

RAPID MICROBIOLOGICAL MONITORING METHODS: THE STATUS QUO

1. INTRODUCTION

The transmission of water-borne diseases is still a matter of major concern, despite worldwide efforts and modern technologies utilised for the production of safe drinking water. This problem is not confined to the developing world where treatment may be non-existent or inadequate. There may also be contamination during storage, a lack of regulations and limited understanding and awareness among the population (AAM, 1996). It may also assume serious proportions in industrial countries (Kramer *et al.*, 1996). Mechanical failure, human error or deterioration in the quality of the source water can lead to the failure of even the best treatment systems and disinfection processes (MacKenzie *et al.*, 1994; Roefer *et al.*, 1996). Rapid and reliable routine monitoring of the microbiological quality of source and treated drinking water will therefore remain of fundamental importance in the control of water-borne diseases (Sobsey *et al.*, 1993).

The purpose of this report is to create a broader understanding among IWA members and other interested parties of developments in the field of rapid methods for microbiological monitoring of water. Microbiological monitoring of source and drinking water is also associated with several other issues of concern that have never been fully resolved.

These include the type and range of microorganisms to be tested for, the required frequency of sampling and the ease of analysis (Pipes, 1990). Although all these issues are of great importance, this report will focus on the need for rapid microbiological analyses and the current state of the art.

The document consists of two parts. First, the report itself gives an overview of the requirements for and developments in rapid microbiological methods. Only the broad issues of concern are addressed since the report is not meant to be an in-depth study.

Secondly, attached to the report is a separate bibliography of literature related to the rapid detection of microbes in source and drinking water. The purpose of this bibliography is

- to create awareness of the work already done in this field,
- to serve as a basis for readers interested in gaining a deeper understanding of it, and
- to stimulate future research into rapid methods.

2. CURRENT MONITORING PROBLEMS

To protect consumers against possible water-borne diseases, ideally the occurrence and levels of all pathogens in drinking water should be monitored. This ideal is, however, not attainable. Many pathogens are only present under specific conditions and, when present, occur at low levels compared with other microorganisms. Most of the methods used for pathogen detection do not give any indication of the level of contamination but only whether or not the specific pathogen is present (Gray, 1994). Many of the detection methods being used at present are also expensive and time-consuming, require sophisticated equipment and expertise, and are not necessarily suitable for routine analysis (WHO, 1996)

To overcome the above-mentioned problems, more readily-detected indicator (index) organisms have been widely used to assess the microbiological quality of drinking and source water (Pipes, 1982). At present, the indicator organisms most widely used are bacteria (coliform bacteria, faecal coliform bacteria, enterococci, etc.). Virus indicators (somatic coliphages, FRNA bacteriophages etc.) are also used (IAWPRC, 1991) and yeasts (*Candida*) have been included in some programmes. For many pathogens such as viruses and protozoan parasites, reliable indicators are not available and lengthy pro-

cedures have to be used for the direct detection of these pathogens (Hibler, 1988; Hurst, 1991; Marshall *et al.*, 1997).

The indicator organisms used to assess the degree of faecal pollution may also be used as model organisms to evaluate the effectiveness of treatment processes, or to provide information on the availability of nutrients in the water to support bacterial growth (WHO, 1996).

3. THE NEED FOR RAPID METHODS

Whether testing directly for pathogens or indirectly by testing for indicator organisms, there is a common need for rapid analyses. Typically, the treatment of drinking water is a continuous process and, owing to constant demand, water is used within hours after treatment. Real-time analysis would be ideal for the management of microbial water quality and the protection of public health.

At present, using conventional methods for the assessment of the bacteriological quality of drinking water, results are only available after 18 to 24 hours. If those results have to be confirmed, another 1 to 2 days may be required. The detection of bacterial pathogens in water can take even longer. Testing water for the presence of specific viruses might even take as long as a few weeks since the standard methodology involves working with cell cultures (APAH *et al.*, 1995). Consequently, information on the microbiological quality of the water supplied to consumers is often available long after the water has been utilized.

The increasing population in many parts of the world has also led to deterioration in the quality of the ground and surface water sources used for the production of drinking water. Surface water is often subject to frequent and dramatic changes in its microbial quality. This is due mainly to intermittent point and diffuse pollution sources within the catchment. Fluctuations may also be seasonal. High microbiological pollution levels have been noted after rainstorms, associated with surface run-off (Venter *et*

al., 1997). Water treatment facilities should have the capability of detecting such fluctuations rapidly in order to adjust the treatment process. If the treatment is not adjusted in time, there may be insufficient treatment or "breakthrough", resulting in contamination of the whole water distribution network (Van der Wende and Charaklis, 1990). Failure of treatment plants, contamination of distribution networks or disruption of water supply services can also be caused by natural disasters or sabotage. In all these cases there is an urgent need for rapid and reliable information on the microbiological quality of drinking water.

Regular fluctuations in water quality and the need for cost-effective operation mean that water treatment works have to optimize treatment processes constantly. In many countries, water suppliers are also required to supply water of a specified microbiological quality and adjustments to the treatment have to be made accordingly. Evaluation of the treatment, especially disinfection, is based mainly on microbial parameters. Rapid analyses of these parameters are needed to determine the effect of changes made to the treatment and to make adjustments whenever necessary.

The lack of rapid methods for the monitoring of source and treated water has not, however, meant an absence of management or control over the microbial quality of drinking water. Water suppliers have often used other measures to protect public health. Typically, they rely on the monitoring of other parameters such as the residual levels of disinfectant as well as turbidity and particle count analysis (APAH *et al.*, 1995).

4. MINIMUM REQUIREMENTS FOR RAPID METHODS

An essential requirement for rapid methods should be the availability of data in the shortest time possible. These methods should be faster than the standard methods currently used. The results of some of the conventional methods can be available within 18

to 24 hours, e.g. the membrane filtration technique for faecal coliforms. For bacterial indicators, the ideal for rapid methods should therefore be to have results within the same working day. Methods providing confirmed results after 24 hours could also be classified as rapid methods when compared with standard methods in which further confirmation of results is required, which may take another 24 to 48 hours. The detection of viruses in water using conventional methods is a lengthy procedure that could take days before results are available. In this case, results within 48 hours may still be considered as rapid (APAH *et al.*, 1995).

Apart from being significantly faster, rapid methods should ideally have sensitivity and specificity equal to those of the standard methods used regularly. Sensitivity remains a major drawback for many of the rapid methods proposed as the number of organisms sought may be below the minimum concentration of microbes essential for direct detection. Some of these methods will only be of use for monitoring wastewater or source water, and will not be sensitive enough to determine the microbial quality of drinking water where much smaller numbers of microorganisms need to be detected (WHO, 1996).

The viability of the organisms detected by rapid methods is as important as with the standard methods. The techniques should have the ability to distinguish between viable and dead microorganisms. Results from rapid methods used for the routine monitoring of water quality should be repeatable and reproducible. This implies not only that the same analyst should be able to get similar results when analysing identical samples, but also that results should be reproducible between analysts in the same laboratory and between various laboratories. Any new method should therefore be subjected to performance testing before it can be used on a routine basis. Ideally the method should perform satisfactorily in a wide range of water supplies of different quality.

It is also essential that laboratories carrying out microbiological examination of water supplies should operate a system of quality assurance which include internal and external analytical control. Internal quality control will focus on factors such as the sterility of equipment, media and reagents as well as the testing of regular positive samples containing known amounts of the organism to be detected by the specific method. External analytical quality control schemes involve the laboratory examining samples prepared by a central organisation. Any evidence of an unacceptable performance in internal or external

schemes should immediately trigger an investigation and remedial action. In order for the development of rapid detection methods to be used on a routine basis, other logistical and economic factors should also be considered. Attention should be given to:

- the cost and availability of reagents,
- the need for special handling of samples,
- the need for dedicated and expensive apparatus,
- the ease of performance and interpretation of results, and
- the training needs of the analyst.

5. CURRENT RAPID METHODS

5.1 Background

Methods for the detection of microorganisms in water can be roughly divided into three categories. Most of the standard methodologies used at present fall within the first group of cultivation techniques. The second group consists of those techniques in which the microorganisms are detected directly in the sample without first culturing the organism. Various techniques are used, including several molecular techniques such as the polymerase chain reaction (PCR). The third group contains the methods based on combinations of the previous two types of techniques.

5.2 Cultivation techniques

Cultivation techniques utilize selective media or as in the case of viruses specific cell lines for growth of the organisms. The targeted organisms are allowed to multiply to a level where the cells can be easily detected by visible growth (turbidity or specific types of colonies). The presence of metabolic products or enzymes could also be used for the detection of specific bacterial groups.

5.2.1 Culture-based techniques for the detection of bacteria

The standard techniques used for the enumeration of bacterial indicators in water, e.g. total coliforms, faecal coliforms or *E. coli*, are culture-based techniques that utilize either the most probable number (MPN) or the membrane filtration technique. Depending on the composition of the media utilized, confirmation of positive results may be an additional requirement (APAH *et al.*, 1995).

The duration of culture-based methods can be improved in two ways. The first would be to use selective media or the detection of specific enzymes or metabolic products that would eliminate the need for additional confirmation tests. There are numerous examples of such improvements, including variations on the presence-absence tests discussed below. The improvement of existing culture media will not be dealt with in this report (Alonso *et al.*, 1996; Brenner *et al.*, 1996). The second way of improving duration would be to shorten the time needed to detect the bacteria, their enzymes or metabolic products.

The original aim in developing the presence-absence test procedure was to provide a simpler and less expensive way of testing water samples where only a limited number of positive samples are expected (Clark, 1990). Over the last ten years, several companies have developed presence-absence tests for the detection of total coliforms, *E. coli* and enterococci (Edberg *et al.*, 1988; Manafi and Kneifel, 1989; Budnick *et al.*, 1996). Recently, variations on these tests based on the MPN technique have also become available (Hernandez *et al.*, 1993). The tests rely on the detection of specific enzyme activities associated with the targeted indicator organism and no further confirmation tests are needed. The results are available within 18 to 24 hours and are therefore faster than those standard methods where confirmation of the results is required, which take 48 to 72 hours. For this reason, the presence-absence tests are often referred to as rapid methods although they may not necessarily be faster than some of the membrane filtration methods using selective media (APAH *et al.*, 1995). For the detection of faecal coliforms, a test providing results after 7 hours has been described (Reasoner *et al.*, 1979).

The specificity of these rapid culture-based methods is usually based on the detection of enzyme activities associated with the specific group of organisms to be detected, e.g. β -galactosidase indicating total coliform bacteria and β -glucuronidase indicating *E. coli* (Manafi *et al.*, 1991; Frampton and Restaino, 1993). *Aeromonas hydrophila* could induce a false positive result for β -galactosidase based tests after prolonged incubation. Specificity has, however, been evaluated on numerous occasions and found to be acceptable when compared to the methods currently used.

The main disadvantage of culture-based techniques is that the growth rate of bacteria under optimal conditions cannot be increased. A certain period always has to be allowed for the growth of

the bacteria. Some efforts at improvement have therefore been aimed at increasing the sensitivity of detecting bacterial growth or enzyme activity. The length of incubation for a fluorogenic presence-absence test for the detection of total and faecal coliforms in surface water could be shortened to 12 h using a spectrofluorometer (Park *et al.*, 1995). It may be possible to detect *E. coli* in potable water within 15 h using indirect impedance (Timms *et al.*, 1996). Fiksdal *et al.* (1994) reported that galactopyranosidase and glucuronidase activities could be detected within 25 minutes with a spectrofluorometer. For the glucuronidase, a sensitivity of 10 to 100 CFU/100 ml was reported, but the sensitivity of the galactopyranosidase assay was poorer.

Using liquid scintillation spectroscopy for the detection of radioactively labelled $^{14}\text{CO}_2$ formed during the growth, faecal coliforms could be detected within 4,5 hours. The sensitivity reported for this technique was as low as 10 cells. Working with radioactive material and the need for a scintillation counter are definite drawbacks of this method (Reasoner and Geldreich, 1989).

A major advantage of the culture-based methods is that there is no doubt about the viability of the organisms detected since they had to grow and multiply in order to be detected. Concern has, however, been expressed about the presence of viable but non-culturable bacteria in samples. Although viable and present in the sample, these organisms will not grow on the media used in the tests and this could lead to false negative results.

In most cases, laboratories responsible for routine microbiological analyses of water samples can easily include the above mentioned culture-based methods, as only limited training of personnel is required. These techniques could easily be incorporated into the existing quality control systems of accredited laboratories performing routine microbiological analyses.

5.2.2 Cultivation methods for the detection of viruses

Cultivation techniques are seldom used for the rapid detection of viruses and protozoan parasites in water as they often need cell-culture systems or live hosts for replication. Work has, however, been done on the rapid detection of coliphages, a viral indicator, in water. With this method, the number of coliphages in 100 ml of water can be estimated within 6 hours, with an estimated sensitivity of $\geq 6/100$ ml (Wentzel *et al.*, 1982).

5.3 Direct detection methods

The basis of these techniques is the direct detection of microorganisms in the water samples, without any prior enrichment. The techniques usually consist of the labelling of the organisms or their components, followed by the detection of the labels. Several techniques can be used to detect the labelled organisms or components, such as spectroscopy, electrophoresis, fluorescence microscopy, flow cytometry and laser scanning.

5.3.1 Non-specific techniques

There are several methods that do not distinguish between species or groups of microorganisms. Methods using fluorochromes, such as acridine orange and 4',6-diamidino-2-phenylindole (DAPI), are non-specific which means that non-viable organisms could also be detected; this restricts their use in the monitoring of microbial water quality (Kepner and Pratt, 1994). There are other techniques that could be used for the detection of active microorganisms in water but they are also non-specific. They provide an indication of the level of active microorganisms (used mainly for bacteria) in the sample and are, therefore, of only limited use in controlling the microbiological quality of water. Bioluminescence, impedance, epifluorescence microscopy and flow cytometry using fluorochromes aimed at detecting viability are among these techniques (Schaule *et al.*, 1993; Diaper and Edwards, 1994; McFeters *et al.*, 1995).

5.3.2 Specific techniques

In recent years, with the rapid development of molecular methods, several techniques with high specificity have been developed for the direct detection of microorganisms in water.

5.3.2.1 Immunological techniques

Immunological techniques for the detection of microorganisms, especially pathogenic organisms, have been widely used in the water field (Hibler, 1988; Obst *et al.*, 1989; Kfir *et al.*, 1993). Antibodies are raised against unique antigens characteristic of the microorganism to be detected. The antibodies are usually linked with an enzyme or fluorescent dye. The fluorescently labelled bacteria or parasites are mostly detected with epifluorescence microscopy. Results can be available within the same day but since each sample has to be evaluated individually

under the microscope, it can take much longer to process a large number of samples.

The processing of fluorescently labelled samples can be accelerated by using techniques such as flow cytometry to detect the labelled organisms (Tyndall *et al.*, 1985; Davey and Kell, 1996). The labelled organisms are sorted by light scattering and fluorescence emitted by the fluorochromes. Histograms showing the number of cells that possess a certain property (size, fluorescence) are given as the output. The sorted cells can be confirmed through microscopy.

The viability of the organisms detected is a matter of great concern and some antibodies used may exhibit cross-reactions which will compromise the specificity of the test (Kfir and Genthe, 1993). The sensitivity of most of the techniques that utilize microscopes for the detection of the organisms is estimated to be in the order of 10^4 cells per slide (Manz *et al.*, 1995). Only small volumes can be examined using microscopy, immunoassays, conductance or flow cytometry, and samples definitely need to be concentrated to detect low levels of microorganisms (Yu and Bruno, 1996).

5.3.2.2 Bacteriophages

Genetically engineered phages containing the *lux* gene have been used for the detection of bacteria in environmental samples. After infection, the targeted organism will emit light that can be detected. Phages containing the *lna* gene have also been used and there have been reports of other methods that do not require genetically engineered phages. Techniques using this approach have been reported to be sensitive and only viable bacteria would be detected (Turpin *et al.*, 1993; Sidorowicz and Whitmore, 1995).

5.3.2.3 Hybridization

Hybridization using various types of probes has been used for the detection of specific pathogenic bacteria, viruses and parasites in water (Abbaszadegan *et al.*, 1991; Dubrou *et al.*, 1991; Knight *et al.*, 1991). Because of its low sensitivity, it has been used mainly for the identification of microorganisms in polluted water and has to a great extent been replaced by PCR-based techniques. It is still used as a method for confirming PCR results (Bej *et al.*, 1991a).

In situ hybridization has been used for the direct detection of bacteria in water samples. Only active bacteria should be detected because the oligonucleotide probe is directed at the rRNA of the

bacterium. After hybridization, the organisms can be detected with a microscope or flow cytometer (Manz *et al.*, 1993; Manz *et al.*, 1995). Oligonucleotide probes for the detection of *Cryptosporidium parvum* have also been described recently (Lindquist, 1997).

5.3.2.4 Laser scanning

Direct methods for the detection of microorganisms usually involve the labelling of organisms or their components, followed by the detection of the labels. Many of the above described procedures rely on the use of a fluorescent label (e.g. fluorescein) which permits detection of the microorganisms using epifluorescence microscopy. However, this technique is labour intensive, time consuming, inaccurate and causes operator fatigue.

The ChemScan RDI instrument (Chemunex, France) has been designed to replace manual microscopic examination of such samples allowing rapid detection and enumeration of fluorescently labelled microorganisms. Organisms are captured by membrane filtration. Labelled and the filter subsequently scanned with a laser. During this analysis, fluorescent events, including labelled organisms are detected by a series of detection units. Finally the signals generated undergo a sequence of computer analyses which distinguish between labelled organisms and fluorescent debris. A visual validation of all results can be made by transferring the membrane to an epifluorescence microscope which is fitted to a motorised stage. This stage, which is controlled by the ChemScan RDI, can be driven to the location of each fluorescent event for a rapid confirmation of all results.

Recent studies have demonstrated that *Cryptosporidium* oocysts and *Giardia* cysts may be detected using laser scanning after labelling with fluorescently labelled monoclonal antibody. In addition further reports suggested that *Escherichia coli* labelled with a specific 16S rRNA probe can also be successfully detected (D Reynolds, personal communication). This detection procedure may be extended for the detection of other organisms, in a variety of applications.

5.3.2.5 Polymerase chain reaction-based techniques

By using the polymerase chain reaction (PCR), a selected gene sequence specific to a group of organisms or a single species can be selectively amplified. The amplified sequence can easily be detected by means of techniques such as elec-

trophoresis, hybridization, high performance liquid chromatography or ELISA. This technique has recently received most of the attention in the development of rapid detection methods. This is due mainly to the excellent specificity, improved sensitivity, applicability to any group of microorganisms and ease of detection of results. It is used mostly for the detection of pathogens in water but can also be used for indicator bacteria (Alvarez *et al.*, 1993).

In the water field, PCR-based methods have been applied extensively for the detection of viruses because they can replace lengthy standard procedures involving the infection of cell cultures (Abbaszadegan *et al.*, 1993; Graff *et al.*, 1993, Schwab *et al.*, 1995). PCR has been used for the direct detection of bacteria in water (Bej *et al.*, 1991a; Toranzos and Alvarez, 1992) and a number of researchers have also used PCR for the detection of protozoan parasites in water (Mahbubani *et al.*, 1991; Mayer and Palmer, 1996, Rochelle *et al.*, 1997a).

The main concern with the use of PCR-based techniques for the direct detection of all types of microorganisms in water is of viability or infectivity. PCR will detect any intact targeted nucleic acid sequence. Studies performed on DNA from pathogenic bacteria showed that non-viable cells could be detected in an environmental water sample for up to three weeks (Josephson *et al.*, 1993). Studies on the survival of viral RNA found that PCR will detect mainly well-protected viral particles and that naked viral RNA does not survive for long periods in water (Tsai *et al.*, 1995). Detection is, however, no assurance of infectivity as it can only be determined using cell cultures or animal models. Some researchers have claimed that the selection of a large DNA target region may result in the detection of only viable organisms (Bej *et al.*, 1991a). The use of reverse transcriptase PCR has also been used for the detection of viable organisms (Rochelle *et al.*, 1997b; Kaucner and Stinear, 1998).

In spite of the improved sensitivity of PCR, its application is limited by small reaction volumes and it can only be used for the direct detection of microorganisms in polluted source water. The technique also needs to be combined with concentration steps. Another problem with PCR-based techniques is that they only supply presence-absence data. Nevertheless, at present different methods for the quantification of PCR products have been developed. PCR can be performed on the basis of the MPN technique, but the number of reactions needed and the costs will increase substantially.

5.4 Combined techniques

In order to utilize the major advantages of both groups of techniques mentioned above, combinations have been developed. For the detection of bacteria, the sensitivity of methods can be improved by incorporating an enrichment step (cultured-based technique). This will also solve the problem of viability as bacteria growing in the enrichment broth will be detected. Enrichment has been combined with PCR on several occasions (Koenraad *et al.*, 1995).

A combined cell-culture-PCR technique was used for the detection of infectious enteroviruses in environmental samples and results were available within 24 hours (Reynolds *et al.*, 1996; Murrin and Slade, 1997). Recently, fluorescently labelled antibodies or PCR have been combined with a cell-culture method to detect infective *Cryptosporidium parvum* oocysts in environmental samples (Slifko *et al.*, 1997, Rochelle *et al.*, 1997b).

A combined immunoassay method using monoclonal antibodies against the enterobacterial common antigen has also been used for the detection of the *Enterobacteriaceae*. An enzyme-linked immunosorbent assay (ELISA) was combined with an enrichment step to improve the sensitivity of the method. The test could be performed in 24 hours (Hübner *et al.*, 1992). Enterococci could be detected within 8 to 20 hours when a culture-based method was combined with rRNA targeted DNA probes. After hybridization of the microcolonies on the filter, the colonies were detected using epifluorescence microscopy (Meier *et al.*, 1997).

5.5 Concentration of samples

For the monitoring of drinking water, the sensitivity required for indicator organisms is often of the order of one organism in 100 ml of water, but for viruses there is a need to sample volumes as large as 100 l (Payment, 1991). To obtain the required sensitivity, many of these methods will incorporate an initial concentration or selective separation procedure or combinations of these. Apart from the normal membrane filtration (Bej *et al.*, 1991b), other techniques have been incorporated, e.g. cross-flow filtration, chromatography and immunomagnetic separation (Sidorowicz and Whitmore, 1995; Paul *et al.*, 1991). The use of some of these techniques is still limited by their cost and applicability in dealing with large volumes.

Immunomagnetic separation has been used for the concentration of *Giardia lamblia* cysts from large water samples. The antibody-magnetite was applied

to concentrate samples obtained after filtration of the original sample. An average recovery rate of 82% was reported (Bifulco and Schaefer, 1993). In addition, the technique has been applied to the capture of *Cryptosporidium* oocysts (Campbell and Smith; 1997). Viruses can also be concentrated in environmental samples using this procedure (Graff *et al.*, 1993; Deng *et al.*, 1994).

6. FUTURE DEVELOPMENTS

At present the ideal of real-time analysis cannot be achieved, but developments during the last ten years have made it possible to detect many indicator organisms and pathogens in water within the same day. Since all techniques have their advantages and disadvantages, the greatest potential lies in combinations of techniques.

Sensitivity is still a major concern, especially when monitoring drinking water. To improve sensitivity, attention should be given not only to culture and direct detection techniques, but also to concentration and separation methods.

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NOTE: This bibliography only contains articles that relate to the detection and or enumeration of microorganisms in water environments. Similar techniques are being used in the clinical field but references to these techniques were not included in the list as this document is primarily aimed at the people working in the water supply industry and not at the researchers involved in the development of these techniques. This list does not contain all articles published in the field, it only contains articles that task force members were aware of during the compilation of the report. Omission of a reference does not mean that the specific article is not of relevance or of a high standard.

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The International Water Association Standards and Monitoring Specialist Group has initiated a Task Force addressing rapid microbiological methods. Within IWA no earlier work has been done on this topic and the main focus of the group was therefore to create a basis for understanding the developments in the field of rapid methods for the monitoring of microbiological water quality. This purpose of the present report: *Rapid Microbiological Monitoring Methods: The Status Quo*, was to fulfill that need.

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Leader: Mr S N Venter, University of Pretoria, South Africa.

Members: Mr V Catalan, Labaqua, Alicante, Spain.
 Ms M du Preez, Environmentek, CSIR, Pretoria, South Africa.
 Mr Everaert, Hach Europe, Floreffe, Belgium.
 Mr P Finch, Anglian Scientific, Cambridge, United Kingdom.
 Dr C Fricker, Thames Water, Reading, United Kingdom
 Mr M Grant, Hach, Ames, Iowa, United States of America.
 Dr A Levine, Utha State University, Logan, Utha, United States of America.
 Ms S Seemendi, Tallinn WSME, Tallinn, Estonia.

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S N Venter
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INTERNATIONAL WATER ASSOCIATION
ALLIANCE HOUSE, 12 CAXTON STREET, LONDON SW1H 0QS, UNITED KINGDOM
TELEPHONE: +44 (0)20 7654 5500
FAX: +44 (0)20 7654 5555
www.iwahq.org.uk