Identification of Microbial Contamination in Water Treatment and Distribution Systems

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Singapore’s national water agency, the Public Utilities Board (PUB), not only carries the responsibility of providing higher-quality and safer water, it performs research and development (R&D) in water technologies to safeguard our water systems. A team of researchers at the Centre for Advanced Water Technology (CAWT) – PUB’s in-house research arm – launches research projects on applications of advanced biotechnology in water science and technology to identify microbial contaminants in water.
Biotechnology Applications in Water Science

Of all the natural resources, water has always been one of the most important. To ensure the safety of critical water supplies, culture-based techniques have been employed by the water industry for decades to assess and predict water microbial contamination. While conventional methods have been accepted as the gold standard for identification of pathogenic microorganisms, such diagnosis takes days or even weeks. This time-lag will not be appreciated with the emergence of possible biological threats in our waters. Moreover, some microbial organisms (e.g., *E. coli*) may transform from an easily culturable state to a viable but non-culturable state (VBNC), thereby falsely suggesting that they are not present when the detection test is carried out using culture method. Furthermore, approximately 99% of microbes in water remain unidentified due to inadequate understanding of the real conditions in which the microorganisms survive. This is a fundamental obstacle towards understanding microbial diversity and activities in water system.

Conventional methods do not adequately assess water quality which is posing an acute problem for public health and the environment. Thus, novel DNA/RNA-based and mass spectrometry (MS) techniques with the merits of fast, specific, reliable, sophisticated and cost-effective detection have been sought by authorities and utilities, today more than ever, to assess water supplies and assist in alerting water facilities. In the E.U. and the U.S.A., water authorities have begun to use these innovation tools for risk assessment, pathogen identification and detection, and source identification.

As with many water agencies around the world, Singapore’s national water agency – Public Utilities Board of Singapore (PUB) is challenged with finding the right balance between supplying high-quality water for the public and protecting watersheds while enabling the full spectrum of water uses. In addition, several R&D projects on the implementation of molecular tools and biological mass spectrometry (BMS) to water quality monitoring in Singapore water system were initiated to help PUB achieve stricter monitoring standards of water supply. These research projects are to make a paradigm shift in routine analysis of raw water and treated water by developing and applying advanced molecular methods and mass spectrometry (MS) techniques. As a result, the microbial population and key pathogenic microorganisms will be monitored on a molecular or mass profiling basis rather than a morphological identification of selected species only.

Due to their complexity and wide-ranging utilities, research works in biology and biotechnology are particularly interdisciplinary. A good research scientist must have a broad knowledge of chemistry, biology and microbiology. Today, many research projects in water fields are not single disciplinary-based. They have overlapped two or even more traditional areas, such as chemistry, microbiology, molecular techniques, physics, spectroscopy, computer science and material science.

CAWT is involved in many research projects in water science and quality. A few of the projects that employ biology, biotechnology, microbiology and chemistry are introduced in this article:

1. Identification of waterborne bacteria by MALDI-TOF-MS coupling with novel separation and purification techniques
2. Fecal contamination and source tracking in catchments by PCR-based approaches

3. Molecular identification of toxigenic cyanobacteria and blooming tracking in reservoirs

4. Elucidation of microbial species in desalination membranes and microbial water quality monitoring using DNA-based molecular technique

**RESEARCH PROJECT**

**Identification of waterborne bacteria by MALDI-TOF-MS coupling with novel separation and purification techniques**

For identification of microorganisms in water, antibody-based methods and genetic methods are increasingly employed as alternates and supplements to conventional culture and biochemical activity test methods. They are sensitive, selective, rapid and labor-saving. However, antibodies are well known to bind to specific microbial proteins and a primer must be selectively designed for the genome of a particular microbe in a PCR method. These technologies are specific to particular microorganisms, thus they are not ideal for one-time wide screening for bacteria in water samples during emergencies.
Biological mass spectrometry (BMS) has now been proven to be a rapid, sensitive, versatile technology for characterization, differentiation and identification of microorganisms, and has numerous applications in clinical isolates, food samples and airborne particulates. Its role as an emerging, promising technology for the screening of microbes has also been widely recognized in recent years and some portable mass spectrometers are available. Scientists all over the world are interested in the potential applications of BMS technology for identification of microbes, especially waterborne pathogens in water, and several R&D projects are underway. The group at CAWT is also working towards this purpose.

How can BMS perform microorganism identification?
Simply, BMS can be regarded as MS for biological molecules. MS is an analytical technology that identifies the chemical composition of a compound or a sample on the basis of mass-to-charge (m/z) ratios of ions formed. In a mass spectrometer, ion source transforms the molecules in a sample into ions with specified masses and charges. Singly charged ions are easily produced in matrix assisted laser desorption/ionization (MALDI). Hence, MALDI has become one of the most powerful ionization techniques for MS analysis and studies of polymers and biomolecules (peptides, proteins, enzymes, nucleic acids, carbohydrates and lipids), imaging studies of biological tissues, and characterization of bacteria, viruses, parasites and fungi.

BMS responds to the chemical compositions of microbes rather than to their three-dimensional structures or biological activities. For a typical microbe, its composition may include hundreds of different proteins. Some
of the proteins may be unique to a particular strain, species or genus and would therefore serve as the basis for the differentiation of strain, species or genus. The identification can be done in two approaches. One of them is mass fingerprinting (or protein profiling) and the other is proteomics.

**Mass fingerprinting approach**
In mass fingerprinting approach, the MALDI mass spectra of the isolates obtained from examined samples are acquired with an appropriate MALDI matrix and under optimal instrument condition (normally in linear positive mode) with a MALDI time-of-flight mass spectrometer (MALDI-TOF-MS). Automated match of the sample spectra against a well-established reference library of characterized microorganisms will yield the identification results as well as related evaluation. So far, most applications have focused on bacterial isolates since other microbes are difficult to grow or ask for stricter safety measures. For mass fingerprinting approach, the key is a universal, robust protocol to improve spectral reproducibility and an established spectral database containing a lot of entries. In some protocols, bacterial cells scraped from culture plate are directly deposited on a MALDI target plate without any treatment prior to being overlaid with MALDI matrix solution and researchers call this the intact cell mass spectrometry (ICMS). ICMS is very simple and does not require sample treatment but the reproducibility with respect to peak intensities among target wells is not so satisfactory. More commonly, bacterial colonies or cells go through a wash procedure before their deposition in the target wells and the spectra acquired with much better reproducibility still belong to whole cells.

**Proteomics-based approach**
Proteomics-based approach follows a different procedure from mass fingerprinting. At first, an intact protein mixture (proteome) is extracted from a microbial isolate with organic solvent under high pressure. The proteome is separated by high performance liquid chromatography (HPLC) and the effluent fractions are spotted in a MALDI plate with an automated MALDI spotter. Offline MALDI MS analysis can give the identities of proteins through searching within a database of proteins using common tools, e.g., MASCOT and SEQUEST. From such known proteins, it is possible to deduce which microbe it is. Instead of offline MALDI analysis, online HPLC-MALDI quadrupole time-of-flight MS (QTOF-MS) can profile the proteome, and differentiate species, serovars and even strains. Other than direct separation of proteome, the proteins are denatured, reduced and digested with trypsin in shotgun proteomics. The peptides obtained are then analyzed with HPLC (or nano LC, multidimensional LC) tandem mass spectrometry and identified by searching within a protein database assembled from theoretical proteomes of bacteria with fully sequenced genomes. The proteomics-based MS can simultaneously identify several bacteria present together in a sample. Some researchers identify in-gel proteins after 2-dimensional gel electrophoresis with HPLC-MS/MS but for bacterial identification purpose, such a combined procedure is tedious.

BMS, especially MALDI-TOF-MS, has been successfully applied to microbial isolates from clinical, food, aerosol and soil. However, it faces some
challenges when directly applied to the identification of microorganisms in drinking water, recreational water and wastewater. Firstly, the current commercially available MALDI spectral database of whole cells cover just a minor part of the waterborne bacteria and protocol-associated spectral entries in some existing databases are not characteristic enough for reliable identification. Secondly, microorganisms are at much lower concentrations in drinking water and sometimes, several species of microbes may be present together. Researchers are devoted to finding solutions to these key problems.

An ongoing research project at CAWT aims to develop a BMS-based technology for the identification of waterborne pathogens bacteria in water treatment and distribution systems. It is based on the combination of MALDI-TOF-MS analysis of microbial isolates and novel separation/purification techniques. We have selected the following bacteria as the targets: Escherichia coli, Salmonella spp., Aeromonas spp., Pseudomonas aeruginosa, Helicobacter pylori, Bacillus spp., Campylobacter spp., Yersinia spp., Staphylococcus aureus and non-tuberculosis mycobacteria.

Both mass fingerprinting and proteomics-based MALDI-TOF-MS as described above are under investigation. A protocol has been developed to achieve reproducible, more informative spectra of bacterial cells (Figure 1). We are developing a MALDI spectral library of whole cells of waterborne bacteria, which is crucial to microbial identification by fingerprinting. Proteomics-based identification is expected to be studied with HPLC-MS/MS and HPLC followed by offline MALDI-TOF-MS. Different spectral data processing algorithms will be compared to extract useful information and minimize background and interference. The reliability, reproducibility, rapidity, etc., will be evaluated.

![Figure 1. MALDI spectra of CLED cultures of two bacterial strains.](image-url)
Proper separation and purification of microorganisms from water will be one of the key factors affecting the ability of MALDI-TOF-MS to identify microbes in water. We are studying separation and purification techniques, including functionalized nanomaterial-based affinity isolation. Recently, we have advanced in magnetic separation of waterborne gram-positive bacteria from water by using vancomycin-attached iron oxide nanoparticles (Figure 2). Captured bacteria are characterized by MALDI-TOF-MS and can be correctly classified against a MALDI spectral database.

In the last phase of this project, we will couple mass fingerprinting and proteomics approaches with proper separation/purification techniques developed to achieve rapid identification of a great number of waterborne bacteria. MALDI-TOF-MS will be compared with PCR and other water testing methods.

We believe that BMS can play more roles in identification of microorganisms and serve as a reliable, versatile, practical technology in water science and quality in the near future.

**Figure 2.** Illustration of the magnetic capture of Bacillus cereus with vancomycin-conjugated Fe₃O₄ nanoparticles.

**RESEARCH PROJECT**

**Fecal contamination and source tracking in catchments by PCR-based approaches**

The focus of this research is to identify the fecal pollution and track categories of contamination in catchments (if any) using PCR-based molecular tools. As the differentiation between human and animal pollution is a great challenge, TaqMan QPCR which is capable of exclusively detecting six human associated *Enterococci*, a regulated bacterial indicator by U.S. EPA for determining the extent of fecal contamination in the marine environment
and recreational waters, was developed and implemented to conduct a full spectrum of investigation for the presence and occurrence of fecal pollution. SYBR Green QPCR and conventional PCR were employed to explore the possibility of *Bacteroides* and *Bifidobacteria* species, exclusive to human, present in site monitoring. By using the strengths of each approach to ascertain inputs and track fates, the potential problem area can be located and successful management solutions can be found to reduce or eliminate the sources. This research program has been planned specifically to generate the essential data that will be used as a baseline for establishing stringent water standards for catchments protection in Singapore.

**RESEARCH PROJECT**

**Molecular identification of toxigenic cyanobacteria and blooming tracking in reservoirs**

Despite the increasing concern on water quality, little sound data is available on the presence and prevalence of potential toxic cyanobacteria in reservoirs globally and locally due to the lack of reliable monitoring tools. To reduce potential sources of pollution, and minimize water, environmental and public health risks, the utilization of both PCR-based method (PCR, QPCR and RT-PCR) for qualitative and quantitative recognition of targeted toxigenic cyanobacteria at species/strain level; and DNA fingerprinting (DGGE and T-FLP) for identification of cyanobacterial at community level were initiated. The record of temporal dynamics and successions of cyanobacteria for bloom tracking with DGGE and T-FLP techniques was proposed. The determination of the abundance of toxigenic species/strains in bloom samples and assessment of the impact of blooms on water quality and human health were sought. The possible environmental condition that may regulate toxin production was probed. As rapid, specific, sensitive, high-throughput and cost-effective tools, molecular techniques permit an increase in the number of sampling sites and sampling frequency when monitoring, and can be used as a sensitive tool for early warning diagnostic.

The use of molecular monitoring technologies for predicting bloom events is a novel technique. When implemented together with existing algae monitoring programs, it should allow PUB to accomplish good water quality and protect public health in Singapore. This research program has also been targeted to generate database for the characterization of harmful cyanobacteria.

**RESEARCH PROJECT**

**Elucidation of microbial species in desalination membranes and microbial water quality monitoring using DNA-based molecular technique**

In water treatment today, desalinated seawater makes an increasing contribution to world water budget and membrane techniques play a vital role in creating valuable water sources. Being one of four national taps for PUB, desalinated water contributes one-tenth of Singapore’s water consumption. However, an inherent problem in a desalination plant is membranes biofouling which has posed a huge and continuing cost to the
desalination industry through reduction of water production, deterioration of quality of potable water, degradation of membrane and increase of O&M costs up to 30%. In addition, biofouling layer in desalination membranes can provide shelter for pathogenic species and contribute pathogenic species to the water supply. Thus, the research project for elucidation of microbial species in desalination membrane and microbial quality monitoring of water by DNA-based molecular techniques (e.g., T-RFLP, 16S rDNA PCR-DGGE, QPCR, multiplex PCR, Nest PCR, RT-PCR, cloning and sequencing) was proposed to address this specific proactive and reactive issues of the water cycles in Singapore.

Here, DGGE and T-RFLP techniques will be used to define microbial community structure. TaqMan and SYBR Green QPCR will be applied for quantification of parasites in source water and biofilm samples. The multiplex PCR, QPCR and Nest-PCR will be chosen to probe selected pathogenic microbial species, including bacteria, viruses and fecal indicator bacteria. RT-PCR will be developed and used to determine microbial activity by elucidating the presence of transcripts in selected gene regions using mRNA as template.

The implementation of a molecular-based platform will not only provide water industries with speedy and sensitive diagnoses for biofilm formation in desalination membrane but also information on the occurrence and concentration of microbial species. These benefits will be of great value for preventing biofouling, reducing potential sources of fouling, and minimizing water and public health risks. Based on the research findings, the system management will be in position to assess the performance of desalination plants, the optimization of disinfection strategies and main cleaning procedures, and work out manageable solution to control or eliminate biofouling. This information can also be used to select intake sites of new desalination plants. The database obtained from this project should also ensure water safety and public health protection.

About the Centre for Advanced Water Technology (CAWT)
The Centre for Advanced Water Technology (CAWT) is an applied water research group established by the Public Utilities Board of Singapore (PUB) to support its water technology business.

With some 40 highly qualified researchers in highly specialized analytical and research laboratories, CAWT has strong in-house expertise to develop and commercialize innovative and cost-effective solutions for water production, water conservation, wastewater recovery and recycling.

CAWT has excellent laboratory facilities and a computerized sample management system dedicated to trace analysis of materials and organic contaminants in water and other matrices. It also has a very strong group of scientists performing applied and fundamental research in the areas of biotechnology, microbiology and chemistry.

CAWT concentrates its strategic resources on technologies that will enhance Singapore’s position in the fields of water supply, wastewater treatment and water reclamation with emphasis on water conservation. The need for water conservation and recycling is also common for most of the regional economies.

The Water Research Analytical Laboratories of CAWT is a leading
world-class research and analytical laboratory in the environmental, water and chemical analytical fields. There are six sections in the laboratory: General Chemistry, Inorganic Chemistry, Organic Chemistry, Microbiology, Biotechnology and PUB Water Science. It is equipped with state-of-the-art instrumentation, and is SAC-SINGLAS accredited and ISO 9001:2000 certified.

The laboratories offer a broad range of environmental and chemical analytical services, including inorganic, organic, radiological, microbiological and biotechnological parameters, to government, academic, commercial and industrial organizations. Specializing in trace and ultra trace level analyses of samples in water, environmental, semiconductor, pharmaceutical, food processing, clean room and other areas, the laboratories’ expertise also extends to research projects and new method developments.

About the Authors

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Dr Yang Zhaoguang, George is currently a Principal Research Scientist and Vice-President of the Centre for Advanced Water Technology (CAWT), Singapore. He has 30 years of R&D experience since graduating from Central-South University of China. He holds a PhD degree from the University of Nevada, Reno and a MSc degree from South Dakota School of Mines and Technology, U.S.A. Dr Yang has extensive R&D experience in analytical chemistry, biotechnology, water quality and security, water and wastewater treatment technologies, with more than 100 publications under his name. He has established several environmental laboratories as well as designed, constructed and managed several large wastewater treatment facilities in the United States and Asia. In CAWT, he performs research, administers and operates research analytical laboratories to conduct drinking water tests, research projects and support regional government agencies and industries.

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