process is usually done in most

treatment plants to add chlorine to disinfect the water and maintain enough residual chlorine in the treated water while it travels through the distribution system. The residual chlorine in water controls pathogens from re-growing, slime, and biofilm growth in the distribution system. To achieve effective chlorination, commonly used chlorine sources are:

- Gaseous chlorine: At a 1 - 16 ppm level.
- Calcium hypochlorite (bleaching powder): At a 0.5 - 5 ppm level.
- Liquid sodium hypochlorite solution: At a 0.2 - 2 ppm level.

Besides chlorination, there are several other methods of water treatment used to a variable extent, including reverse osmosis, UV disinfection, filtration, ozonisation, halogenation (with iodine and bromine), and silverization (silver nanoparticles). Sanosil, a technology of water treatment, uses a combination of hydrogen peroxide and silver to disinfect water. An ideal method of water disinfection/treatment should not leave any noxious (hazardous to health) byproducts behind after treatment. Even chlorination produces trihalomethane (THM) and haloacetic acid (HAA) if the water has higher values of organic contaminants like humic acid and fulvic acid (mainly present in rainwater harvesters and run-off water accumulated in ponds, lakes, and other surface water reservoirs used as a source of drinking water). THM is a carcinogen and should not be present in water beyond 0.1 ppm levels.

Another oxidizing agent used for water disinfection is potassium permanganate ($KMNO_4$), commonly used at a 1-3 ppm level; for open well water, it is used at a 1000-2000 ppm level. It can remove impurities like off-taste and odor-causing substances, limit the amount of THM and HAA formation (byproducts of halogenation), as well as soluble manganese and iron. However, if excess is added, it causes pink discoloration of water and an increase in manganese concentration; the permitted limit for manganese in water is 0.05 ppm.

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