

Original papers

Mycological monitoring of selected aquatic ecosystems in the context of epidemiological hazards. Drinking water.

Anna Biedunkiewicz, Katarzyna Kowalska, Łukasz Schulz, Kamila Stojek, Maria Dynowska, Elżbieta Ejdyś, Ewa Sucharzewska, Dariusz Kubiak

Department of Mycology, Faculty of Biology and Biotechnology, University of Warmia and Mazury, Oczapowskiego 1A, 10-917 Olsztyn, Poland

Corresponding author: Anna Biedunkiewicz; e-mail: alibi@uwm.edu.pl

ABSTRACT. Many species of microfungi are reported in aquatic ecosystems with different frequency. Their number constantly fluctuates depending on the concentration of environmental and anthropogenic factors. Drinking water, tap and bottled, is essential for the proper functioning of the human body. It is also the main component of food and hence it should be safe for human health and free of contaminants. The mycological purity of tap water in two large cities in the region (Olsztyn and Ostrołęka) and a small village (Gągławki) as well as bottled, medium-mineralized and curative water stored under different conditions were tested. The laboratory investigations followed a pathway applied in diagnostic mycological laboratories. The conducted tests demonstrated that microfungi were found in tap water originating from the cities and in bottled water. The rural water supply system was free from contaminations. Eighteen species of microfungi were identified in tap water from Olsztyn and 9 species in tap water from Ostrołęka. In bottled water, 13 fungal species were detected. *Exophiala spinifera* and *Debaryomyces hansenii* were recorded in the water supply systems of both cities, while one common species, i.e. *Aspergillus fumigatus*, was identified in tap water from Ostrołęka and in bottled water. The conducted studies have significant practical implications, for instance in sanitary and epidemiological water evaluation and in medicine in the context of analysing the quality of drinking water in reference to health resorts and nosocomial infections.

Key words: microfungi, drinking water, epidemiological hazards

Introduction

Mycological monitoring of different aquatic ecosystems carried out for many years have identified constant presence of microfungi in the complete profile of water [1,2]. Their number is determined by a variety of factors yet it clearly increases proportionally to progressive degradation of the environment and incremental anthropogenic pressure. The most of microfungi found in different types of water are potential etiological factors of dangerous superficial, organ and systemic mycoses and their appearance in the aquatic environment indisputably poses an epidemiological hazard [3].

Water is essential in our life. Its consumption determines the proper functioning of the body. It fills the interior of the cells and constitutes the

environment for all processes in the body. It is also the main quantitative component of food [4]. If used for consumption purposes, it should be safe for human health and thus meet all basic microbiological and chemical requirements specified by the Minister of Health, the World Health Organization (WHO) and the legislation of the European Union (EU). Water delivered to recipients by different systems of distribution is subject to continuous, initial microbiological control by sanitary public services and water treatment plants. On its way to a recipient or by improper storing of different water types, its quality deteriorates as biological contaminants appear, including microfungi.

The objective of the studies was thus to evaluate the mycological purity of drinking water (tap and

bottled) both quantitatively and qualitatively and to assess the epidemiological hazard that micro-biologically contaminated water poses.

Materials and Methods

The studies of tap water were carried out in the province of Warmia-Mazury in two cities: Olsztyn (176,402 residents) and Ostrołęka (53,572 residents), and in a small village Gałąwki (260 residents). For the analyses of bottled water, still, sparkling, spring and curative varieties that are widely available on the market were used. The physicochemical and microbiological tests of tap water were performed in WOD-KAN Olsztyn and the Water Treatment Plant in Ostrołęka.

In Olsztyn, 10 stations were selected. They were located at recipients' premises and regularly distributed on four areas of deep wells (Karolin, Kortowo, Likuzy, Zachód). In Ostrołęka, there were 24 stations in the area of the Water Treatment Plant (WTP) "Leśna" and WTP "Kurpiowska". In Gałąwki, two locations were established located within two independent deep wells. One thousand millilitres (1000 ml) of tap water was taken from all stations according to the standards [5] during a year at monthly intervals. In total, 432 samples of tap water were collected.

The studies also included bottled water varieties. In total, 28 L of bottled water were filtered (12 L of still and sparkling water of four varieties and 4 L of curative water). Bottled water was filtered each time at a volume of 250 ml and then stored at 4°C and 22°C after a sip had been drunk. The action was repeated three times at one day intervals. The samples of drinking water were then processed in the same manner.

In order to isolate and determine the number of microfungi in water, the membrane filters method was applied. In addition, impressions were taken from faucet aerators and seals from taps located in recipients where water was sampled for testing. The samples of water (1000 ml) were filtered through a Millipore membrane filter (0.45µm) with a vacuum pump (under pressure below 0.2 bars). The filters were placed in a vessel filled with 20 ml of 0.9% sterile NaCl and shaken for 30 minutes to eliminate mould spores from filter pores. Next, the procedure was two-directional: the filters were put on a separate plate with solid Sabouraud's medium and 1 ml of suspension was cultured on another Sabouraud's medium and distributed with a sterile

smoother. The plates were incubated at 37°C for 48–72 hours. Thereafter, the growth of microfungi was controlled, grown colonies were counted, morphological types of mycelium were segregated and categorized (yeast-like fungi and mould), passages onto new Sabouraud's media with chloramphenicol were performed and slides stained with methylene blue were prepared. Microscopic and gross features were used to identify fungal species (yeast-like fungi and mould) and in the case of yeast-like fungi, biochemical characteristics were also included (zymogram, auxanogram and CHROMagar Candida GRASO). Microfungi were successively observed under an optic microscope Olympus BX51 (200, 400, 600× magnification). Impression smears stained with methylene blue with lactophenol were prepared from mould cultures according to Gerlach [6].

Yeast-like fungi and mould were identified based on specialist keys: de Hoog [7], Raper et al. [8], Raper, Fennel [9], Kurtzman, Fell [10], and Howard [11]. Photographic documentation was prepared and the identified fungi were catalogued in the Department of Mycology, Faculty of Biology and Biotechnology University of Warmia and Mazury in Olsztyn.

Results

Eighteen species of yeast-like fungi from 8 genera and one mould species were isolated from drinking water from Olsztyn – 66% of positive results, whereas 6 yeast-like species from 6 genera and two mould species from 2 genera were identified in drinking water from Ostrołęka – 6% (Table 1). *Exophiala spinifera* and *Debaryomyces hansenii* were detected in tap water from both cities. In Ostrołęka, *E. spinifera* was recorded once while in Olsztyn this species was detected on four stations within one of the deep wells and its number amounted to 690 CFU/dm³. In tap water from the village Gałąwki not found any fungi.

After physicochemical, microbiological and mycological analyses, it was found that before fungi occurred in the water samples, the concentration of Cl had dropped by 0.2–0.6 mg/L. The occurrence of fungi was accompanied by the highest increase in iron, ammonia, manganese and nitrites whereas following the isolation of fungi, in the subsequent samples the total number of psychrophilic (10–300%) and mesophilic bacteria (15–600%) increased, the concentration of ammonium

Table 1. List of species of microfungi found in the tested drinking water with regard to the classification of BSL*

No	Species	BSL*	Tap water		Bottled water		
			Olsztyn	Ostrołęka	Sparkling water	Still water	Healing water
1	<i>Acremonium kiliense</i> Grütz, 1925	2	x				
2	<i>Arthrographis</i> spp.	ns		x			
3	<i>Aspergillus fumigatus</i> Fresen, 1863	2	x	x	x	x	
4	<i>Aspergillus versicolor</i> Tirab, 1908	1			x	x	
5	<i>Candida guilliermondii</i> Langeron & Guerra, 1938	1					x
6	<i>Candida intermedia</i> Langeron & Guerra, 1938	ns	x				
7	<i>Candida krusei</i> Berkhout, 1923	2	x				
8	<i>Candida parapsilosis</i> Langeron & Talice, 1932	1		x			
9	<i>Candida pelliculosa</i> Redaelli, 1925	1	x				
10	<i>Candida versatilis</i> S.A. Mey. & Yarrow, 1978	ns	x				
11	<i>Cystofilobasidium lari-marini</i> Fell & Tallman, 1992	ns	x				
12	<i>Debaryomyces carsonii</i> Y. Yamada, K. Maeda, I. Banno & Van der Walt, 1992	ns	x				
13	<i>Debaryomyces hansenii</i> Lodder & Kreger-van Rij, 1984	ns	x	x	x		
14	<i>Exophiala castellani</i> Iwatsu, Nishim. & Miyaji, 1984	2	x				
15	<i>Exophiala jeanselmei</i> McGinnis & A.A. Padhye, 1977	2	x				
16	<i>Exophiala spinifera</i> McGinnis, 1977	2	x	x	x		
17	<i>Kluyveromyces lactis</i> Van der Walt, 1965	ns	x				
18	<i>Kluyveromyces marxianus</i> Van der Walt, 1965	ns	x				
19	<i>Moniliella suaveolens</i> Arx, 1972	1	x				
20	<i>Oosporidium margaritiferum</i> Stautz, 1931	ns	x				
21	<i>Paecilomyces</i> sp.	ns			x		x
22	<i>Penicillium aurantiogriseum</i> Dierckx, 1901	ns					x
23	<i>Penicillium brevicompactum</i> Dierckx, 1901	ns					x
24	<i>Penicillium chrysogenum</i> Thom, 1910	1					x
25	<i>Penicillium citrinum</i> Thom, 1910	1					x
26	<i>Penicillium griseofulvum</i> Dierckx, 1901	1					x
27	<i>Penicillium piceum</i> Raper & Fennell, 1948	ns					x
27	<i>Penicillium rugulosum</i> Thom, 1910	ns				x	
29	<i>Penicillium spinulosum</i> Thom, 1910	1					x
30	<i>Priceomyces carsonii</i> M. Suzuki, 2010	ns		x			
31	<i>Rhodotorula</i> spp.	ns		x			
32	<i>Saccharomycopsis capsularis</i> Schønning, 1903	ns		x			
33	<i>Scopulariopsis fusca</i> Zach, 1934	1		x			
34	<i>Sporobolomyces lactophilus</i> Nakase, Itoh, M. Suzuki & Bandoni, 1990	ns	x				
35	<i>Sporobolomyces shibatanus</i> Verona & Cif, 1939	ns	x				
36	<i>Trichoderma longibrachiatum</i> Rifai, 1969	1				x	
	Total		18	9	5	4	9

Explanations: bold – isolated from all studied types of water; ns – not specified; * – according to de Hoog [7], and Kurtzman and Fell [10]

compounds (0.41%), manganese (0.45%) and iron (0.04%) increased or exceeded the allowed threshold, yet water pH dropped to 6.3–6.5.

In general, microfungi were detected in 33% of the samples of bottled water. In total, 201 fungal isolates were obtained, of which 200 were moulds (99.5%) representing 12 species from 4 genera and 1 species of yeast-like fungus (0.5%). Water was most often contaminated with *Aspergillus fumigatus*, which was dominant, and *A. versicolor*. In the case of curative water, there were a variety of moulds identified only after the first filtration, such as *Penicillium rugulosum*, *P. spinulosum*, *P. griseofulvum*, *P. brevicompactum*, *P. aurantiogriseum*, *P. citrinum*, *P. chrysogenum*, and *P. piceum*. In addition, yeast-like fungi (*Candida guilliermondii*) were detected only in curative water (Table 1).

Considering the biosafety level (BSL) classification, of 13 isolated fungal species, only *Aspergillus fumigatus* belongs to BSL-2 and the remaining 12 species are categorized as BSL-1.

Comparing the microbiological purity of bottled still and sparkling waters that were stored at two temperatures (4°C and 22°C), there was a substantially higher number of isolates in still water stored at 22°C (95 isolates) than in sparkling water stored at 4°C (9 isolates) (Fig. 1).

Discussion

Potentially pathogenic fungi are common in aquatic ecosystems yet they are not covered in monitoring and control studies of tap water. The presence of microscopic fungi in drinking water and distribution systems is unfavourable as it is associated with its secondary contamination that results from damages to pipes caused by prolonged utilization and release of compounds that are substrates for growth and development of these fungi.

Microorganisms enter the water supply system together with treated water in which the amount of chlorine used for disinfection was suboptimal to eliminate all microbes [12,13]. They develop mainly in chemically corroded pipes when internal biological overgrowth and biofilm are present [12]. In the mature biofilm, cells communicate with each other and produce extracellular polymers (EPS) which cover cells and thus facilitate colonization [14]. They impact resistance of its structure and density as well as provide a barrier against mechanical damage to the membrane, preventing the access of a disinfectant to the interior of a cell cluster. Over time, the biofilm ages and cells start to separate from its surface layer, which is caused by

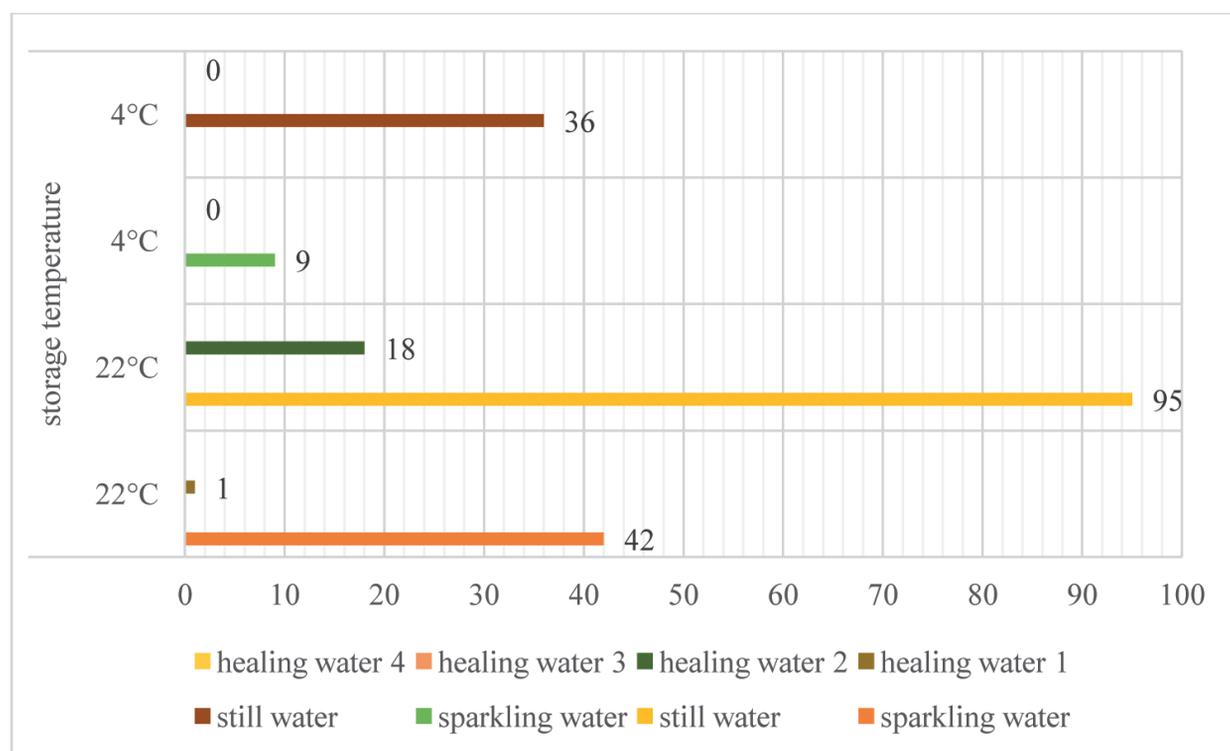


Fig. 1. Number of isolated microfungi in various types of bottled waters stored in two temperature ranges

an excessively high intensity of water flow and the presence of disinfectants which damage the structure of cells [14].

The development of biofilm in a water supply system largely depends on the type of material that was used to produce a given installation [13]. Currently, polyvinyl chloride (PCV), polyethylene (PE), polypropylene (PP) and poly-1-butene (PB) are most often used, in the belief that a smooth surface will eliminate the problem of biological sediment formation and protect against microbiological corrosion. In the 1990s, it was found that these materials are biodegradable and thus create an opportunity for microbiological biofilm formation. It is mainly caused by chemically active metabolites, such as organic acids, and enzymatic reactions that speed up corrosion induced by microorganisms [12,14]. Hyphal fungi additionally intensify biocorrosion by the growth of mycelium. These processes result in damage to the surface structure, i.e. cracks, scratches, and changes in the chemical structure as well as contribute towards formation of free radicals and intermediary metabolites that present a risk to the health and life of recipients of water from such water supply systems [14]. The growth and development of microscopic fungi in water supply systems causes changes in the taste and smell of water and generates technological problems associated with the destruction of water distribution systems [12].

Water for drinking may be derived from natural sources, underground intakes, wells and surface waters. However, to make it ready for consumption, they must be appropriately treated and should meet the legal regulations that guarantee that water is drinkable [15].

Nowadays, bottled water is used not only for drinking in its raw form, but it is also used to prepare different varieties of beverages at home. This raw ingredient is widely available and thus a consumer should be informed about types, uses and method of storage at home. Such information can usually be found on the label [16].

All bottled water varieties are colloquially called "mineral water". This term is incorrect as natural mineral water is one of three types of bottled water. The other two are natural spring water and table water [16]. Curative waters are an entirely separate category. All types of water should be adequately labelled, including the intake data (name and location), bottling plant (name and address), variety name (natural mineral water, natural spring water or

table water) and a brand name as well as the mineral content characteristics [17]. Natural mineral waters, due to the quantity of dissolved mineral compounds, are divided into high-, medium- and low-mineralized. The latter can be included in the spring water category. Curative waters are a separate variety. They have pharmacodynamic properties because they contain a large amount of some mineral compounds or the so-called specific ingredients. These waters help in different disease conditions. Depending on what they contain and in what amount, they provide therapeutic support in diabetes, in problems with excretion and elimination, in gastric disturbances and may improve general metabolism.

The medium-mineralized water that was analysed contained from 500 to 1500 mg of mineral compounds/L. The most important characteristic of this variety is that it does not disturb the electrolyte balance in the body. It can be drunk in unlimited amounts to satiate thirst. They do not have any curative properties, yet thanks to a minor amount of mineral salt, they are tastier than spring waters.

The impact of fungi found in drinking water on the quality of water and human health is poorly investigated [18]. Regardless of the fact that mould is common in drinkable water, it is thought that they do not generate problems for public health as the majority of these microorganisms is not pathogenic to humans [19]. Bottled drinking water is rarely free of microorganisms. It usually contains autochthonous organisms that are a minor health problem [20] yet, for instance, *Penicillium citrinum* may present a toxicological hazard since this microbe is capable of producing mycotoxins [21].

Aspergillus fumigatus was the most common fungal species isolated from drinking water originating from the cities and from bottled water. Moulds were detected in all types of crude tap water, after the first-degree treatment and in treated water. *A. fumigatus* is a thermophilous species. It is the main causative agent of respiratory aspergillosis in patients with impaired immunity (the so-called "farmer's lung") [22]. It is pathogenic to most vertebrates and some invertebrates. In humans, it may cause infections of the digestive, genital, cardiovascular and nervous systems and the urinary tract and skeletal muscles [23].

Yeasts were identified only in tap water originating from individual recipients. Their frequency was impacted by the season. At the turn of spring and summer, the highest increase of these

fungi was recorded (12 colonies were isolated from 8 samples) while at the turn of autumn and winter 10 colonies were isolated from 6 samples.

The presence of fungi in tap water has been reported worldwide. In his studies, Ramirez-Toro [24] isolated yeast-like fungi from *Candida*, *Cryptococcus*, *Rhodotorula* and *Phaeococcus* genera and moulds, such as *Aspergillus niger*, and fungi from *Fusarium* and *Trichoderma* genera. Warris et al. [25] tested tap water supplied to the paediatric unit that transplanted bone marrow in one of the hospitals in Oslo, Norway. The researchers found that mould was present in 94% of water samples, including 49% of samples collected from faucets (1.9 CFU/500 ml), in 5.6% of samples from showers (1.0 CFU/500 ml) and in 38.8% of samples from the main pipe supplying water to the unit (2.1 CFU/500 ml). *Aspergillus fumigatus* was the most common fungus isolated from samples (3.1 CFU/500 ml). In Portugal, *Acremonium* (38.8%) and *Penicillium* (40.6%) were most often identified in tap water. Gançalves et al. [26] demonstrated that bacteria and yeasts compete with moulds for nutrients by producing toxins that inhibit the growth of the latter. It was also observed that hyphal fungi were more common in colder months. In Portugal, temperatures in the winter are moderate and precipitation is abundant. Fungi from *Aspergillus* genus were a minor percentage, which may be related to an excessively low temperature (25°C) for incubation.

Several studies have proven that microfungi are found in chlorinated water and in surface water treated with coagulation, filtration and chlorination procedures. The results of these studies have shown that water disinfection with the above-mentioned methods does not protect against the growth of microscopic fungi [12]. This has also been demonstrated by current and previous personal studies. Common fungi were identified in tap water from Olsztyn and Ostrołęka. In the water samples from Olsztyn, up to 66% of them were positive. Yeast-like fungi (81.3%) were detected in the vast majority thereof. Over a half were so-called "black fungi", among which three species of *Exophiala* genus were identified: *E. castellani* (BSL-2), *E. jeanselmei* (BSL-2) and *E. spinifera* (BSL-2). Each of these species belongs to fungi that are potentially pathogenic to humans [27,28]. Fungi from *Exophiala* genus are the causative agents of phaeohyphomycoses and infections with these fungi may result in subcutaneous infections such as

mycetoma and *chromoblastomycosis* [23].

Yamaguchi et al. [29] compared the prevalence of yeasts and moulds in tap water and in bottled water. Yeasts were detected in 36.6% of bottled mineral water and in 11.6% of tap water. Twenty-one (21) samples (35.0%) of bottled mineral water and two samples (3.3%) of tap water yielded positive results for moulds. A substantially higher number of microfungi were detected in samples of bottled water, which clearly corresponds to the results of personal studies. Bottled waters are contaminated with both yeasts and moulds and further growth thereof may be impacted by storage conditions.

It is believed that the growth of fungal spores in bottled water into visible colonies is not possible, as such water contains too low concentrations of nutrients for these organisms [30]. Moulds can probably use, for their growth, compounds that are released from the polyterephthalate ethylene or plasticizers of which PET-type bottles are made [31].

Fungi, as euryecological microorganisms [32], are capable of growth across a wide spectrum of temperatures. Temperature may exhibit an inhibitory action only after exceeding the optimal value for fungal growth [19]. In the case of tested samples of bottled water stored at 4°C and 22°C, there was a difference in the number of isolated fungi. A storage temperature below room temperature was more favourable for the microbiological quality of this type of water [33]. The room temperature (22°C) favoured the growth of microfungi. It is thus recommended to store bottled water after opening at lower temperatures, approximate 4°C. A period of storage after opening may have a minor impact on the presence of fungi, although it is advised to adhere to manufacturer's recommendations and drink water within 12 hours after opening.

The content of CO₂ is an additional factor that stabilizes the microbiological quality of bottled water. For mineral and spring waters, still varieties may be more susceptible to the presence and growth of microfungi. The highest number of isolates, i.e. as many as 95 (52%), were identified in still water stored at 22°C while the least, i.e. 9 (5%), was from sparkling water stored at 4°C.

The conducted studies confirmed the presence and survival of microscopic fungi in drinking water of different varieties. It presents a direct threat to human health. According the regulation of the

Minister of Health of March, 29, 2007 [34] on the quality of drinking water for humans, water treatment plants (WTP) are obliged to supply tap water with specific criteria. Paragraph 2 specifies that “Water is safe for human health if it is free of pathogenic microorganisms...”. However, in that case, microscopic fungi were not considered as a potential hazard and indicator of water purity. Further in the document it is written that water should meet “the basic microbiological requirements specified in Annex 1” yet this annex lacks references to microfungi. This is a serious problem, as water recipients are not only exposed to fungal infections, but they are not aware of this threat. In addition, it should be noted that fungal microbiota in water distribution systems cause technical problems related to faster pipeline decay. Directives that treat the presence of microscopic fungi as a biological indicator of drinkable water purity are not in force in any of the EU member countries. *Candida albicans* is the only indicator of purity in open and closed watering-places approved in Canada and the USA for nearly 40 years [3].

The conducted studies demonstrated that only a thorough and complete biological evaluation including microfungi is an important addition to physicochemical water quality. The growth of microfungi in drinking water is arguably impacted by a lack of water biological stability, biocorrosion, increased use of disinfectants in water distribution systems, secondary contamination, obstruction of pipelines (water residues) and improper storage of opened bottled water.

The conducted studies have a significant practical implication in, for instance, sanitary and epidemiological water evaluation and in medicine in the context of analysing the quality of drinking water in reference to health resorts and nosocomial infections.

Regular mycological evaluations (including legal regulations) that confirm the presence of potentially pathogenic fungi in drinking water and the assessment of sanitary and the epidemiological hazards they pose will allow for a reliable determination of the quality of water supplied to humans.

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Received 1 July 2014

Accepted 10 August 2014