Bacterial growth and biofilm formation in household-stored groundwater collected from public wells

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ABSTRACT

The research was aimed at assessing changes in the number of bacteria and evaluating biofilm formation in groundwater collected from public wells, both aspects directly related to the methods of household storage. In the research, water collected from Cretaceous aquifer wells in Toruń (Poland) was stored in a refrigerator and at room temperature. Microbiological parameters of the water were measured immediately after the water collection, and then after 3 and 7 days of storage under specified conditions. The microbiological examination involved determining the number of heterotrophic bacteria capable of growth at 22 and 37 °C, the number of spore-forming bacteria, and the total number of bacteria on membrane filters. The storage may affect water quality to such an extent that the water, which initially met the microbiological criteria for water intended for human consumption, may pose a health risk. The repeated use of the same containers for water storage results in biofilm formation containing live and metabolically active bacterial cells.

Key words | bacteria, biofilm formation, groundwater storage, health risk, public health

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INTRODUCTION

Providing safe drinking water is considered a priority in any civilized society. Due to the fact that organoleptic properties (smell, taste) of tap water in Poland are often poor, many people use public wells. The quality of water from these sources is evaluated by a water company as part of internal control and is monitored by the appropriate sanitary inspector. At collection, the water must meet the physico-chemical and bacteriological criteria specified in the Ministry of Health Regulation of 29 March 2007 on the quality of water intended for human consumption, as amended on 20 April 2010 (Dz.U.2007.61.417 2007; Dz.U.10.72.466 2010).

Although groundwater, particularly that pumped from deep wells, is generally thought to be advantageous when compared to surface water with respect to quality (e.g. suspended solids and microbial contamination), it always contains a number of microorganisms (10^3–10^7 cells/mL) (Kotowski & Burkowska 2011; Itävaara *et al.* 2011; Roudnew *et al.* 2012; Krawiec & Walczak 2012).

Water collected from springs and groundwater wells is seldom treated, that is, chlorinated or ozonated, and can therefore contain microbes including numerous opportunistic pathogens, well adapted to oligotrophic environments such as water intakes (Olańczuk-Neyman 2001; Feuerpfeil *et al.* 2009).

Water from public groundwater wells is usually collected into plastic containers (cans, bottles) and stored at home to be used within the following days. Biofilm formation was observed on all surfaces which came in contact with non-sterile water (Singh *et al.* 2006; Flemming 2011; Sakurai & Yoshikawa 2012). Biofilm-forming bacteria, including pathogens, are far less sensitive to environmental factors, the quality which guarantees their survival even under extremely unfavorable conditions (Tamayo *et al.* 2010; Xu *et al.* 2011). In recent years it has been conclusively established that biofilm in water supply systems may constitute a temporary or long-term habitat for hygienically relevant microorganisms (Wingender 2011). The assumption

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that biofilm in containers with drinking water may constitute a source of pathogenic microorganisms and thus a source of water contamination (Wingender & Fleming 2011) leads to the conclusion that the repeated use of containers may pose a health hazard.

The aim of our research was to assess changes in the number of bacteria and evaluate biofilm formation in groundwater collected from public wells, both aspects directly related to water storage.

MATERIALS AND METHODS

Hydrogeologic characteristics of the investigated water

In the vicinity of Toruń, Upper Cretaceous aquifers are located at a depth of 40–50 m in the Vistula valley and at over 100 m on the upland. Well efficiency ranges from several m^3/h to nearly 120 m^3/h. Although water from Upper Cretaceous aquifers is known for its good quality, it often requires iron and manganese removal (Lidzbarski & Prussak 2009). Toruń municipality owns five public Cretaceous aquifer wells exploited by Towarzystwo Wodociągowe Spółka z o.o. (Waterworks Association Ltd). Another well, the property of the Provincial Children's Hospital, is also located in Toruń.

Determining the methods of household water storage

To determine the most frequently used methods of household water storage we conducted a survey among 100 residents of Toruń who use this type of well. The survey contained the following questions: How often do you use this kind of well? For what purposes do you use the water from this well? Where do you store the water from this well? How long do you store the water? In what type of container do you store it? How many times do you use the same container? How do you prepare the container before reusing it? The results of the survey presented in Table 1 enabled us to define research requirements.

Table 1 | The results of the survey (the total numbers of respondens = 100)

Questions	Answers in %	
How often do you use this kind of well?	3 or more times a week	31
	1–2 times a week	42
	2–3 times a month	23
	1 time a month or less	4
For what purposes do you use the water from this well?	To drink without boiling	31
	To drink after boiling	51
	To prepare meals after boiling	18
Where do you store the water from this well?	At room temperature	67
	In the refrigerator	33
How long do you store the water?	1–3 days	68
	4–7 days	32
In what container do you store it?	Plastic container	100
	Glass container	0
	Other container	0
How many times do you use the same container?	1 time	2
	2–3 times	17
	4–5 times	44
	More than 6 times	37
How do you prepare the container before reusing it?	Only the pouring out of the old water	32
	Rinsing the containers with tap water	68

Water sampling and water storage

Water for microbiological analysis was collected from the Cretaceous aquifer wells located in ul. Bażyńskich (the name of the street in Toruń) and at the Provincial Children's Hospital. Basic physico-chemical and microbiological parameters of the water are presented in Table 2. Water collected from public wells in Toruń is not treated, that is, chlorinated or ozonated. Water, collected five times at weekly intervals, each time in the same 5 L plastic bottles, was then stored in a refrigerator and at room temperature. Microbiological parameters were measured immediately after the water collection, then after 3 and 7 days of storage under specified conditions. The entire procedure was repeated five times using the same bottles. Before every consecutive sampling the reusable plastic bottles were vigorously rinsed (twice) with approximately 1 L of tap water.

Microbiological analysis

The following microbiological parameters were determined immediately after the water collection, then after 3 and 7 days of storage under specified conditions:

Table 2 | Basic physico-chemical and microbiological parameters of the water

	The well		
Parameter	Ul. Bażyńskich	Provincial Children's Hospital	Acceptable concentration
Turbidity (NTU)	<0.10*	0.73	1
Color (mg/L)	5	5	15
Smell	Acceptable	Acceptable	–
Taste	Acceptable	Acceptable	–
pH	8.2	7.9	6.5–9.5
Conductivity (μS/cm)	631	630	2,500
NH_4^+ (mg/L)	0.56	<0.05	0.5
Fe (μg/L)	164	48	200
Mn (μg/L)	<25	<25	50
Coliform bacteria (CFU/100 mL)	0	0	0
E. coli (CFU/100 mL)	0	0	0

*Data of the district sanitary-epidemiological station in Toruń (03.06.2013). NTU = nephelometric turbidity units; CFU = colony-forming units

The number of heterotrophic bacteria capable of growth at 22 and 37 °C

The number of heterotrophic bacteria capable of growth at 22 and 37 °C was determined through the inoculation of water samples of 1 mL on nutrient agar in accordance with PN-EN ISO 6222.

The number of spore-forming bacteria

To detect the presence of spore-forming microorganisms the 100 mL water samples were pasteurized in a water bath at 80 °C for 20 minutes, then samples of 1 mL were inoculated on nutrient agar (pour plates method) and incubated for 5 days at 22 °C. Subsequently, the grown colonies were counted.

The total number of bacteria

One hundred mL water samples were fixed with 40% sodium formaldehyde to achieve the final concentration of 4%. The total number of bacteria was determined using the method of direct count on membrane filters (Zimmermann 1981). Bacteria stained with acridine orange were counted under an epifluorescence microscope Nikon H550S at 1,000× magnification.

Evaluating biofilm formation rate on plastic bottles

Biofilm formation rate was evaluated on 2 cm^2 fragments of the plastic bottles used in the research. Fragments of a new clean bottle were used as control samples.

Determining biofilm formation rate by the crystal violet method

A biofilm formation rate was assessed using a modified method described by Stepanovic *et al.* (2007). Fragments of bottles (2 cm^2, five replicates for each bottle), dried at 60 °C for 60 minutes, were immersed for 15 minutes in 1% solution of crystal violet, rinsed three times with distilled water and dried at room temperature. Then fragments of bottles with stained biofilm were placed in chemically clean test tubes filled with alcohol (2.5 mL). After rinsing off the crystal violet-stained biofilm, the absorbance was measured at a wavelength of 590 nm using a Hitachi spectrophotometer U-190.

A biofilm formation rate on the plastic bottles was evaluated using the Stepanovic *et al.* (2007) method according to the equation:

$$ODc = OD \text{ of control} + 3 \times SD \text{ of control},$$

where OD is optical density; SD is standard deviation; $OD \leq ODc$, no biofilm formation; $ODc < OD < 2 \times ODc$, weak biofilm formation; $2 \times ODc < OD < 4 \times ODc$, moderate biofilm formation; $4 \times ODc < OD$, strong biofilm formation.

Evaluating hydrolase acivity in the biofilm

General hydrolase activity in the biofilm was determined using fluorescein diacetate (FDA), a non-specific substrate for hydrolases, and applying the modified method described by Adam & Duncan (2001), who studied the activity of soil enzymes. They maintain that the incubation time for soil samples should not exceed 40 minutes, otherwise there is the risk of proliferation of microorganisms (making the results unreliable). In our research the risk of proliferation of microorganisms does not

increase with the increased incubation time because the number of microorganisms in our water samples was very low (compared to the number of microorganisms in soil samples), and the samples did not contain any nutrients. The research and control samples of the plastic bottles (2 cm^2, five replicates for each bottle) were placed in tubes containing 5 mL phosphate-buffered saline. After adding 50 μL FDA the samples were incubated in the dark for 3 hours at 30 °C. The fluorescence intensity was measured using a Hitachi F-2500 spectrophotometer, at the emission wavelength of 505 nm and at the excitation wavelength of 480 nm.

Assessment and interpretation of bacterial viability by using the LIVE/DEAD method

Fragments of the bottles (five fragments of 1 cm^2 from each bottle) covered with the aqueous solutions of dyes of LIVE/DEAD® BacLight™ Bacterial Viability Kit were incubated for 15 minutes in the dark at room temperature. After the excess dye was removed, the samples were placed on microscope slides and viewed under oil immersion at 1,000× magnification using a Nikon H550S epifluorescence microscope with the appropriate filter set (470–490 nm excitation filter, 520 nm barrier filter). For each fragment 20–40 fields of view were evaluated (depending on how evenly the microorganisms were distributed). Color micrographs were taken with a digital image processor (Olympus XC50) using the software package CellB v. 3.1). The numbers of bacteria on the investigated slides were evaluated using MultiScan Base software. Viable cells were fluorescent green while non-viable cells were fluorescent red.

RESULTS

Survey results

The respondents used public groundwater wells several times a week (73%). Most respondents used the water only after boiling it (69%), but almost one third drank the water without boiling it first (31%). All respondents (100%) stored the water in plastic containers. Most people stored the water at room temperature (67%), for a period of 1–3 days (67%) up to 1 week (33%). Almost all respondents admitted to reusing the container in which they stored the water (98%): some used it four or five times (44%), some used it more than six times (37%), rinsing the container with tap water before reusing it (68%).

The number of heterotrophic bacteria capable of growth at 22 and 37 °C

Higher numbers of both groups of heterotrophic bacteria (capable of growth at 22 and 37 °C) were recorded in the water samples from the well located at the Provincial Children's Hospital, stored in the refrigerator and at room temperature (Figure 1). All differences were statistically significant ($p < 0.05$).

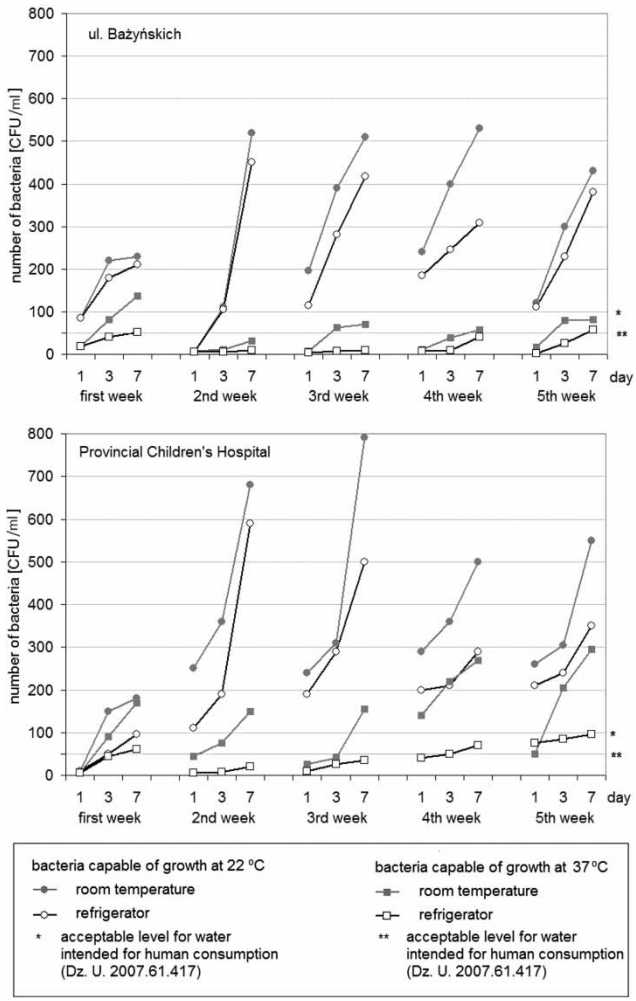

Figure 1 | The number of heterotrophic bacteria in household-stored groundwater collected from public wells in Toruń.

The number of microorganisms in both groups (growing at 22 and at 37 °C) was higher in water stored at room temperature than in the refrigerator. The differences were statistically significant: $p < 0.05$ for bacteria grown at 22 °C and $p < 0.001$ for bacteria grown at 37 °C. Both the water stored in the refrigerator and at room temperature appeared to be a better environment for bacteria growing at 22 °C than for bacteria growing at 37 °C. In the majority of tests, the number of bacteria was many times higher in cultures incubated at 22 °C ($p < 0.001$).

The number of heterotrophic bacteria capable of growth at 37 and 22 °C increased with water storage time: the highest numbers were usually recorded on the last day of the weekly research cycle. The differences were statistically significant ($p < 0.001$ for bacteria capable of growth at 22 °C and $p < 0.05$ for bacteria grown at 37 °C). This tendency was observed for the samples from both wells, in the majority of tests taken during the 5-week research period. When the same container was reused, the numbers of bacteria increased in subsequent research cycles.

The number of heterotrophic bacteria capable of growth at 22 and 37 °C in the studied water samples immediately after their collection into clean bottles was not higher than permissible according to the Regulation of the Ministry of Health of 29 March 2007 (50 CFU/mL for bacteria growing at 37 °C, 100 CFU/mL for bacteria growing at 22 °C). However, after only 3 days of household storage, especially at room temperature, all samples failed to meet the criteria specified in the above regulation. Furthermore, water freshly collected into the bottles used repeatedly before frequently failed to meet the requirements for water intended for human consumption.

The number of spore-forming bacteria

The number of spore-forming bacteria (Table 3) in the water samples from both wells was low; in 76% of the samples the number was ≤1 CFU/mL. Only in two samples did the number exceed 10 CFU/mL.

The total number of bacteria

The water from the well in ul. Bażyńskich contained a higher total number of bacteria than the water from the well at the Provincial Children's Hospital (Figure 2). The differences were statistically significant ($p < 0.05$). In all samples the highest number was recorded on the seventh day of the third week. In the water stored at room temperature the number of bacteria was generally higher than in the water stored in a refrigerator but the differences were statistically significant ($p < 0.05$) only in the samples from the well in ul. Bażyńskich. In most cases (ul. Bażyńskich – in the third, fourth and fifth week, the Provincial Children's Hospital – all weeks), the total number of bacteria increased with water storage time and was highest on the seventh day of each week of the research.

Table 3 | The number of spore-forming bacteria (CFU/mL) in water samples from wells

		Provincial Children's Hospital		Ul. Bażyńskich	
Week	Day	Room temperature	Refrigerator	Room temperature	Refrigerator
1	1	2	–	1	–
	3	9	7	5	0
	7	3	1,66	6,33	2
2	1	0	2,33	0	0
	3	0	0,33	0	0
	7	2,33	14	12,66	0
3	1	0	0,66	0,66	0
	3	1	0	0,66	1,33
	7	0,33	0,33	0	0,33
4	1	0,66	0,33	1,33	0,33
	3	0,33	0	0	0
	7	0	0	0,66	0,66
5	1	0	0,66	0	1
	3	0	1	0	0,33
	7	0	0,66	0	0

Biofilm formation on the surface of plastic bottles

For the control sample with the average OD value = 0.0086, ODc was 0.013, considered a threshold below which there is no biofilm production. The average optical density of the samples from all four bottles was higher than 2ODc, which indicates at least moderate biofilm formation. The highest OD (0.056) was recorded in the sample from the bottle stored in the refrigerator, containing water from the well at ul. Bażyńskich. The result was higher than 4ODc, which indicates strong biofilm formation (Figure 3(a)).

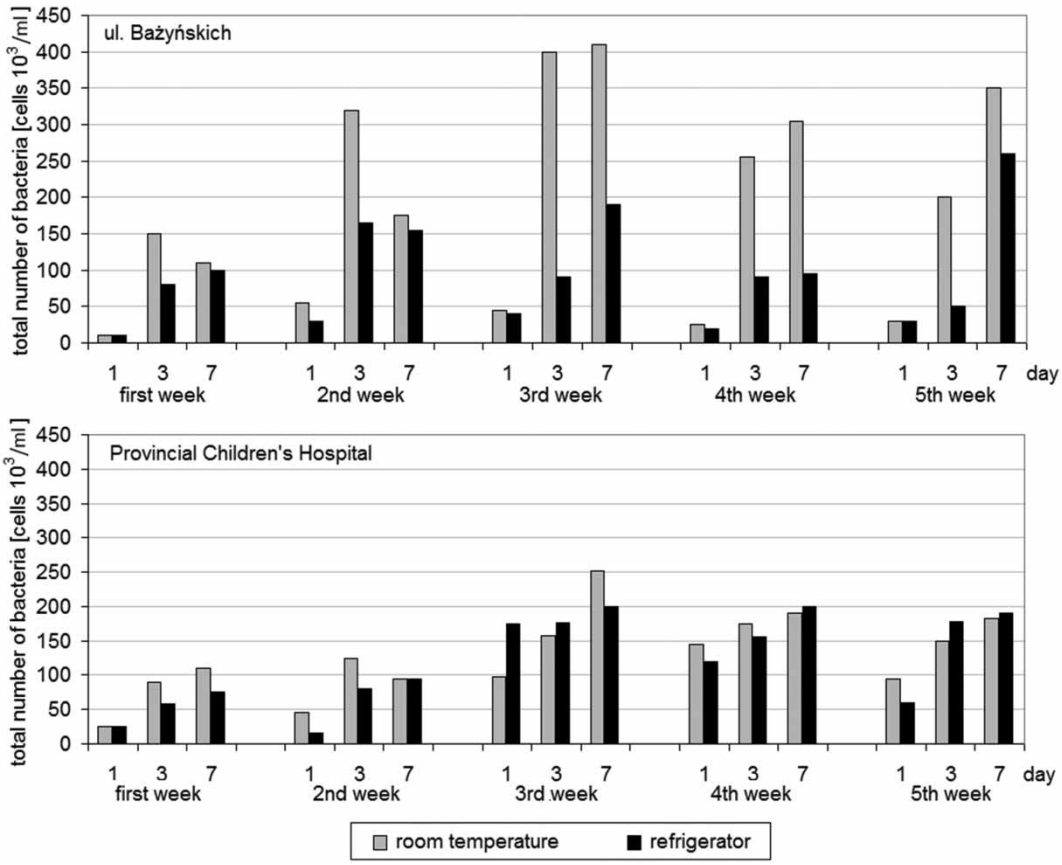

Figure 2 | The total number of bacteria in household-stored groundwater collected from public wells in Toruń.

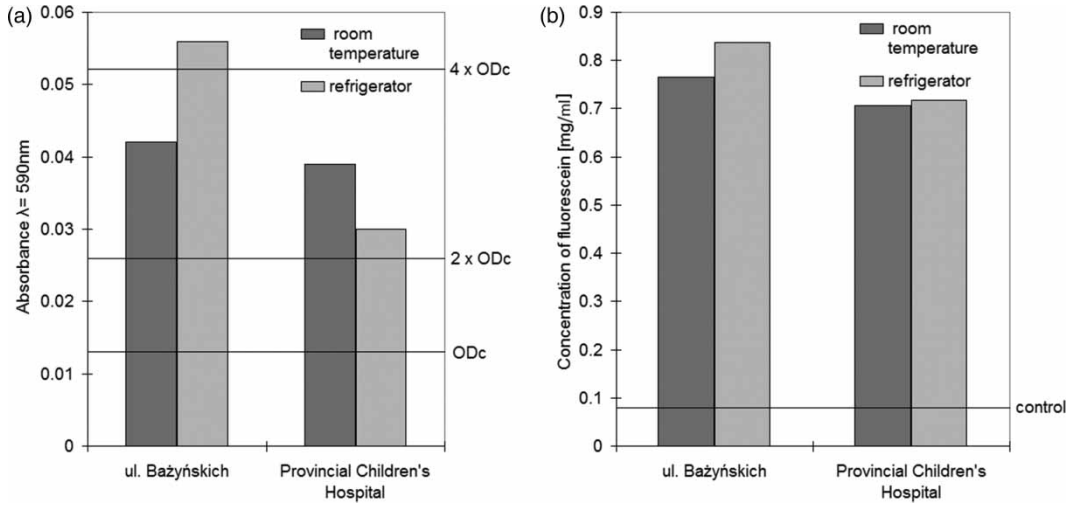

Figure 3 | Biofilm formation (a) and hydrolase activity in the biofilm (b) on the surface of the plastic bottles.

Hydrolase activity in the biofilm

The average concentration of fluorescein released in all the investigated samples was higher than in the control sample (0.079 mg/mL). The highest value (0.838 mg/mL) was recorded for the refrigerated sample containing water from the well in ul. Bażyńskich. The comparison of hydrolase activity and the amount of biofilm indicates a positive correlation with the correlation coefficient $R = 0.91$. The high correlation between biofilm formation (the amount of biofilm) and hydrolase activity indicates the presence of metabolically active cells in the biofilm.

LIVE/DEAD cell staining

Twenty-seven percent of bacterial cells in all investigated samples stained green, which indicates the presence of live and metabolically active bacterial cells in the biofilm formed on the bottle fragments (Figure 4).

DISCUSSION

In Poland groundwater constitutes about 68.5% of water distributed through the water supply system. Two-thirds of the groundwater in the supply system undergoes treatment, with the side effect being the deterioration of its organoleptic properties; a vast majority of consumers complain about poor quality of water, accentuating its bad taste and/or smell (Wojtyła-Buciora & Marcinkowski 2010). In a response to these complaints, many cities in Poland provide access to Oligocene (Warsaw), Jurassic (Krakow) or Quaternary wells (Lodz). The results of the survey indicate that Toruń residents frequently use public Cretaceous wells.

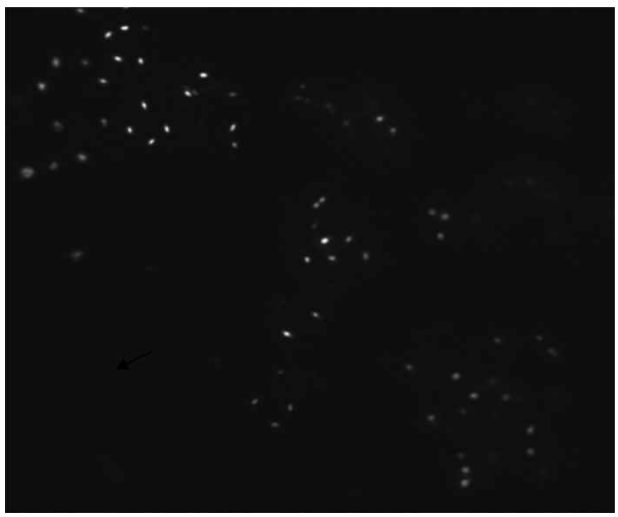

Figure 4 | The biofilm formed on the bottle fragments stained by using the LIVE/DEAD method (microscope Olympus BX50, magnification 1,000×, live bacteria – white, dead bacteria – grey).

Groundwater always contains a certain number of indigenous microorganisms, mainly psychrophilic and oligotrophic (Olańczuk-Neyman 2001; Walczak & Krawiec 2014). Immediately after water collection the number of heterotrophic bacteria capable of growth at 22 °C was low and the quality of water collected from both wells complied with the standards specified in the Regulation of the Minister of Health of 29 March 2007 (Dz.U. 2007.61.417 2007).

Available data indicate that the total number of bacteria identified in groundwater under a microscope ranges from 10^3 to 10^7 cells in 1 mL (Bray *et al.* 2011; Kotowski & Burkowska 2011; Walczak & Krawiec 2014). In the present research the number ranged from 1.8×10^4 to 4.07×10^5, which is not considered high when compared to the range mentioned above. In our research the total number of bacteria identified under a fluorescence microscope increased with storage time, which was stastistically significant ($p < 0.05$ by analysis of variance (ANOVA)). Bray *et al.* (2011) noted a significant increase in the total number of bacteria after 7-day storage at a temperature ranging from 10 to 20 °C, and a less significant increase after 7-day storage at 5 °C.

Microorganisms found in groundwater most commonly belong to psychrophiles whose optimum temperature is below 20 °C, thus the water from both wells stored in a refrigerator and at room temperature constituted a better environment for bacteria growing at 22 °C than for bacteria growing at 37 °C. The water stored at room temperature contained a higher number of both groups of heterotrophic microorganisms than the water stored in a refrigerator. However, even the refrigerated water contained heterotrophic bacteria. The results obtained by Olańczuk-Neyman (2001) indicate that at temperatures below 10 °C microbiological processes in tap water run with high efficiency.

Increased numbers of bacteria were recorded in the subsequent research cycles using the same container. Further studies confirmed biofilm formation of metabolically active

microbial cells on the surface of the bottles. Microorganisms attach within minutes to most inanimate solid surfaces immersed in natural water, where they may grow to form biofilms (Brümmer *et al.* 2000; Czaczyk & Myszka 2007) and can survive under conditions in which the survival of single cells would be difficult or even impossible (Furowicz *et al.* 2010; Jägevall *et al.* 2011; Diaz Villanueva *et al.* 2011). Fecal indicator bacteria (e.g. *Escherichia coli*), pathogenic bacteria (e.g. *Campylobacter* spp.), opportunistic bacteria (e.g. *Legionella* spp., *Pseudomonas aeruginosa*), enteric viruses (e.g. adenoviruses, rotaviruses, noroviruses), and parasitic protozoa (e.g. *Cryptosporidium parvum*) can attach to already existing biofilms and survive for a period of several weeks (Wingender & Fleming 2011; Walczak *et al.* 2013). Biofilm formed on repeatedly used bottles may contain pathogenic microorganisms and become a potential source of contamination, affecting the quality of water, which met all the microbiological criteria at the time of its collection. (Tamayo *et al.* 2010; Wingender & Fleming 2011; Ahmed *et al.* 2013).

Regular groundwater quality monitoring at public wells is necessary to ensure the safety of users and reduce the risk of waterborne epidemics. Moreover, consumers should be warned about dangers associated with drinking water that contains a higher than permissible number of microorganisms. In order to take proper safety precautions users should be informed how household storage (time and conditions) affects water microbial quality.

CONCLUSIONS

Household water storage affects the growth rate of heterotrophic bacteria. In the present research their number increased with time, especially in the samples stored at room temperature. Inadequate storage may affect water quality to such an extent that the water, which initially met the microbiological criteria for water intended for human consumption, may pose a health risk. It is therefore recommended that water collected from public wells should be stored in refrigerators for no longer than 3 days. The repeated use of the same containers for water storage leads to the formation of biofilm, which contains live and metabolically active bacterial cells. Prior to reuse, plastic containers should be thoroughly washed with detergent, rinsed several times, and dried to remove biofilm.

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