ABSTRACT: The paper investigates the presence of spore-forming bacteria of the genus *Bacillus* among aerobic mesophilic bacteria found in water samples collected from the City of Novi Sad groundwater supplies. Microbiological examination included the samples from three groundwater supplies: *Štrand* (19 samples), *Petrovaradinska ada* (10 samples) and *Ratno ostrvo* (12 samples). Apart from the analysis of groundwater, the research also included microbiological examination of water from the City of Novi Sad water supply system collected from three localities - Liman 1, Slana bara and Stari grad. All samples were inoculated onto Plate Count Agar and low nutrient medium R2A.

The presence of *Bacillus* spp. was detected in all three groundwater supplies. Comparing the samples obtained from the three sites, a significantly higher number or percentage of the genus *Bacillus*, in comparison with aerobic mesophilic bacteria, was detected in the groundwater supply *Štrand* using R2A medium. No correlation was detected between the total count of aerobic mesophilic bacteria and *Bacillus* spp.

KEYWORDS: aerobic mesophilic bacteria, *Bacillus* spp., groundwater, City of Novi Sad

INTRODUCTION

According to the World Health Organization, drinking water quality is one of the main indicators of the wealth and health conditions of a country’s population [World Health Organization and United Nations Children’s Fund, 2004]. Apart from its physiological, hygienic and toxicological importance,
Water has a considerable epidemiological significance. Humans can become infected either directly, by consuming polluted drinking water and using it for food preparation, or indirectly, by applying polluted water for recreational purposes. The knowledge concerning the most common genera of bacterial contaminants is of great significance, not only for water itself, but for groceries of various origin. In this respect, the presence of spore-forming bacteria can be of great importance [Petrović, 1996].

Spores are primarily formed by gram positive rods, mainly the species belonging to the genera *Clostridium* and *Bacillus*, as well as by certain cocci and gram negative rods [Holt et al., 1994]. The genus *Bacillus* is a frequent contaminant of water, milk and dairy products, wheat, bread and pasta. Some of the common contaminants of food, including drinking water as well, are aerobic mesophilic bacterium *Bacillus subtilis*, aerobic thermophilic spore-forming bacterium *Bacillus stearothermophilus* and aerobic mesophilic spore-forming bacterium *Bacillus cereus* [Kramer and Gilbert, 1989].

The data gathered by the Institute of Public Health of Vojvodina point to a frequent cultivation of *Bacillus* spp. in purified and chlorinated water from the City of Novi Sad water supply system. Accordingly, *Bacillus* spp. appeared as the most commonly cultivated microorganisms in the tested samples of purified and chlorinated drinking water in 2011 [Ekobilten, 2008; Ekobilten, 2009; Ekobilten, 2011].

The City of Novi Sad water supply system uses the groundwater supply sites located in the vicinity of the Danube River. Three groundwater supply sites are in use: Štrand and Ratno ostrvo on the left bank of the river and Petrovaradinska ada on the right bank. A great cause for concern is the fact that, in spite of technological processing of groundwater, the presence of spore-forming bacteria of the genus *Bacillus* has been continually detected in a considerable number of the tested samples of drinking water from the City of Novi Sad water supply system [Ekobilten, 2008; Ekobilten, 2009; Ekobilten, 2011].

The aim of this study was to determine whether and to what extent bacterial spores of the genus *Bacillus* are detected in samples of the City of Novi Sad groundwater supplies. Also, the aim of this study was to determine if this phenomenon can be associated with a sampling site and the presence of large numbers of aerobic mesophiles. Furthermore, the study examined the possible presence of *Bacillus* spp. in purified and chlorinated drinking water distributed by the City of Novi Sad water supply system.

**MATERIAL AND METHODS**

The samples were collected in January, February and March 2011. The groundwater samples were collected from Štrand, Petrovaradinska ada and
Ratno ostrvo supply sites. The analysis included 19 samples collected from Štrand groundwater supply: Š1, Š3, Š5, Š7, Š8, Š9, Š10, Š16, Š17, Š18, Š19, Š20, Š21, BHD-1-2-20, P1, P2, P5, P8, and P10. From Petrovaradinska ada, 10 samples were collected: PA1, PA6, PA7, PA9, PA11, PA11a, PA12, PA13, PA14, and PAzbirni, while 12 samples were taken from Ratno ostrvo: BHZ1, BHZ3, BHZ5, BHZ7, BHZ9, BHZ11, Mp6, Md6, PJC1, PJC9, PPJC6, and PPJC10.

Apart from the groundwater samples, the study also included the analysis of three drinking water samples collected from different localities of the City of Novi Sad water supply system. The samples were designated as SG, SB and L1.

The analysis focused on the total number of *Bacillus* spp. and the total count of aerobic mesophilic bacteria in all of the collected samples. The total count of aerobic mesophilic bacteria was determined using Plate Count Agar (PCA) (Torlak) and low nutrient medium R2A (HiMedia), while the inoculation was performed using standard spread plate technique directly from the sample and from appropriate dilution in two repetitions, the inoculum quantity being 0.5 ml. With the samples inoculated onto PCA the incubation temperature was 37 °C, while in the case of R2A medium the temperature was 22 °C. The incubation period in both cases was 48 hours. After the incubation period, the counting of all grown colonies was performed. In order to determine the number of spore-forming bacteria of the genus *Bacillus*, the water samples were treated in the water bath at the temperature of 85 °C during 15 minutes prior to the inoculation. As for the inoculation process, the used media and the incubation temperature, the procedure was identical to the one applied to aerobic mesophilic bacteria. The incubation period lasted for 5 days. After the incubation period, the grown colonies were counted and the detection of *Bacillus* spp. was conducted with microscopic examination of preparations stained by Gramm and Schaeffer-Fulton technique.

Differences in number and percentage of *Bacillus* spp. found in groundwater samples were processed by means of Kruskal-Wallis and Mann-Whitney test. The tests, along with the figures and correlation analysis were processed by STATISTICA v. 10.0 [StatSoft, Inc. 1984 – 2011].

**RESULTS AND DISCUSSION**

Out of 19 samples collected from the Štrand spring, *Bacillus* spp. were present in 18. Only the sample P5 showed no colonies of these bacteria in any of the applied media. The greatest number of *Bacillus* spp. colonies on PCA were detected in sample Š7, with the recorded value of 250 CFU/ml. A slightly lower number was detected in samples BHD-1-2-20 (199 CFU/ml) and Š9 (176 CFU/ml), while the complete absence of spore-forming *Bacillus* genus bacteria was in samples Š21 and P5 (Figure 1). As for R2A medium, the number
of Bacillus spp. was the largest in sample Š9 (289 CFU/ml), while sample P5 showed total absence of the bacteria (Figure 1). In relation to the number of aerobic mesophilic acteria, the percentage of Bacillus spp. colonies on PCA ranged from 0 to 95.4%, while on R2A medium it spanned from 0 to 29.9% (Figure 1). In samples Š3, Š9, P1 and P8 on R2A medium, as well as in samples P1 and BHD-1-2-20 on PCA, the percentage of Bacillus colonies could not be determined due to the abundance of aerobic mesophilic bacteria.

Figure 1. The total number of Bacillus spp. in samples from Štrand groundwater supply site isolated on PCA and R2A (left) and the percentage of Bacillus spp. among aerobic mesophiles on PCA and R2A (right)

In the case of Petrovaradinska ada supply site, the presence of Bacillus spp. was recorded in all of 10 samples included in the analysis. The highest number of Bacillus spp. colonies on PCA was detected in sample PA-14, 22 CFU/ml, while the samples PA-1, PA-9 and PA-11 showed the smallest number of the bacteria (Figure 2). As for R2A medium, the largest number of Bacillus spp. appeared also in PA-14 sample, the recorded value 6 of CFU/ml, while the samples PA1, PA6, PA9, PA11 and PA12 showed total absence of the bacteria (Figure 2). The percentage of Bacillus spp. colonies in relation to the number of aerobic mesophiles grown on PCA and R2A ranged 0.01% – 3.33% and 0% – 2.22%, respectively (Figure 2). In the case of sample PA14 on R2A, due to the abundance of aerobic mesophilic bacteria, the percentage of Bacillus spp. colonies could not be determined.
In 12 samples collected from Ratno ostrvo, the presence of spore-forming bacteria of the genus *Bacillus* was detected in 9 samples. The sample BHZ5 showed the highest number of *Bacillus* spp. colonies on PCA - 20 CFU/ml, while in samples BHZ3, BHZ11, PJC9 and PPJC6 none of the colonies was detected. As for R2A medium, the greatest number of the colonies was recorded in sample Md6 (14 CFU/ml), while in samples BHZ3, Mp6, PJC9 and PPJC6, *Bacillus* spp. colonies were absent (Figure 3). The percentage of *Bacillus* spp. in relation to the number of aerobic mesophiles on PCA ranged from 0% to 3.5%, while in the case of R2A, the recorded value was rather low ranging from 0% to 0.43% (Figure 3).
In case of PCA application, no statistical differences were observed regarding the number of *Bacillus* spp. members detected in three groundwater supply sites (Kruskal-Wallis, $X^2 = 6.536579$, df = 2, $p>0.001$). However, with the use of low-nutrient R2A medium, statistical analysis pointed to a significant difference between the bacterial counts obtained for the three supply sites (Kruskal-Wallis, $X^2 = 15.16343$, df = 2, $p<0.001$). A considerably higher number of *Bacillus* spp. on R2A was registered in the samples taken from Štrand supply site, while the values obtained for other two sites showed no statistical differences (Mann-Whitney U Test, $p>0.01$). Comparing all three supply sites, it can be concluded that the sample with the largest count of *Bacillus* spp. detected on PCA was the one that originates from Štrand supply site. It was the sample Š7 with a total of 250 *Bacillus* spp. colonies detected. When applying on R2A medium, the sample Š9, collected from the same site, showed 289 colonies of spore-forming bacteria, which was also the highest number of *Bacillus* spp. detected in all three supply sites. With reference to all three supply sites, no statistical differences were observed in the percentage of *Bacillus* spp. present among aerobic mesophiles detected on PCA (Kruskal-Wallis, $X^2 = 5.793854$, df = 2, $p>0.01$). However, with the application of R2A,
the analysis pointed to a significant difference in percentage of the bacteria detected among aerobic mesophiles (Kruskal-Wallis, \(X^2 = 16.11641\), df = 2, \(p<0.01\)). A higher percentage of *Bacillus* spp. detected on R2A medium was recorded in the case of Štrand supply site, while the other two sites showed no significant differences in percentage of the detected bacteria (Mann-Whitney U Test, \(p>0.01\)). The results referring to the number of aerobic mesophilic bacteria recorded in the samples from all three supply sites and grown on PCA and R2A can be seen in Figure 4.

No correlation was detected between the number of aerobic mesophilic bacteria and *Bacillus* spp. number recorded for the groundwater supply sites (Figure 4).

Table 1. shows the total count of *Bacillus* spp. and aerobic mesophilic bacteria detected in samples collected from various localities of the city water supply system.

Table 1. The total count of *Bacillus* spp. and aerobic mesophilic bacteria detected in the samples from the water supply system

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aerobic mesophilic bacteria CFU/ml (PCA)</th>
<th><em>Bacillus</em> spp CFU/ml (PCA)</th>
<th>Aerobic mesophilic bacteria CFU/ml (R2A)</th>
<th><em>Bacillus</em> spp CFU/ml (R2A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>5</td>
<td>0</td>
<td>784</td>
<td>0</td>
</tr>
<tr>
<td>SB</td>
<td>6</td>
<td>0</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>L1</td>
<td>3</td>
<td>0</td>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>

The results presented in Table 1. clearly demonstrate that *Bacillus* spp. were not detected in any of the samples originating from the city water supply system.

The present study points to a high frequency of *Bacillus* spp. in the City of Novi Sad groundwater supplies. These results can be explained by the ubiquity of the bacteria in the environment and by a high resistance of the bacterial spores as well [Hosni et al., 2011]. *Bacillus* spp. members have been isolated from both shallow groundwater supplies [Chapelle et al., 1988] and from samples collected from a considerable depth [Boone et al., 1995]. Their viability in the form of spores in the process of drinking water treatment has been reported as a permanent problem [Bloomfield, 1999; Setlow, 2000; Dalmacija et al., 1996; Zhang et al., 2006; Morrow et al., 2008]. Some studies on the presence of bacterial spores [Sagripanto and Bonificino, 1996] compare the efficacy of glutaraldehydes, formaldehydes, hydrogen peroxide, peracetic acid, cupric ascorbate and natrium hypochlorite in destroying *Bacillus subtilis* spores under various conditions. Each of the above agents were used
considering pH, temperature and period of time. Only three of the chemicals, natrium hypochlorite, peracetic acid and cupric ascorbate, managed to destroy 99.9% of the spores, 30 minutes after incubation at the temperature of 20 °C. Glutaraldehydes inactivated 90% of the spores, while hydrogen peroxide and phenol destroyed a small proportion of spores in the suspension [Sagripanto and Bonificino, 1996].

Referring to Ekobilten, a publication comprising data gathered by the Institute of Public Health of Vojvodina, the presence of Bacillus spp. has been reported in a large number of samples from the City of Novi Sad water supply system [Ekobilten, 2011]. In the present study, the samples of water from the city water supply system showed no presence of Bacillus bacteria. However, as the results of the Institute of Public Health of Vojvodina point to a frequent cultivation of these bacteria in purified and chlorinated water from the city water supply system, this may cast doubt on the efficacy of the applied water treatment technology.

CONCLUSION

The presence of Bacillus spp. was detected in groundwater samples from all three supply sites. The percentage of Bacillus spp. detected among aerobic mesophilic bacteria widely varied, from 0 to 95%. Comparing all three water supply sites, a significantly higher total count and percentage of the genus Bacillus, in relation to the number of aerobic mesophilic bacteria, was detected in the samples from Štrand groundwater supply site using R2A medium. No correlation was detected between the number of aerobic mesophilic bacteria and Bacillus spp. number. Bacillus spp. were absent from the samples collected from the City of Novi Sad water supply system.

REFERENCES


BACILLUS SPP. У ПОДЗЕМНИМ ВОДАМА НОВОСАДСКИХ ИЗВОРИШТА ВОДЕ ЗА ПИЋЕ

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РЕЗИМЕ: У овом раду праћена је заступљеност спорогених бактерија рода Bacillus у оквиру аеробних мезофилних бактерија у узорцима вода новосадских изворишта. Микробиолошка испитивања вршена су на узорцима изворишта Штранд (19 узорака), Петроварадинска ада (10 узорака) и Ратно острво (12 узорака). Поред микробиолошке анализе вода изворишта вршена је микробиолошка анализе воде из дистрибуционог система водовода града Новог Сада са локалитета Лиман 1, Слана бара и Стари град. Узорци су инокулисани на подлоге PCA и R2A.
У подземној води сва три изворишта констатовано је присуство врста рода *Bacillus*. Посматрано за сва три изворишта значајно већа бројност и процентуална заступљеност представника рода *Bacillus* у односу на аеробне мезофилне бактерије регистрована је у води изворишта Штранд коришћењем R2A подлоге. Између бројности аеробних мезофилних бактерија и бројности бактерија рода *Bacillus* у извориштима подземних вода није утврђена корелација.

КЉУЧНЕ РЕЧИ: аеробне мезофилне бактерије, *Bacillus sp*, Нови Сад, подземне воде