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USE OF THE DIRECT AND CULTIVATION METHODS IN THE BACTERIOLOGICAL EXAMINATION OF WATER IN WATER SUPPLY SYSTEM

S. Ćirić, Z. ilić, B. Milošević, Z. Spasić, J. Stojković

Faculty of Agriculture, Lešak, Republic of Serbia
Corresponding author: slavica.ciric@open.telekom.rs
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Abstract: Bacterial abundance in the water used for drinking by humans and animals is a parameter that is closely linked to their health. Therefore, application of a method of detection of bacterial count which will indicate the real status of this parameter, as authentically as possible, is necessary. The bacterial load of water in certain stages of water supply using direct and cultivation methods was investigated. In the cultivation method, the water samples were inoculated on PCA and on R2A medium, and incubated for 7 days at 37 °C and at room temperature. Direct method included filtration of samples stained with acridine orange and counting of bacteria on the filters under the epifluorescence microscope. Cultivation and direct methods showed the lowest bacterial count in the tank of Bagdala II, 0 cfu/ml and 611 bacteria/ml, respectively, and maximum abundance in raw water, 157 cfu/ml and 1,378,698 bacteria/ml, respectively. Statistical analysis showed that significantly higher count of bacteria was recorded on R2A medium compared to PCA, and at room temperature than at 37 °C. By index TBC/AMB, the native raw water was classified as more polluted water when applying the results obtained at room temperature compared to the results obtained at 37 °C. The most realistic bacteriological status of all water types was obtained using the direct method of quantification of bacteria. However, for routine monitoring of water in water supply system, as well as for the examination of bacterial regrowth in the distribution network, it is the best to apply the cultivation method which involves inoculation of samples on R2A medium and incubation at room temperature.

Key words: water supply, PCA medium, R2A medium, epifluorescence microscopy

Introduction

One of the basic and most important parameter of quality of water that is used for drinking by humans and animals is the quality from microbiological point

of view. This parameter of water quality is closely linked to the health of humans and animals, because the drinking water was and still is one of the main link in the transmission of infections and communicable diseases. Microbiological stability of water is provided by the knowledge of source water quality, control of treatment process and by stability and distribution system integrity.

There is no single microbiological parameter based on which it can be established whether all the stages in the processing and distribution of water work correctly in all situations, but as the main indicator the total bacterial count is used. The number of bacteria is determined by indirect (cultivation) and direct methods. Each method has certain advantages and disadvantages, and choice of method depends upon the situation, the existing conditions and requirements. In Serbia, the total count of bacteria referred to as the total number of aerobic mesophilic bacteria. It is determined by cultivation on high-nutrient medium after incubation for 48 h at 37°C *(Sl. list SFRJ, 33/87; Sl. list SRJ, 42/98)*. The low-nutrient media (such as R2A) and prolonged incubation (5-7 days) at room temperature which has been shown to detect significantly more bacteria than the standard method *(Ćirić, 2009)* are favored in the developed countries. On the other hand, any so far known cultivation method detects less than 1% of bacteria compared to the direct method *(Bloomfield et al.,* 1998).

The aim of this study was to determine the bacteriological status of water in certain stages of the watersupply system of Kruševac, using direct and indirect methods. Also, the objective was to define the most appropriate methods and combination of cultivation conditions depending on the aspect that we want to examine. This is the first time that the water in the watersupply system of Kruševac is examined by direct method.

Materials and Methods

During the one-year examination, the fifty samples from each of the eight main points in the watersupply system of the town of Kruševac were taken. These points were:

- The native raw water - S1 (water which is taken from the hypolimnion of the catchment basin of Reservoir Ćelije and sent to the Drinking Water Treatment Plant for processing);
- Chlorinated raw water tank - S2 (water after prechlorination);
- Settler - S3 (water after coagulation and sedimentation);
- Ozonator - S4 (water after ozonation);
- The final water tank - S5 (water after final chlorination, which is sent to the distribution network);
- City tank Bagdala I - S6;
- City tank Bagdala II - S7;
- Distribution network - S8 (water from the endpoints of network, ie. tap water).

In each water sample the bacterial count was determined using cultivation and direct methods. For cultivation method, the samples were inoculated by pour plate technique on high-nutrient PCA (Plate Count Agar, Merck) medium and on low-nutrient R2A (Reasoner's 2 Agar, Merck) medium. Three plates of each inoculated medium were incubated at 37 °C and at room temperature (22 °C). The amount of inoculum for all samples was 1 ml, while for the native raw water, 10^{-1} and 10^{-2} dilutions were inoculated also. The bacteria were counted after 7 days of incubation for both temperatures. Plates incubated at 37 °C were previously protected from drying with parafilm. For the native raw water, the bacterial count was carried out on plates inoculated with the lowest dilution that could be read, and the obtained number was converted to 1 ml of sample.

By direct method, the number of bacteria was determined using epifluorescence microscope after staining of samples with acridine orange *(Ćirić, 2009)*.

On the basis of the results, the percentage of the bacterial count, obtained by cultivation methods, in the total bacterial count, obtained by direct method, was determined. TBC/AMB index was also calculated. This index is the ratio of total bacterial count (TBC) and the count of aerobic mesophilic bacteria (AMB) obtained by cultivation method *(Petrović et al., 1998)*.

By the statistical method of variance analysis, the significance of difference between the media and incubation conditions, as well as between the cultivation and direct bacteriological methods was tested. Statistical analyses were performed using software STATISTICA v. 8, StatSoft, Inc.

Results and Discussion

Average numbers of bacteria obtained by direct and indirect methods are shown in Figures 1 and 2.

Using the cultivation methods, the highest count of bacteria was recorded in the native raw water (S1) on R2A medium at room temperature (157 cfu/ml) and

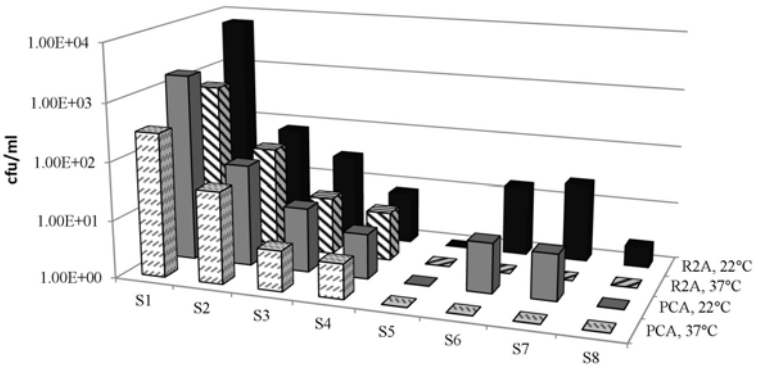

Figure 1. Mean count of aerobic mesophilic bacteria in certain stages of water supply system obtained by cultivation methods

the lowest number of bacteria was recorded in the water of tank Bagdala II (S7) (0 cfu/ml) on both media, at 37 °C (Figure 1). Analysis of variance showed that significantly more bacteria grew on R2A medium compared to the PCA ($p<0.05$), and at room temperature compared to 37 °C ($p<0.05$).

The highest average value of the total number of bacteria obtained by the direct method of quantification was also recorded in the native raw water (1,378,698 bacteria/ml), and lowest in the water tank Bagdala II (611 bacteria/ml) (Figure 2). At all sampling points, the direct count of bacteria was highly significant greater than the number obtained by cultivation methods ($p<0.01$).

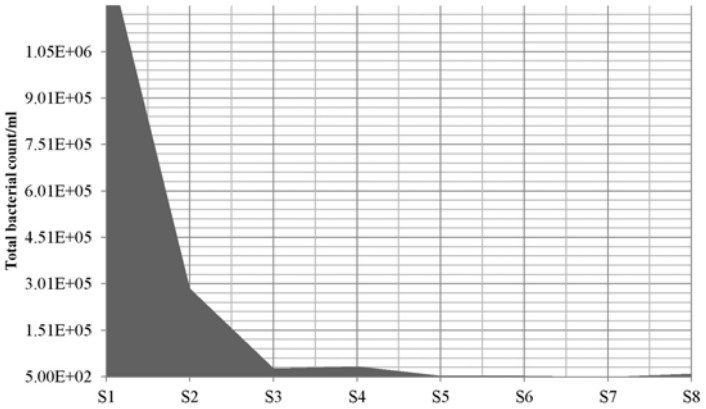

Figure 2. Mean values of total bacterial count at sampling sites obtained by direct method

Percentages of aerobic mesophilic bacteria in the total bacterial count in the tested waters are shown in Figure 3. The largest percentage of aerobic mesophiles was obtained in the water tank Bagdala II on R2A medium at room temperature (4.42%), and least in the same tank, but at 37 °C on both media, whereas, otherwise, it was not detected the presence of bacteria in the water. In the native raw water, which comes from the surface water, the highest percentage of aerobic mesophiles in the total number was 0.80% and was obtained by applying the number of bacteria detected on R2A at room temperature. This result is consistent with previous studies of surface waters *(Ćurčić, 2003)*.

TBC/AMB index values for the tested waters are shown in table 1. In very clear waters this index does not show the real situation; its significance is expressed primarily in surface waters. Thus, the values of this index obtained applying the number of bacteria on each medium at 37 °C, classified the native raw water into clean waters *(Petrović et al., 1998)*. When in this relation the number of aerobic mesophiles obtained on both media at room temperature was used, native

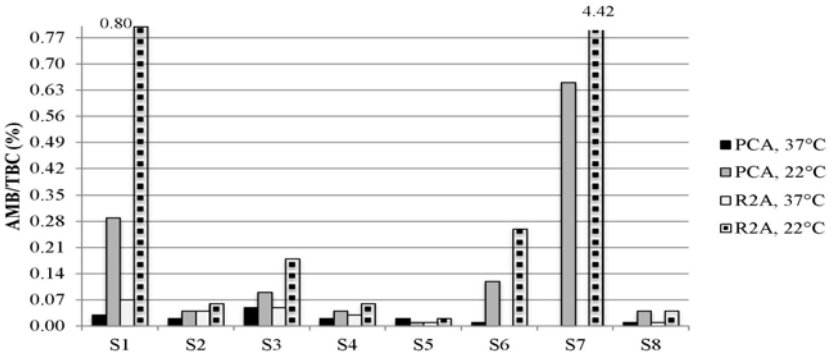

Figure 3. Percentage of aerobic mesophilic bacteria (AMB) in the total bacterial count (TBC)

water was classified as moderately polluted water. Previous testing of this water *(Ćirić, 2003; Ćirić, 2009)*, as well as the categorization according to Kohl *(Petrović et al., 1998)*, have shown that a more realistic estimation of its quality has been achieved by applying the results obtained at room temperature. In the case of assessment of the water treatment efficiency, the applied methods were correlated. Specifically, the percentage of reduction in the number of bacteria presented in the native raw water at the end of the treatment process, in final water, ranged from 99.71% to 99.99%. It is a very narrow range, so that the application of any tested methods give a realistic assessment of the effectiveness of treatment.

Table 1. TBC/AMB Index

TBC/AMB Index	Sampling site							
	S1	S2	S3	S4	S5	S6	S7	S8
TBC/AMB (PCA, 37°C)	3,071	5,738	1,980	4,938	6,553	13,867	0	15,276
TBC/AMB (PCA, 22°C)	350	2,785	1,155	2,304	15,290	832	153	2,673
TBC/AMB (R2A, 37°C)	1,471	2,758	1,848	3,142	15,290	0	0	15,276
TBC/AMB (R2A, 22°C)	125	1,827	555	1,819	4,587	378	23	2,673

The final water is distributed to end users through a distribution network, but does not retain its quality. The most common problem of water quality in distribution systems is the "regrowth" of bacteria *(Laurent et al., 1993)*. This process is largely the cause of many illnesses incurred using this water for drinking, increased corrosion of distribution pipes and problems with taste and odor of water *(Block, 1992; Critchley et al., 2001)*. Regrowth occurs due to recovery of bacteria from the stress (the effect of disinfectants, the lack of nutrients, etc.), and/or is caused by bacteria in biofilms. Results obtained on PCA

medium at 37°C showed that, in general, there was no regrowth in the network, while all other methods showed that it existed. The direct method showed an increase in the number of bacteria in the network in relation to the final water by a factor 2.3. Identical factor of increase in bacterial number in the network was shown by the results recorded on R2A medium at 37°C, while the larger factor (4.0) was shown by the results from the same medium at room temperature.

Conclusion

These examinations have shown that the most realistic number of bacteria in all types of water was obtained by applying the direct method of quantification.

If there is a need for determining the effectiveness of water treatment, it can be done by any of the methods applied in the work, depending on specific conditions, laboratory equipment, time limitations for obtaining results, economic situation, etc.

In the case of routine monitoring, to assess the bacteriological status of any water, it is better to use the cultivation methods because they are easier to perform and cost-effective compared to the direct methods. It should be noted that the application of PCA medium and incubation at 37°C gives the least informative results.

For the examination of regrowth of bacteria in the distribution network, it is best to apply a method that involves inoculation of samples on R2A medium and their incubation at room temperature for 7 days.

Primena direktne i kultivacionih metoda u bakteriološkim ispitivanjima vode u procesu vodosnabdevanja

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Rezime

Bakterijska abundanca u vodi koju za piće koriste ljudi i životinje predstavlja parametar koji je najuže povezan sa njihovim zdravljem. Zato je neophodna primena metode određivanja brojnosti bakterija koja će što realnije pokazati stvarno stanje ovog parametra. Ispitivano je bakteriološko opterećenje vode u pojedinim fazama procesa vodosnabdevanja primenom direktne i odgajivačkih metoda. U okviru odgajivačkih metoda uzorci vode su zasejavani na PCA i na R2A medijum, i inkubirani 7 dana na temperaturi od 37°C, kao i na sobnoj temperaturi. Direktna metoda obuhvatala je filtraciju uzoraka obojenih akridin oranžom i brojanje bakterija na filterima pod epifluorescentnim mikroskopom. Odgajivačke i direktna metoda pokazuju najmanju brojnost

bakterija u rezervoaru Bagdala II, 0 cfu/ml i 611 bakterija/ml, respektivno, a najveću brojnost u sirovoj vodi, 157 cfu/ml i 1.378.698 bakterija/ml, respektivno. Statistička analiza je pokazala da se značajno veći broj bakterija dobija na R2A podlozi u odnosu na PCA, kao i na sobnoj temperaturi u odnosu na 37°C. Indeks UBB/AMB svrstava nativnu vodu u klasu više zagađenih voda kada se u odnosu primene rezultati dobijeni na sobnoj temperaturi u odnosu na rezultate dobijene na 37°C. Najrealnije bakteriološko stanje svih tipova voda dobija se primenom direktne metode kvantifikacije bakterija. Međutim, za rutinski monitoring vode u procesu vodosnabdevanja, kao i za ispitivanje "ponovnog rasta" bakterija u distributivnoj mreži najbolje je primeniti odgajivačku metodu koja uključuje zasejavanje uzoraka na R2A medijum i njihovu inkubaciju na sobnoj temperaturi.

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